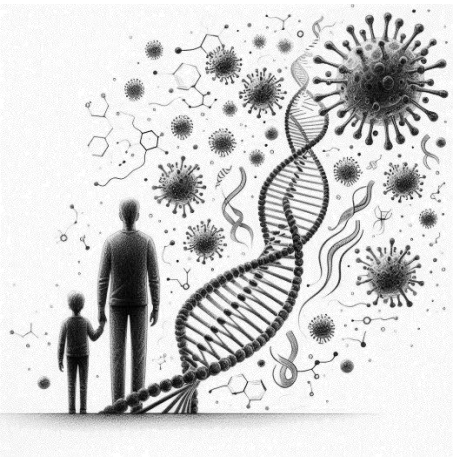
UNIVERSITÀ
DI PAVIA

Dipartimento di Scienze Clinico-Chirurgiche,
Diagnostiche e Pediatriche

Genetic analysis and pathogenetic aspects of emerging and re-emerging respiratory viral infections



Federica Anna Maria Giardina

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
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Table of contents

Abstract of the research

Respiratory viruses are one of the leading causes of morbidity and mortality worldwide especially for children, immunocompromised subjects and older people. Flu, hRSV, hRVs, EVs, hAdVs, MPV, PIVs, seasonal hCoVs, and SARS-CoV-2 are the main viruses as etiologic agents of respiratory syndromes. Clinical syndromes of viral respiratory infections may range from mild to severe and are characterized by common symptoms such as fever, cough, pharyngitis or laryngitis. However, with few exceptions, there are no antivirals, and hence therapy is mainly supportive.

This research project aimed to perform genetic analysis and investigate the pathogenetic aspects of emerging and re-emerging respiratory viral infections.

The analysis focused on SARS-CoV-2, rhinoviruses, echovirus 11, influenza and adenovirus-related infections.

Multi-lineage circulation was demonstrated for SARS-CoV-2 and molecular signature associated with the viral progression from the upper to lower respiratory tract. Multiple genotypes were identified when investigating the epidemiology of adenoviruses and rhinoviruses; moreover, the ability of rhinoviruses to cause prolonged and severe infections was assessed. Following the circulation of echovirus 11 among newborns and infants, molecular analysis on the recombinant origin of the novel strain allowed the identification of echovirus 6 as a parental strain for the partial P3 region of the viral genome. Lastly, influenza A(H3N2) circulation during the 2021-2022 season was analyzed. Sequencing of hemagglutinin protein revealed the presence of 65 mutations in 59 amino acid sites against the vaccine strain, thus suggesting the circulation of drifted strains in comparison to the one included in the vaccine.

Abbreviations

Flu/IV: Influenzavirus; hRSV: human Respiratory Syncytial virus; hRV: human Rhinoviruses; EVs: human Enteroviruses; hAdVs: human Adenoviruses; MPV: Metapneumovirus; PIV: Parainfluenzavirus; seasonal hCoVs: seasonal Coronaviruses; SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2; SARS-CoV: Severe Acute Respiratory Syndrome Coronavirus; MERS-CoV: Middle East Respiratory Syndrome Coronavirus; hBoV: human Bocavirus; NPIs: non-pharmaceutical interventions; URTI: upper respiratory tract infection; LRTI: lower respiratory tract infection; ARDS: acute respiratory distress syndrome; ICU: intensive care unit; CAP: community acquired pneumonia; HPAI: highly pathogenic avian influenza; HIV: human Immunodeficiency virus; HSCT: hematopoietic stem cells transplantation; WHO: World Health Organization; FDA: Food and Drug Administration; PA protein: polymerase acidic protein; PCR: polymerase chain reaction; RT-PCR: reverse transcription polymerase chain reaction; NGS: next-generation sequencing; COVID-19: Coronavirus infectious disease 2019; PANGOLIN: Phylogenetic Assignment of Named Global Outbreak LINeages; ECDC: European Centre for Disease Prevention and Control; VEWG: Virus Evolution Expert Working Group; BAL: bronchoalveolar lavages; MVs: minority variants; NTD: N-terminal domain; VOC: Variant of concern; ACE: angiotensin converting enzyme; NICU: neonatal intensive care unit; CMV: Cytomegalovirus; WGS: whole genome sequencing; E11: Echovirus 11; E6: Echovirus 6; NPEV: non-polio enterovirus; HA: hemagglutinin protein; GLIViRe: Working Group on Respiratory Virus Infections; AMCLI: Italian association of Clinical Microbiologist; RBD: Receptor Binding Domain; ISS: Istituto Superiore di Sanità.

Chapter 1

General Introduction

In early 2020, Lombardy (North Italy – 10 million inhabitants) was the first Italian region to be heavily affected by the SARS-CoV-2 pandemic. Since the identification of the first Italian case of SARS-CoV-2 on February 20th, more than 26 million cases have been laboratory-confirmed in Italy and more than 190 thousand deaths identified. (Ministero della Salute, www.salute.gov.it). Worldwide, more than 775 million cases have been reported with 7 million related deaths (10%) (World Health Organization, https://data.who.int/dashboards/covid19/cases), raising higher awareness on respiratory viruses.

The main viruses causing respiratory syndromes in humans are influenza A (FluA), influenza B (FluB), respiratory syncytial virus (hRSV), metapneumovirus (hMPV), parainfluenzaviruses (hPIVs), seasonal coronaviruses-NL63, -OC43, -HKU1 and 229E (hCoVs), rhinoviruses (hRV), enteroviruses (EVs), adenoviruses (hAdVs) and bocavirus (hBoVs). Among them, some have specific circulation patterns (e.g. in the winter season) while some other circulates year-round (De Francesco *et al.*, 2021).

Historically, different respiratory virus-related pandemics have been observed. Indeed, only in the 20th century, at least three pandemics caused by influenza A viruses have been reported. The first one, the so-called "Spanish Flu" was caused by an influenzaA /H1N1 strains and occurred in between 1918 and 1919, with more than 50 million of deaths worldwide. In 1957-58, another influenza pandemic was observed, caused by an H2N2 strain that first emerged in East Asia and then rapidly spread to other continents. Ten years later, in 1968, an H3N2 strain caused a further pandemic: first cases were identified in US and an excess of mortality was reported during the winter season 1968-69. Furthermore, an influenza A strain (A/H1N1/pmd09) caused a pandemic also in recent years (2009-2010) after a recombination event between human, swine and avian strains. The novel H1N1 strain spread very rapidly in the human population, especially children and young adults who did not have pre-existing immunity against the infection (Neumann et Kawaoka, 2022).

However, although influenza virus is one of the main respiratory viruses, also coronaviruses caused large outbreaks in the pre-SARS-CoV-2 era. Before 2002, coronaviruses were known to be pathogens for animals and humans, causing mild respiratory infections. But in 2002 in China, a novel coronavirus, later named SARS-CoV (Severe Acute Respiratory Syndrome Coronavirus), caused a large cluster of pneumonia. About 8000 cases were detected with a mortality rate of 10% (Peiris *et al.*, 2003). In 2012, in Saudi Arabia, another new coronavirus named MERS-CoV (Middle East Respiratory Syndrome Coronavirus) caused an outbreak of severe respiratory syndromes, with a total of 1800 cases confirmed and a mortality rate of 35% (Zumla *et al.*, 2015) (Fig.1).

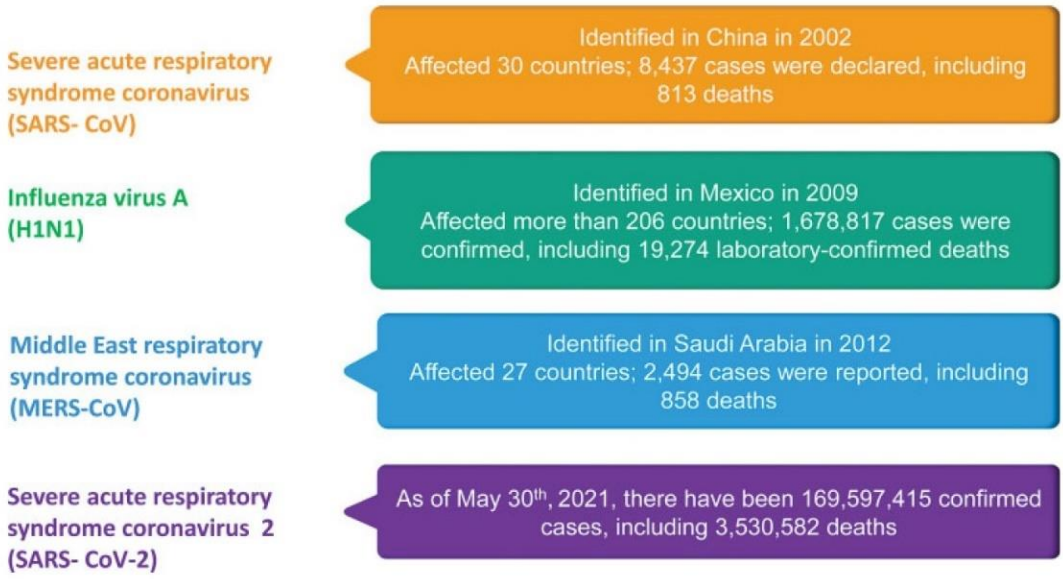

Figure 1. Influenza pandemic and coronaviruses-related outbreaks in 21st century (Cilloniz *et al.*, 2022).

Therefore, these data suggest that respiratory viruses, mainly the ones originated from zoonotic events, could be able to cause large epidemics inhuman population and spread very rapidly.

Moreover, these data confirmed that respiratory viral infections are one of the leading causes of morbidity and mortality worldwide, mainly in children, elderly or immunocompromised subjects. Indeed, the estimated mortality rate in Europe in 2019 due to viral respiratory infection was 146.040 cases (WHO, 2023). Moreover, the upsurge of the SARS-CoV-2 pandemic has also raised attention on respiratory viruses and their burden on healthcare and social systems, including hospitals and commercial activities.

In this context, the advances in new diagnostic tools and new molecular technologies are essential to able the active surveillance of respiratory viruses.

1.1 Epidemiology of respiratory viruses

Respiratory viruses can be transmitted from an infected subject by direct or indirect contact through aerosol, droplets, or fomites (Brankston et al., 2007). They can infect people of all ages, but some categories, such as infants and children, individuals over 65 years old, immunocompromised subjects, or those with pre-existing comorbidities, are at higher risk of being affected (Nguyen-Van-Tam *et al.*, 2022).
Several factors contribute to establishing the seasonality of respiratory viruses. For example, viral stability and transmissibility in different humidity and temperature conditions or changes in human behavior (e.g. indoor/outdoor activities) play a crucial role in viral circulation (Moriyama *et al.*, 2020). In temperate climates, viruses like influenza A/B, hRSV or seasonal hCoVs circulate predominantly during the winter season with peaks of cases between January and February. However, out-of-season influenza circulation is also documented (Gerna *et al.*, 2009; Loconsole *et al.*, 2022). Other viruses like hAdV can be detected all year round. HRV instead circulates through the entire year but has two distinct peaks in spring and fall while respiratory EVs can typically be detected from late spring to early fall (Fig.2).

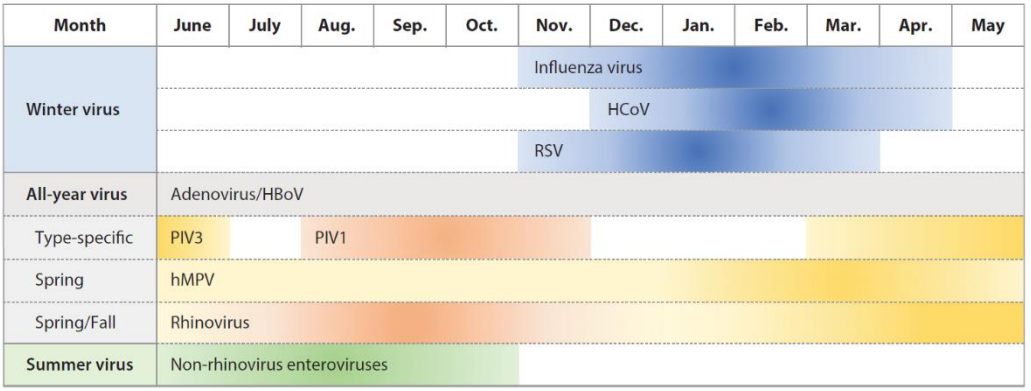

Figure 2. Schematic representation of respiratory virus circulation in pre-SARS-CoV-2 era (Moriyama *et al.*, 2020).

As reported by Visseaux and colleagues in a study regarding the prevalence of respiratory viruses in the hospitalized adult population in the period 2012-2016, the three respiratory viruses with the highest prevalence rates were picornaviruses (hRV and EVs), influenza and hCoVs (34.3%, 26.6% and 11.7%, respectively). They were followed by hRSV, with a prevalence of 9.7%, hPIVs (8.1%), MPV (5.7%), hAdV (2.7%) and hBoV (1.3%). Positivity rates in different age groups were similar, ranging from 27.2% to 31.8%. However, the detection rates of different viruses diverged in age groups, with picornaviruses being the most detected in all age groups except for the group 20-30 years and >80 years, in which influenza was the most prevalent (Visseaux *et al.*, 2017).

Regarding respiratory infections in the pediatric population, a Cypriot study analyzing the epidemiology of respiratory viruses in hospitalized pediatric patients under the age of 12, reported that hRSV was the most commonly detected, with a prevalence of 30.4%, followed by hRV (27.4%), together accounting for almost 60% of all cases. Conversely, all other respiratory viruses were detected with a moderate-to-low frequency: 11,6% for influenza A/B, 7.3% for hAdVs, hBoV in 5.7%, EVs and PIV3 in 5.4%. MPV and hCoVs were detected in less than 4% of cases (Richter *et al*., 2016). Similar results were observed in Italy, France, Poland and China (Leli et al., 2021; Fillatre et al., 2018; Cieślak et al., 2018; Yu et al., 2018).
In the pre-pandemic era, respiratory virus circulation was stable, with very similar detection rates in different years. However, the epidemiological characteristics of circulation changed at the beginning of the pandemic. In fact, the implementation of non-pharmaceutical interventions (NPIs) during the early phase of the SARS-CoV-2 pandemic caused a decrease in non-SARS-CoV-2 respiratory virus detection (Palmas et al., 2024) and a disrupted circulation (Chow *et al.*, 2023). Indeed, the use of facial masks, social distancing or stay-at-home restriction measures to prevent the spread of SARS-CoV-2 had also a huge impact on the typical epidemiology (De Francesco *et al.*, 2021).

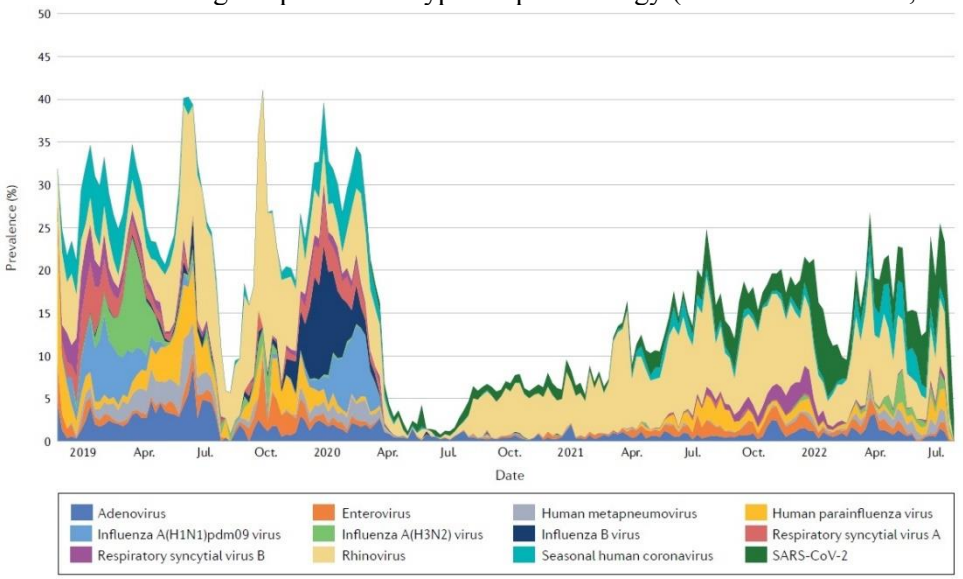

Figure 3. Incidence of community respiratory viruses and SARS-CoV-2 during the period 2019-2022 (Chow *et al.*, 2023).

Figure 3 reports the incidence of worldwide community respiratory viruses and SARS-CoV-2 during the period 2019-2022. influenza circulation significantly decreased in the early phase of the pandemic and remained low until 2021-2022 winter season, when a slight increase was documented in comparison the previous winter season (WHO, 2022). hRSV circulation was also affected by the pandemic because the number of confirmed cases decreased significantly (Tempia *et al.*, 2021; Casalegno *et al.*, 2021;). However,

an interseasonal circulation of hRSV was documented during the pandemic in Japan, probably due to the partial resumption of social activities (Ujiie *et al.*, 2021). Like influenza and hRSV, also seasonal hCoVs, hPIVs and MPV detection rates were affected by the use of NPIs (Olsen *et al.*, 2021; Park *et al.*, 2021). Conversely, hRV/EVs and hAdVs cases decreased very rapidly during the period March-May 2020 but rebounded to pre-pandemic rates of detection during the second and third waves of the pandemic (Ippolito et al., 2021; Olsen *et al.*, 2021). A possible explanation of these un-affected circulations may rely on the absence of envelope which makes these viruses more resistant under harsh environmental conditions in comparison to enveloped viruses (Kıymet *et al.*, 2021). Results reported in the Italian study by De Francesco and colleagues confirmed these data: during the pandemic, positivity rates for influenza, hRSV, hPIVs and hCoVs significantly decreased; however, hAdVs positivity rate decrease was not statistically significant. On the contrary, a statistically significant increase was documented for hRV (De Francesco *et al.*, 2021). The usual seasonality of respiratory viruses circulation began to be restored in 2022-2023 winter season (Cho *et al.*, 2024).

1.2 Clinical syndromes of respiratory viral infections

The infection of respiratory viruses may cause high morbidity and mortality rates also in otherwise healthy subjects, ranging from asymptomatic or pauci-symptomatic to extremely severe.
The severity of viral respiratory infections is multi-factorial and depends on both the host and virus characteristics (e.g. immunological status, viral transmissibility). Among the others, one of the main factors is the circulation rates in the previous seasons: it has been demonstrated that lower circulation rates of influenza in 2020-2021 winter season had contributed to lower specific immunity and thus was statistically correlated to more severe infections in the next winter season (Baker *et al.*, 2020).
The main clinical manifestation of most respiratory viruses is an upper respiratory tract infection (URTI). The incubation period before the onset of symptoms is 1-3 days (Park *et al.*, 2018) even if it may be longer (5-7 days) for SARS-CoV-2 (Cimolai, 2021).
The main symptoms of the infection, which is generally self-limiting in immunocompetent individuals (Wang et al., 2021; Volpe *et al.*, 2023), are fever, colds, rhinosinusitis, pharyngitis and laryngitis. However, all respiratory viruses could cause severe clinical syndromes of lower respiratory tract (LRTI) such as pneumonia, tracheitis, bronchitis, bronchiolitis or even Acute Respiratory Distress Syndrome (ARDS), sometimes requiring ICU admission and mechanical ventilation. In fact, respiratory viral infections are the main cause of ICU hospitalization in the USA (Walter and Wunderink, 2017). Besides, it is estimated that 20-40% of community-acquired pneumonia (CAP) and 50% of CAP in pediatric patients with laboratory-confirmed microbiological diagnosis are caused by respiratory viruses (WHO, 2013; Bianchini *et al.*, 2020). As regards hospital-acquired pneumonia (HAP), viruses cause 15-30% of cases in adults and up to 60% in children (Thorburn *et al.*, 2012; Hei *et al.*, 2018; Vanhems *et al.*, 2016).
Respiratory viruses also can cause prolonged infection in immunocompromised individuals. Gooskens and colleagues reported several cases of prolonged excretion of influenza virus (longer than 2 weeks) in a cohort of immunocompromised patients. All of the patients were experiencing lymphocytopenia and 75% developed a lower respiratory tract infection (LRTI). Moreover, viral clearance was associated with lymphocyte count reconstitutions (Gooskens *et al.*, 2009). Prolonged infections have also been described in a cohort of healthy/asymptomatic children in the USA: from birth to the age of 2 years, in 23.4% of cases a prolonged infection was detected and the majority of them was caused by hBoV and hRV (39% and 33%, respectively). These data suggest that immunocompromission due to underlying disease or immaturity of the immune system due to young age play a key role on viral shedding and prolonged infection in both asymptomatic and symptomatic subjects (Teoh *et al.*, 2024; Piralla *et al.*, 2015).
About 30-40% of hospitalized patients with laboratory-confirmed influenza are diagnosed with pneumonia. Among them, the majority are children under <5 years of age, adults >65 years of age, immunocompromised or with chronic lung or heart disease (Kalil and Thomas, 2019). Among all influenza strains, the ones of swine or avian origin, such as H5 or H7 strains classified as high pathogenicity avian influenza viruses (HPAI),

are able to cause severe infection in humans (Wang *et al.*, 2021), even if sporadic. The first case of H5 HPAI infection in humans was reported in 1998, in a 3-year-old boy diagnosed with pneumonia and respiratory distress syndrome (Claas *et al.*, 1998). Recently, further human H5N1 cases have been documented in the United States, in the context of a large outbreak involving both cattle and companion animals (Uyeki *et al.*, 2024; Burrough *et al.*, 2024; Ly, 2024).

HRSV is one of the leading causes of children's hospitalization for respiratory infections. It is estimated that globally in 2019, hRSV had caused 3.6 million cases of hospitalization among children (Li *et al.*, 2019). HRSV is indeed the main cause of bronchiolitis and pneumonia in young children. In particular, pre-term newborns, infants <6 months of age or with congenital heart/lungs disease are at high risk for hRSV infection leading to bronchiolitis (Hon *et al.*, 2023). Furthermore, hRSV infection is a major health problem for older adults (>65 years) and accounts for high rates of hospitalization for pneumonia (sometimes exceeding those for influenza), with exacerbation of chronic obstructive pulmonary disease, heart failure and asthma (Falsey *et al.*, 2005; McClure *et al.*, 2014; Prasad *et al.*, 2021; Branche *et al.*, 2022).

HRVs are normally known as the causative agents for the "common cold" but they can cause severe infections (Drysdale *et al.*, 2017; Aydin *et al.*, 2019). Some studies reported that, among all hRV genotypes, the ones belonging to species C are statistically correlated to more severe respiratory syndromes like pneumonia (Calvo *et al.*, 2009; Cox *et al.*, 2013). Moreover, hRV infection is associated with asthma exacerbation, especially in children (Lerman *et al.*, 2023). Together with hRV, also EVs can cause severe respiratory infections mainly in children and neonates (Pellegrinelli *et al.*, 2021). Moreover EVs can also disseminate causing extra-respiratory infections like otitis, hepatitis or encephalitis (Seppälä *et al.*, 2016; Piralla *et al.*, 2023; Roh *et al.*, 2023).

Generally, seasonal hCoVs are considered lowly pathogenic viruses, causing mild URTI. However, in high-risk patients, such as individuals with underlying conditions, they can cause severe infections (Trombetta et al., 2016). In contrast, SARS-CoV, MERS-CoV and SARS-CoV-2 are considered highly pathogenic viruses, able to cause severe clinical syndrome as pneumonia. In particular, hCoV infection may damage other organs and tissue including the gastrointestinal tract, spleen, lymph nodes, brain, skeletal muscle and heart (Channappanavar and Perlman, 2017; Huang *et al.*, 2020). Moreover, SARS-CoV-2 is associated with two specific symptoms, anosmia and ageusia, that have never been associated with respiratory viral infections before (Vaira et al., 2020).

Non-influenza respiratory viruses like MPV, hAdV and hPIVs often lead to LRTI in immunocompromised patients (Azoulay *et al.*, 2020; Collins *et al.*, 2020). Several studies have documented severe pneumonia cases caused by MPV, hAdV or hPIVs strains in HIV+ patients, solid organ transplantation (SOT) or hematopoietic stem cell transplantation (HSCT) subjects or patients with chronic obstructive pulmonary disease or chronic heart disease (Lynch and Kayon, 2016; Branche and Falsey, 2016; Fragkou *et al.*, 2020).

1.3 Multiple respiratory viral infections

Since several respiratory viruses can co-circulate in the same period, individuals may be infected simultaneously by different viruses. Several studies have reported different co-detection rates for respiratory viruses. For example, in a study by Da Conto and colleagues, it was reported that in over 20% of cases, more than two viruses were simultaneously detected. HRSV, hAdV and hCoVs were most commonly detected in co-infection cases (Da Conto *et al.*, 2019). De Francesco and colleagues, instead, evaluated the epidemiology of respiratory viruses before and during SARS-CoV2-pandemics. Before 2020, hRSV was detected in 58% of cases of co-infection, while during the pandemic SARS-CoV-2 and hRV were the only ones detected in co-infection. This can be explained by the different viral circulation during the pandemic (De Francesco *et al.*, 2021).

However, although several epidemiological data on co-infections are reported in the scientific literature, the impact of mixed viral infection on clinical outcomes remains unclear and may depend on the specific combination of viruses involved (Antalis *et al.*, 2018). In the study by Antalis and colleagues, the authors evaluated the correlation between co-infection rates and clinical outcomes. Mixed infections accounted for 20% of all cases detected with hRSV and influenza A being the most detected in co-infection. Mixed infections were statistically associated with younger age and higher fever rates but, unexpectedly, were not statistically associated with higher hospitalization rates. Although not statistically significant, mixed infections appeared more frequent than single infections in patients with underlying comorbidities (Antalis *et al.*, 2018).

The recent pandemic has promoted clinical research about mixed infections with SARS-CoV-2 and other respiratory viruses. An *in vitro* study reported the simultaneous infection of a cell line with SARS-CoV-2 and influenza has promoted SARS-CoV-2 infectivity. Moreover, mixed infection of SARS-CoV-2 and influenza in mice resulted in increased SARS-CoV-2 viral loads and more severe lung damage. Furthermore, analyses were conducted on other respiratory viruses, yet none demonstrated the same impact as influenza on SARS-CoV-2 infection (Bai et al., 2021). As reported by Krumbein et al., in a clinical setting, influenza, hRV and hAdV were the most frequently detected in SARS-CoV-2-positive patients, especially in pediatric patients. Mixed infections were associated with dyspnea and higher mortality rates (Krumbein et al., 2022).

The data presented highlight the importance of mixed infection, particularly in the context of pediatric patients and individuals with comorbidities. Further studies are required to enhance our understanding of the clinical implications of such infections, including their impact on therapeutic management.

1.4 Treatment and prevention of respiratory viral infections

Treatment options for respiratory viral infections are relatively scarce and the therapy is mainly supportive. However, for some of them, there are few therapeutic options (Cilloniz *et al.*, 2022). Table 1 reports the main therapeutic options for respiratory virus infections.

Antiviral therapy	**Recommendation**
Oseltamivir and other neuraminidase inhibitors (NAI)	Influenza A/B
Influenza A/B	Influenza A/B
Ribavirin, palivizumab, nirsevimab	hRSV
Cidofovir	Adenovirus
Remdesivir	SARS-CoV-2
Corticosteroids/Dexametasone	Severe cases of SARS-CoV-2

Table 1. Summary of the main therapeutic options for respiratory viral infections.

The first-line antiviral medications employed to treat influenza infection are neuraminidase inhibitors such as oseltamivir. Neuraminidase is one of the two major glycoproteins on the surface of the influenza virion and plays a pivotal role in the formation and release of new virions. Oseltamivir is active against influenza A and influenza B and it is first-line treatment recommended for severe influenza cases and in patients with a high risk of complications (Javanian *et al.*, 2021; Bulloch, 2023; Chan and Hui, 2023). However, the circulation of oseltamivir-resistant strains has been reported (Meijer *et al.*, 2009; Skog *et al.*, 2023). Along with oseltamivir, also zanamivir is a neuraminidase inhibitor, used both for treatment and prophylaxis of influenza infections (Dreitlein *et al.,* 2001; Jackson *et al.*, 2011).
In October 2018, a novel antiviral drug, named baloxavir marboxil, was approved by Food and Drug Administration (FDA) (Food and Drug Administration, 2018). Baloxavir marboxil is a polymerase acidic (PA) protein inhibitor and blocks influenza proliferation by inhibiting the initiation of mRNA synthesis. Baloxavir showed high efficacy in alleviating influenza symptoms and reducing viral loads in shorter time than placebo or other antiviral drugs (Hayden *et al.*, 2018). Moreover, baloxavir was also evaluated with good results as a prophylaxis treatment for those in contact with patients with confirmed influenza infection (Ikematsu *et al*., 2020).
The main antiviral drugs used for hRSV treatment and prevention are ribavirin, palivizumab and nirsevimab. Ribavirin blocks viral polymerase preventing the replication of vira RNA (Tejada et al., 2022). Palivizumab is instead monoclonal antibody active against hRSV F protein preventing membrane fusion. However, palivizumab is mostly used as a prophylactic measure against hRSV infection but it also can be used to treat severe cases of infection and prevent complications (Caserta *et al.*,

2023), especially in pre-term newborns or those with respiratory or cardiovascular disease.
Cidofovir is the antiviral option for hAdV treatment; it has been successfully used to treat hAdV-related pneumonia in immunocompetent adults and pediatric patients (Barker *et al.*, 2003; Zhao *et al.*, 2020; Le *et al.*, 2020) and seems to be associated with lower risk of respiratory failure (Ko *et al.*, 2020). However, cidofovir is characterized by high levels of nephrotoxicity (Lynch *et al.*, 2016).
There is no specific therapy for SARS-CoV-2 treatment. The first form of anti-SARS-CoV-2 treatment, in the very early phase of the pandemic, was the convalescent plasma, containing high titers of neutralizing antibodies, collected from patients who had recovered from the infection (Perotti *et al.*, 2020). Nowadays, SARS-CoV-2 positive patients could be treated with a cocktail of medications including polymerase inhibitors such as remdesivir (Wang et al., 2020) together with anti-inflammatory drugs and corticosteroids to enhance effectiveness.
However, the best form to treat a respiratory infection is to prevent it by vaccination.
Vaccines are available against influenza and SARS-CoV-2 and the first data about hRSV vaccine are reported.
Influenza vaccine may be trivalent or quadrivalent, containing proteins of two influenza A strains (H1N1 and H3N2) and one or both influenza B strains (Victoria and Yamagata) (https://gisaid.org/resources/human-influenza-vaccine-composition/). It has to be administered preferably before influenza circulation (around mid-October) but it can also be administered during the winter season. Vaccine administration is recommended for high-risk subjects such as infants <6 months of age, older people >65 years of age, pregnant women or those with comorbidities.
As prevention measures for hRSV infection, FDA has recently approved new bivalent pre-Fusion F vaccines to be administered to older adults (>60 years) and pregnant women to prevent hRSV infection in infants (Walsh *et al.*, 2023; Kampmann *et al.*, 2023).
Moreover, anti-hRSV measures include also the administration of nirsevimab, an anti-F monoclonal antibody with extended half-life that was approved for the first time in 2022 in Europe (Keam, 2023) by FDA in 2023 (https://www.fda.gov/drugs/novel-drug-approvals-fda/novel-drug-approvals-2023). Nirsevimab efficacy has been tested in pre-term (born from 29 weeks of gestation onward) and term infants who were approaching their first hRSV season: it is reported that nirsevimab protected infants from severe hRSV infection and hospitalization (Hammitt *et al.*, 2022; Drysdale *et al.*, 2023).
Four anti-SARS-CoV-2 vaccines have been approved and their efficacy ranges from 70% to 95% (Polack et al., 2020; Baden et al., 2020; Sadoff et al., 2021; Voysey et al., 2021). Two of them are based on mRNA coding for the Spike protein (Polack et al., 2020; Baden et al., 2020) while the others are based on viral vector always coding for the Spike protein (Sadoff et al., 2021; Voysey et al., 2021) (Table 3). Since SARS-CoV2 has evolved very rapidly, with different variants circulating at the same time, the vaccines have been updated based on the new antigenic characteristics of spike protein.
Table 2 reassumes the main characteristics of anti-hRSV and anti-SARS-CoV-2 vaccines and prevention measures.

Name	Commercial name	Type of drug	Target	Efficacy
RSVPreF	Abrysvo	Vaccine	Pregnant women	81.8%
RSVPreF3	Arexvy	Vaccine	Adults >60 years	85.7%
Nirsevimab	Beyfortus	Monoclonal Antibody	Newborns	83.2%
BNT162b2	Pfizer-BionTech	mRNA vaccine	General population	95%
mRNA-1273	Moderna	mRNA vaccine	General population	94%
Ad26.COV2.S	Johnson&Johnson	Recombinant vaccine	General population	70-85%
ChAdOx1	Astrazeneca	Recombinant vaccine	General population	70%

Table 2. Characteristics of anti-hRSV and anti-SARS-CoV-2 vaccines and prevention measures.

1.5 Diagnosis of respiratory viruses

The clinical symptoms of a viral respiratory infection (e.g. fever, cough, laryngitis) are aspecific and may be due to different viruses, especially during those periods like winter season with a simultaneous circulation of many viruses. Thus, a clinical diagnosis based on the patient's respiratory syndrome is not sufficient to identify the etiologic agent of the infection but the diagnosis needs to be confirmed by laboratory testing.
Before the advent of modern molecular diagnostic assays, diagnosis of viral infection was based on cell culture, where samples collected from patients were inoculated (Landry *et al.*, 1997). After a few days of incubation, necessary for the infection of cells, the presence of a cytopathic effect was suggestive of the infection. This approach for the diagnosis of viral infection was time-consuming and labor-intensive and could be affected by several factors such as the viability of sample, the use of correct cell line, the viral load, possible bacterial/fungal contamination and the personal expertise (Landry *et al.*, 1997; de Crom *et al.*, 2013).
Nowadays, molecular assays as the real time PCR or real time RT-PCR are the gold standard for the diagnosis of viral infection (Kim *et al.*, 2009; Dominguez and Blodget, 2019). They are based on the exponential amplification of a target region through the annealing of specific primers and probes on the viral genome. These assays use fluorescence dye whose signal intensity reflects the amount of amplified DNA (Kubista *et al.*, 2006; Walter and Wunderink, 2017).
Molecular assays can be designed as singleplex or multiplex. In the first case, a single virus is targeted in a single reaction, while in multiplex assay multiple viruses are targeted in the same reaction tube. Thus multiplex assays allow simultaneous analysis of more than one target reducing the turnaround time, but their design and optimization are more complex, requiring higher expertise. Moreover, the costs of multiplex reaction and the sensitivity may be affected (Parker *et al.*, 2015).
Molecular assays can also be designed as qualitative or quantitative. The result of qualitative assays indicates the presence or absence of the targeted virus in the clinical specimen; the quantitative assays instead, through the testing of standard samples whose concentration is already known, allow the monitoring of viral loads and their kinetics. This is very important in case of multiple respiratory viruses detection to define which one is predominant and causing the clinical syndrome but also in the therapeutic treatment setting to monitor the efficacy of antiviral therapies through the viral loads decrease.

1.6 Molecular surveillance of respiratory viruses

The advent of molecular testing for viral infections provided new tools for routine diagnostic procedures. However, both Sanger sequencing or Next Generation sequencing (NGS) represent a very powerful tool for the molecular surveillance of viruses and can be applied in different fields of virological research.
One of the main applications of sequencing has an epidemiological purpose and includes the genotyping of circulating strains (Wang *et al.*, 2015; Pscheidt *et al.*, 2021).
Moreover, the NGS application for complete genome sequencing can provide a deeper insight into viral evolution. For example, a study by Selleri and colleagues reported the use of NGS techniques for the analysis of influenza quasispecies in a cohort of 32 patients with mild-to-severe infection leading to the identification of D222 polymorphism in the hemagglutinin gene which is associated with the enhanced binding to α2,3-linked sialic acid, suggesting a greater ability to bind the lung cells and hence a more severe outcome of the infection (Selleri *et al.*, 2013). Notably, SARS-CoV-2 pandemic has highly promoted the use of NGS techniques in clinical laboratories. Thus, a lot of data have been reported in the scientific literature about SARS-CoV-2 evolution, not only referring to those proteins on the surface of the virions such as the spike, which was the main focus in the early phase of the pandemic, but also on the non-structural proteins (Charre *et al.*, 2020; John *et al.*, 2021; Cecchetto *et al.*, 2023).
The new frontier of NGS technologies in the virological field is the metagenomic approach which is not amplicon-based. It so potentially allows for obtaining a complete genome sequence of all microorganisms present in the specimen (Liang et al., 2021). Based on its characteristics, the metagenomic approach could also be used in the diagnostic procedure to identify pathogens in samples that are apparently negative for all viruses tested or for identifying emerging viruses (Slavov, 2022; Li *et al.*, 2022).

Chapter 2

Aim of the Research

Respiratory viral infections are one of the main causes of morbidity and mortality worldwide, especially in children, older adults and immunocompromised subjects. The main viruses as etiologic agents of respiratory syndromes are influenza, hRSV, hRV, EV, hAdV, MPV, PIV, seasonal hCoV, SARS-CoV-2.
Some of them, such as influenza or hRSV, circulate mainly during the winter season while some others (hRV, hAdV or SARS-CoV-2) may be detected year-round, with peaks of cases in spring and fall. The implementation of NPI measures occurred during SARS-CoV-2 pandemic had also an impact on the circulation of other respiratory viruses and hRV and hAdV were the only viruses to be detected during the first months of the pandemic.
Respiratory viruses infections have common characteristics such as fever, cough, pharyngitis or laryngitis and may range from mild to severe: it is estimated that influenza is the main cause of severe syndrome among adults and hRSV among children.
Therapeutic options for respiratory viral infections are scarce and therapy is mainly supportive. However, severe infections associated with influenza viruses and hRSV may be treated with specific antivirals such as neuraminidase inhibitors and anti-F monoclonal antibodies, respectively.
In this setting, the active surveillance of respiratory viruses is of paramount importance to evaluate their circulation and impact on the human population, to track their molecular evolution and antiviral resistance and to establish guidelines for disease control.
The aim of this research was to evaluate the genetic and pathogenetic markers of emerging and re-emerging respiratory viral infections. In particular, this thesis is divided in different sections:

I) The first section is on SARS-CoV-2 variants and their epidemiology throughout different pandemic waves and in the pediatric population. Moreover, the intra-patient evolution of SARS-CoV-2 quasispecies was evaluated through the analysis of paired samples collected from upper and lower respiratory tract of patients.

II) The second part is on *Picornaviridae* family, with a focus on the molecular epidemiology of hRVs and their ability to cause severe and prolonged infections. Furthermore, a deeper analysis on the recombinant origin of echovirus 11 strain associated with severe hepatitis in neonates was performed.

III) The third part is focused on the molecular epidemiology of influenza virus A/H3N2 strains circulating in the 2021-2022 winter season.

IV) The fourth and last part reports the results of a multicentric study about the epidemiological impact of adenoviruses as the causative agent of respiratory infections in children and adults.

Chapter 3

Spread of multiple SARS-CoV-2 lineages April-August 2020 anticipated the second pandemic wave in Lombardy (Italy)

Monica Tallarita, Federica A.M. Giardina, Federica Novazzi, Stefano Gaiarsa, Gherard Batisti Biffignandi, Stefania Paolucci, Francesca Rovida, Antonio Piralla, Fausto Baldanti

Role: Co-first author

Author's contribution: Methodology, writing, review and editing

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Spread of multiple SARS-CoV-2 lineages April-August 2020 anticipated the second pandemic wave in Lombardy (Italy).

Monica Tallarita[1], Federica A.M. Giardina[1], Federica Novazzi[1], Stefano Gaiarsa[1], Gherard Batisti Biffignandi[2], Stefania Paolucci[1], Francesca Rovida[1], Antonio Piralla[1], Fausto Baldanti[1,2]

[1] *Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy;*

[2] *Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy.*

ABSTRACT

During the initial phase of the pandemic (20 February–4 April 2020), we conducted an investigation into the temporal and geographical evolution of the virus in Lombardy. Our findings revealed the circulation of at least seven distinct lineages, which exhibited varying distributions within the region. The present study monitored the molecular epidemiology of SARS-CoV-2 between two pandemic waves (April-August 2020) to track the circulation of new variants. The majority of SARS-CoV-2 strains (70.8%) belonged to lineages B, B.1, B.1.1 and B.1.1.1. Furthermore, five strains belonging to four lineages were already reported in Italy (B.1.1.148, B.1.1.162, B.1.1.71, and B.1.425). Moreover, 21 SARS-CoV-2 strains belonging to six lineages not previously observed in Italy were identified. No variants of concern were identified. A total of 152 amino acid changes were observed among spike gene sequences, representing 11.3% of the total. Of these, 26 (17.1%) occurred in the receptor-binding domain region of the spike protein. The results of this study indicate that the transmission of the virus continued throughout the period of lockdown, rather than suggesting the reintroduction of novel lineages following the lifting of restrictions. The application of molecular epidemiology in Italy should be encouraged in order to enhance understanding of the transmission of the disease and to have a significant impact on disease control.

Keywords: COVID-19, molecular epidemiology, NGS, SARS-CoV-2, whole genome sequencing.

1. INTRODUCTION

Since the initial report of the novel coronavirus disease 2019 (COVID-19) in China on 30 December 2019 [1,2], SARS-CoV-2 has been spreading rapidly worldwide. As of 5 February 2021, there have been 104 million confirmed infections and more than 2 million deaths have been reported worldwide [1]. Lombardy, with a population of 10 million inhabitants, is the most densely populated and affected region in Italy during the first wave, with more than 90,000 cases at the end of May 2020 [3,4]. The population density, coupled with the high level of transportation links to Europe, creates the conditions to host and favor the spread of a highly transmissible virus such as SARS-CoV-2. During the initial phase of the pandemic (20 February–4 April 2020), we conducted a temporal and geographical analysis of the virus in Lombardy [5]. This study has documented the circulation of at least seven lineages, which exhibited differential distribution patterns within the region. The initial wave of the pandemic showed as sharp down at the end of May 2020. However, a considerable number of cases were reported (exceeding 10,000 in June–August 2020) and international road transport to and from several EU states was re-established after 1 June 2020 [4,6]. In the present study, the molecular epidemiology of SARS-CoV-2 was monitored between two pandemic waves in order to track the circulation of new variants.

2. MATERIALS AND METHODS

A total of 89 respiratory samples with SARS-CoV-2 cycle threshold values below 24 were sequenced. Clinical samples were collected between 15 April and 20 August 2020 and tested positive for SARS-CoV-2, as previously described [5,7]. Total RNA was extracted from nasopharyngeal swabs using the QIAamp Viral RNA Mini Kit, followed by purification with Agencourt RNA Clean XP beads. Virus genomes (GISAID EPI_ISL_1133145-1133202 and 1166095-1166108) were generated by a multiplex approach using version 1 of the CleanPlex SARS-CoV-2 Research and Surveillance Panel (Paragon Genomics, Inc.), according to the manufacturer's protocol, starting with 50 ng of total RNA, followed by Illumina sequencing on a MiSeq platform. Furthermore, the NGS data were analyzed using an in-house pipeline to compare our sequences with those retrieved from GISAID EpiCoV™ database. Lineages were assigned from the alignment file using the Phylogenetic Assignment of Named Global Outbreak LINeages (PANGOLIN) tool, version 1.07 (https://github.com/hCoV-2019/pangolin) [8]. The study protocol was approved by the local Research Ethics Committee of Fondazione IRCCS Policlinico San Matteo (P_20200085574). This study was conducted in accordance with the principles outlined in the 1964 Declaration of Helsinki. In accordance with Italian governmental regulations on observational retrospective studies, informed consent was waived.

3. RESULTS

A total of 3212 SARS-CoV Italian sequences were available in the GISAID EpiCoV™ database (https://www.epicov.org/epi3/) as of 5 February 2021. A comparison between these sequences was performed and presented in Table 1, which shows the SARS-CoV strains. A lineage analysis of SARS-CoV-2 conducted using the Pangolin web application indicated that the B lineages were the most prevalent in Lombardy (Figure 1). In detail, the majority of SARS-CoV-2 strains (63/89; 70.8%) belonged to lineages B, B.1, B.1.1 and B.1.1.1. Five strains were grouped into clade A, including sequences from China and numerous strains from South East Asia, Japan, South Korea, Australia, and the USA. The distribution of lineages in the early phase of the pandemic exhibited a similar pattern until August [5]. This may indicate that the initial introduction of multiple lineages in Lombardy was followed by local transmission events during the period of lockdown. Furthermore, five strains belonging to four lineages that have previously been identified in Italy (B.1.1.148, B.1.1.162, B.1.1.71, and B.1.425) were also detected. Finally, 21 SARS-CoV-2 strains belonging to six lineages not previously observed in Italy were detected (Table 1). This finding demonstrated the introduction of additional lineages in the context of a predominant circulation of previously introduced lineages.

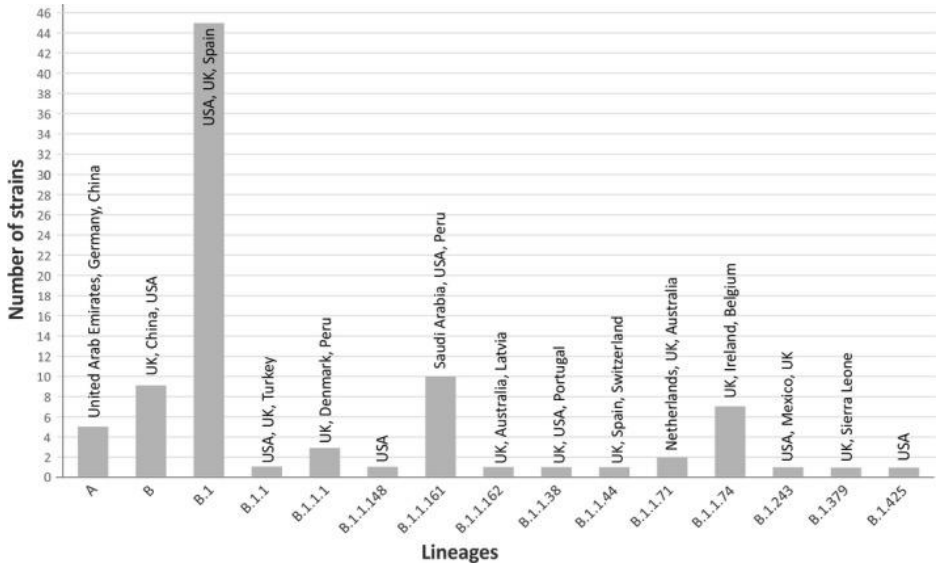

Figure 1. Distribution of SARS-CoV-2 strains according to lineages assigned by Pangolin COVID-19 Lineage Assigner online tool ((https://github.com/hCoV-2019/pangolin). The most common countries in which each lineage was identified are reported above the bars).

Lineages	This study (no.)	Italian sequences (no.)	Most common countries	Earliest date	Count	Description*
A	5	16	United_Arab_Emirates 19.0%, Germany 13.0%, China 13.0%, United States of America 6.0%, Japan 5.0%	30-12-2019	1312	Root of the pandemic lies within lineage A
B	9	21	United Kingdom 36.0%, United States of America 16.0%, China 13.0%, Spain 4.0%, Singapore 3.0%	24-12-2019	4976	Base of this lineage also lies in China, with many global exports, two distinct SNPs `8782TC` and `28144CT` define this lineage
B.1	45	743	United States of America 46.0%, United Kingdom 13.0%, Canada 5.0%, Spain 4.0%, France 3.0%	24-01-2020	50516	A large European lineage that corresponds to the Italian outbreak.
B.1.1	1	29	United States of America 27.0%, United Kingdom 23.0%, Canada 6.0%, Germany 6.0%, Netherlands 5.0%	24-02-2020	2766	European lineage
B.1.1.1	3	150	United Kingdom 76.0%, Denmark 2.0%, Italy 2.0%, United States of America 2.0%, Switzerland 2.0%	02-03-2020	7604	UK/ Europe lineage
B.1.1.148	1	1	USA 89.0%, Canada 5.0%, Germany 2.0%, UK 2.0%, Mexico 1.0%	16-05-2020	87	USA lineage
B.1.1.161	10	0	Saudi_Arabia 24.0%, UK 21.0%, Switzerland 10.0%, Czech_republic 10.0%, Denmark 4.0%	09-03-2020	202	Saudi Arabian lineage
B.1.1.162	1	57	United Kingdom 23.0%, United States of America 15.0%, Japan 10.0%, Canada 9.0%, Spain 6.0%	26-02-2020	1927	Australian/UK lineage
B.1.1.38	1	0	United Kingdom 23.0%, United States of America 15.0%, Japan 10.0%, Canada 9.0%, Spain 6.0%	24-03-2020	260	UK lineage
B.1.1.44	1	0	United Kingdom 56.0%, Spain 20.0%, United States of America 11.0%, Denmark 2.0%, Switzerland 1.0%	23-03-2020	630	UK/Spain lineage
B.1.1.71	2	4	Netherlands 27.0%, United_Arab_ Emirates 21.0%, UK 17.0%, Australia 8.0%, Belgium 7.0%	06-03-2020	146	The Netherlands lineage
B.1.1.74	7	0	United Kingdom 87.0%, United States of America 5.0%, Peru 3.0%, Ireland 1.0%, Russia 1.0%	23-05-2020	268	Northern Irish lineage
B.1.243	1	0	United States of America 98.0%, Mexico 1.0%, Canada 0.0%, Switzerland 0.0%, South_Korea 0.0%	23-03-2020	5256	USA lineage
B.1.379	1	0	United Kingdom 91.0%, France 3.0%, United States of America 1.0%, Sierra_Leone 1.0%, Germany 1.0%	16-03-2020	67	UK lineage, previously B.1.5.30
B.1.425	1	1	United States of America 90.0%, Turkey 10.0%, Italy 0.0%		296	USA lineage (UT), reassigned from part of B.1.370

Table 1. Lineages identified in the study.

Since the summer of 2020, a series of SARS-CoV-2 variants of concern (VOC) (e.g., VOC 202012/01 and 501Y.V2) have been identified by the European Centre for Disease Prevention and Control (ECDC) as potentially increasing in pathogenicity due to several amino acid changes [9]. Some of these variants were associated with increased infection

rates in the UK (VOC 202012/01) and later in other countries. Conversely, a few (VOC501Y.V2 and Brasil) were associated with reduced neutralization by plasma. During the period from 15 April to 20 August 2020, no evidence of these variants was observed. A total of 152 amino acid (aa) changes were observed among spike gene sequences, representing 11.3% of the total number of observed changes. However, only 10 of these changes (6.6% of the total number of observed changes) were observed in at least two SARS-CoV-2 strains. These included T29I, E281G, Q564R, F565S, D614G, S640A, N641H, and K964R. It is noteworthy that only 26/152 (17.1%, 2.0% of the total) changes occurred in the receptor-binding domain (RBD) region of the spike protein.

4. DISCUSSION

The phylogenetic analysis of the viral sequences collected during the initial phase of the pandemic in Italy, mainly originated from Lombardy, indicated the clear circulation of at least seven distinct SARS-CoV-2 lineages [5]. Similar results were also observed in a subsequent study, which included 460 Italian strains that had been previously reported [10]. Furthermore, a more extensive study was conducted to describe the circulation of SARS-CoV-2 in the European region [11]. The genomes generated in the present study provide additional insight into the SARS-CoV-2 lineages and variants circulating in Lombardy during the period between the first and the second pandemic wave. The results of the analyses suggest that additional lineages were introduced in Lombardy during the summer. However, these introductions did not result in further transmission of the virus within the community, or alternatively, a limited transmission may have occurred. The segregation of specific lineages was observed, which is likely due to the implementation of stringent lockdown measures during the three-month state of emergency (March to May 2020). Several lineages, mainly observed in other countries, have been identified, with a few of these never reported in Italy before. None "high-risk" variants were observed to circulate in Lombardy during the study period. Nevertheless, the recent circulation of UK variants (B.1.1.7) associated with a more transmissible virus has prompted a re-evaluation of the global epidemiology of SARS-CoV-2. It is recommended that the ongoing vaccine campaign should be supported by real-time surveillance of circulating variants in order to monitor the emergence of mutations associated with poor antibody recognition. Finally, the vast majority of mutations within the spike gene region were observed in a single strain, and only the D614G change appears to be fixed in the SARS-CoV-2 population analyzed. Furthermore, the majority of these mutations are located outside the RBD region, which has a limited impact on antibody recognition.

5. CONCLUSION

This study indicated that limited but ongoing within-region transmission occurred during the initial phase of the SARS-CoV-2 pandemic in Lombardy, suggesting that transmission continued throughout the period of lockdown, rather than indicating the

reintroduction of novel lineages following the lifting of restrictions. Nevertheless, it is critical to acknowledge the extremely limited amount of genomic data from Italy in comparison to other regions, which has significantly limited the strength of the conclusions that can be drawn here. It is therefore vital that Italy establish a more robust genomic epidemiology infrastructure of any future significant outbreaks of emerging infectious diseases.

REFERENCES

1. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. Lancet Infect Dis. 2020;20:533-534.

2. Wu F, Zhao S, Yu B, et al. A new coronavirus associated with human respiratory disease in China. Nature. 2020;579:265-269.

3. Eurostat. The statistical office of the European Union statistics. https://ec.europa.eu/Eurostat.web/cities/data/database (Accessed 4 April 2021).

4. Regione Lombardia - Dashboard COVID 19 update of cases. https://www.regione.lombardia.it/wps/portal/istituzionale/HP/servizi-e-informazioni/cittadini/salute-e-prevenzione/coronavirus/dashboard-covid19 (Accessed 4 April 2021).

5. Alteri C, Cento V, Piralla A, et al. Genomic epidemiology of SARS-CoV-2 reveals multiple lineages and early spread of SARS-CoV-2 infections in Lombardy, Italy. Nat Commun. 2021;12:434.

6. European Union. https://ec.europa.eu/info/live-work-travel-eu/coronavirus-response/travel-during-coronavirus-pandemic_en. (accessed 4 Apr 2021).

7. Piralla A, Ricchi M, Cusi MG, et al. Residual SARS-CoV-2 RNA in nasal swabs of convalescent COVID-19 patients: Is prolonged quarantine always justified? Int J Infect Dis. 2021;102:299-302.

8. Rambaut A, Holmes EC, O'Toole Á, et al. A dynamic nomenclature proposal for SARS-CoV-2 lineages to assist genomic epidemiology. Nat Microbiol. 2020;5:1403-1407.

9. European Centre for Disease Prevention and Control. Risk Related to Spread of New SARS-CoV-2 Variants of Concern in the EU/EEA, First Update – 21 January 2021. ECDC; 2021. https://www.ecdc.europa.eu/en/publications-data/covid-19-risk-assessment-spread-new-variants-concern-eueea-first-update (Accessed 4 April 2021).

10. Di Giallonardo F, Duchene S, Puglia I, et al. Genomic epidemiology of the first wave of SARS-CoV-2 in Italy. Viruses. 2020;12:1438.

11. Alm E, Broberg EK, Connor T, et al. Geographical and temporal distribution of SARS-CoV-2 clades in the WHO European Region, January to June 2020. Euro Surveill. 2020;25:2001410.

Chapter 4

An overview of SARS-CoV-2 variants circulating in the 2020-2022 period in Lombardy

Federica A.M. Giardina, Guglielmo Ferrari, Federica Zavaglio, Stefania Paolucci, Francesca Rovida, Giulia Campanini, Laur Pellegrinelli, Cristina Galli, Elena Pariani, Federica Bergami, Alice Nava, Elisa Matarazzo, Silvia Renica, Diana Fanti, Valeria Cento, Claudia Alteri, Francesco Scaglione, Chiara Vismara, Carlo Federico Perno, Antonio Piralla, Fausto Baldanti

Role: First author

Author's contribution: Conceptualization, validation, data analysis, investigation, resources, data curation, writing-original draft.

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An overview of SARS-CoV-2 variants circulating in the 2020-2022 period in Lombardy

Federica A.M. Giardina[a], Guglielmo Ferrari[b], Federica Zavaglio[b], Stefania Paolucci[b], Francesca Rovida[a,b], Giulia Campanini[b], Laura Pellegrinelli[c], Cristina Galli[c], Elena Pariani[c], Federica Bergami[b], Alice Nava[d], Elisa Matarazzo[d], Silvia [Renicae], Diana Fanti[d], Valeria Cento[f,g], Claudia Alteri[e], Francesco Scaglione[e], Chiara Vismara[d], Carlo Federico Perno[h], Antonio Piralla[b], Fausto Baldanti[a,b].

[a] Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy;

[b] Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy;

[c] Department of Biomedical Sciences for Health, University Of Milan, Milan, Italy;

[d] Microbiological Analysis Unit, ASST Grande Ospedale Metropolitano Niguarda; Milan, Italy;

[e] Department of Oncology and Hemato-oncology, University of Milan, Milan, Italy;

[f] Department of Biomedical Sciences, Humanitas University, Milan, Italy;

[g] IRCCS Humanitas Research Hospital, Milan, Italy;

[h] Multimodal Research Area, Unity of Microbiology and Diagnostic Immunology, Bambino Gesù Children Hospital IRCCS, Roma, Italy.

ABSTRACT

Since the beginning of the pandemic, SARS-CoV-2 demonstrated high genetic variability. All variants that have sustained pandemic waves have exhibited multiple mutations, particularly in the spike protein, which could affect viral pathogenesis. A total of 15,729 respiratory samples, collected between December 2020 and August 2022, have been included in this study. This study reports the circulation of SARS-CoV-2 variants in Lombardy region over a two-year period. Alpha, Delta, and Omicron variants became predominant, accounting for the majority of cases, whereas the Beta or Gamma variants mostly caused local outbreaks. Next-generation sequencing revealed the presence of several mutations and a few deletions in all of the main variants. Indeed, 147 mutations were identified in the Spike protein of Omicron sublineages, with 20% of these occurring in the receptor-binding domain region.

Keywords: Epidemiology; Next-generation sequencing; SARS-CoV-2; Variants of concern.

1. INTRODUCTION

Since its initial identification at the end of 2019, SARS-CoV-2 spread rapidly on a global scale. Italy, and in particular Lombardy region (10 million inhabitants), was one of the first European countries to be significantly affected by the pandemic. Full genome sequencing of hundreds of SARS-CoV-2-positive respiratory samples revealed that the early phase of the pandemic was sustained by seven different lineages, with different geographical distributions within the Lombardy region [1]. The number of circulating lineages increased during the summer of 2020 [2]. Concurrently, the first notewhorty amino acid change in the Spike protein (D614G) was linked to increased viral loads in clinical specimens [3]. However, concerns about the emergence of lineages carrying new amino acid substitutions emerged at the end of 2020, following the initial identification of B.1.1.7 (Alpha variant) in the United Kingdom [4]. A series of novel lineages emerged, including B.1.351, P.1, and B.1.617.2 (designated Beta, Gamma, and Delta variants, respectively), which were identified in South Africa, Brazil, and India, respectively [5, 6, 7]. All of these lineages exhibited novel mutations. In particular, those that occurred in the receptor-binding domain had a significant impact on viral pathogenesis, resulting in high transmissibility rates, severe clinical syndromes, or immune evasion [8, 9]. The objective of this study was to assess the prevalence and circulation of these lineages in Lombardy between September 2020 and August 2022. Additionally, the study aimed to elucidate the molecular characteristics of novel variants through next-generation sequencing.

2. METHODS

2.1 Study Design

In order to perform regional surveillance of circulating variants, specimens were collected in 67 hospitals in all Lombardy provinces between December 2020 and August 2022. All samples were tested for the presence of the SARS-CoV-2 genome. Positive samples with a cycle threshold value <30 were then referred to Fondazione I.R.C.C.S. Policlinico San Matteo, Pavia, Italy, as one of the reference centres for SARS-CoV-2 genotyping. In particular, in accordance with the recommendations of the Italian Ministry of Health, genotyping was recommended for individuals who tested positive following anti-SARS-CoV-2 vaccination, those who had been reinfected, and those who had returned to Italy from any country with a high prevalence of an emerging variant. Furthermore, genotyping was recommended if (1) the number of cases was increasing, and (2) the viral transmissibility or virulence was rising (Italian Ministry of Health, 0003787-31/01/2021-DGPRE-DGPRE-P). Furthermore, a retrospective analysis was conducted between September and December 2020 to investigate the circulation of SARS-CoV-2 variants. The majority of respiratory samples were collected at the

following institutions: Fondazione I.R.C.C.S. Policlinico San Matteo in Pavia, Grande Ospedale Metropolitano Niguarda in Milan, and the University of Milan. Sequences investigation of clinical samples was approved by the Ethics Committee of our institution (P_20200085574) [10].

2.2 SARS-CoV-2 RNA detection

A total of 315,697 respiratory samples were analysed at Fondazione Policlinico San Matteo in Pavia between September 2020 and August 2022. RNA was extracted using the MGISP-960 automated workstation and the MGI Easy Magnetic Beads Virus DNA/RNA Extraction Kit (MGI Technologies, Shenzhen, China). The presence of SARS-CoV-2 RNA was determined using SARS-CoV-2 variants ELITe MGB® kit (ELITechGroup, Puteaux, France; cat. no. RTS170ING), which targets the *ORF8* and *RdRp* genes. The reactions were conducted on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Mississauga, ON, Canada).

2.3 Molecular Screening for detection of SAS-CoV-2 variants

Genotyping was conducted using a set of multiplex real-time PCR assays that were specifically designed to target specific mutations. To identify Alpha cases, including those with the E484K mutation, a multiplex real-time PCR assay targeting the N501Y and E484K mutations was performed. To detect the Delta variant, mutations T478K and L452R were employed as targets. Finally, Omicron cases were identified by multiplex real-time PCR targeting mutations in positions 417, 484, and 501, as well as the deletion 368 to 370. The primers and probes employed for genotyping are detailed in Table 1.

2.4 Sanger sequencing

To confirm the results of the real-time PCR, the Spike gene was amplified and sequenced in 10% of the samples, randomly chosen. The Spike gene was amplified in accordance with the methodology described by Gaiarsa et al. [11]. In brief, RNA was subjected to a one-step reverse transcription polymerase chain reaction (RT-PCR) using the SuperScript IV One-Step RT-PCR System (Thermo Fisher Scientific, Waltham, MA, USA), using primer SARS-2-S-F3 and SARS-2-S-R3 (Supplementary Table S1) for full gene amplification (approximately 4000 base pairs). The polymerase chain reaction (PCR) parameters were as follows: 55 °C for 10 min, 98 °C for 2 minutes, 42 cycles at 98 °C for 10 sec, 60 °C for 10 sec and 72 °C for 3 min, with a final extension at 72 °C for 5 min. The sequencing reaction of the appropriate PCR products was conducted using the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, CA, USA) on a 3130xl Genetic Analyzer. The reaction was conducted with the same primers used in the one-step RT-PCR and seven additional internal primers (see Supplementary Table S1). The resulting sequences were then analysed using the Sequencer 5.0 software.

Target	Primers/Probes	Sequence
B.1.1.7 + B.1.1.7/E484K (Alpha variant)	E484K-For	TGAAATCTATCAGGCCGGTAGC
	N501Y-Rev	GTACTACTACTCTGTATGGTTGG
	N501-probe	FAM-AACCCACTAATGGTGTTGG-MGB
	501Y-probe	VIC-AACCCACTTATGGTGTTGG-MGB
	484K-probe	Cy5-GTAATGGTGTTAAAGGTTT-MGB
B.1.617 (Delta variant)	VAR-IND-For	AGGCTGCGTTATAGCTTGGAATT
	VAR-IND-Rev	AGTAACAATTAAAACCTTCAACACCATTAC
	452R-probe	FAM- CCGGTATAGATTGTTTAGGAAG-MGB
	478K-probe	HEX-CCGGTAGCAAACCT-MGB
B.1.1.529 (Omicron variant)	S-E484KFor	TGAAATCTATCAGGCCGGTAGC
	S-N501YRev	GTACTACTACTCTGTATGGTTGG
	S417For2	CTCTGCTTTACTAATGTCTATGCA
	S-417Rev2	CGCAGCCTGTAAAATCATCTGG
	S368L-S370PFor	GGAAGAGAATCAGCAACTGTGTTG
	S368-S370Rev	TCTGCATAGACATTAGTAAAGCAGAGATC
	S-501Y	VIC-AACCCACTTATGGTGTTGG-MGB
	E484A	CY5-CCTTGTAATGGTGTTGCAGG-MGB
	S-417N	TEXASRED-CAAACTGGAAATATTG
	S368-S370	FAM-CCTATATAATCTCGCACCATT-MGB

Table 1. Primers and probes used to identify SARS-CoV-2 Alpha, Delta, and Omicron variants.

2.5 Whole genome sequencing

Genomic libraries were prepared using the NEBNext® ARTIC SARS-CoV-2 library Prep kit (New England Biolabs) from 8 µL of viral RNA, in accordance with the manufacturer's instructions. The libraries were quantified using the Qubit™ 1X dsDNA HS kit (Invitrogen, Carlsbad, CA, USA) on a Qubit 4 Fluorometer, normalised at the same concentration, and then pooled together. The pool was denatured with 0.2 M NaOH and then diluted to 10 pM. Sequencing was conducted using the MiSeq Reagent Kit V2 300 cycles (Illumina, San Diego, CA) on the MiSeqDx platform. The Fastq files were analysed using the Illumina® DRAGEN COVID Lineage App, which facilitates sequence alignment and variant identification.

3. RESULTS

3.1 Patients

A total of 30,244 respiratory samples were positive for SARS-CoV-2 during the study period. Among them, 15,729 samples were included in this study. The median age of patients was 46 years (range 1 day–106 years), with 7,900 males (50.3%) and 7,829 females (49.7%). The samples were collected in several hospitals in Lombardy provinces, including both inpatients and outpatients. In detail, 5,919 samples (37.6%) were collected in Pavia province, 3,956 (25.2%) in Milano, 2,476 (15.7%) in Mantua, 1,534 (9.8%) in Lecco, 698 (4.4%) in Lodi and 609 (3.9%) in the Cremona area. A total of 537 samples (3.4%) were collected in the provinces of Bergamo, Brescia, Como, Monza Brianza, Sondrio, and Varese.

3.2 Variant identification

A total of 14,075 SARS-CoV-2-positive samples (89.5% of the total) were successfully genotyped by real time-RT-PCR or sequencing. The Delta variant was the most prevalent, identified in 8,091 samples (57.5%), followed by the Omicron variant (2,719 cases, 19.3%) and the Alpha variant (2,333 cases, 16.6%). The early variants, which included all lineages that were circulating in the Lombardy region prior to the emergence of the Alpha variant, were identified in 798 cases (5.7%). Of these, B.1.177 was detected in 67% of cases, B.1.160 in 7.5%, B.1.1.161 in 4.1%, B.1.177.52 and B.1.177.75 in 3.4% and 3.2%, respectively. All other lineages, including Beta, Eta, Gamma, Mu, and variants, were present at a frequency of less than 3%. A series of local clusters of these variants were observed, with a total of fewer than 20 cases identified in each province. Genotyping was unsuccessful in 1,654 samples (10.5%) due to the low viral load present.

3.3 Chronological circulation of SARS-CoV-2 variants

As reported in Figure 1, at the beginning of the study period, the only lineages identified were those designated as early variants. In December 2020, the Alpha variant was identified for the first time, accounting for 12.4% of all cases included in the study (December 2020–August 2022). Subsequently, in February 2021, the Alpha variant became the predominant strain, responsible for 49.2% of all cases. During the same period, the circulation of the Beta, Eta, and Gamma variants was observed. These variants were detected in 23, 48, and 49 cases, respectively (0.14%, 0.3%, and 0.3%), between February and June 2021. In May, the Delta variant was identified in eight positive samples (2%) out of 372 samples sequenced in May 2021. From June onwards, the number of strains belonging to the Delta variantwas 10-fold higher than in May, accounting for 27.5% of all cases tested in June. Following a period of co-circulation (between June and July 2021) of the Alpha and Delta variants, the Alpha variant was no longer detected in August, and the Delta variant became the sole variant until December 2021. In December 2021, the first case of the Omicron clade (B.1.1.529) was identified in 900 individuals (26%) out of 3,461 patients tested. While the number of cases caused

by Delta decreased in February 2022, the number of cases due to the Omicron variant increased rapidly, becoming the predominant variant until the end of August 2022.

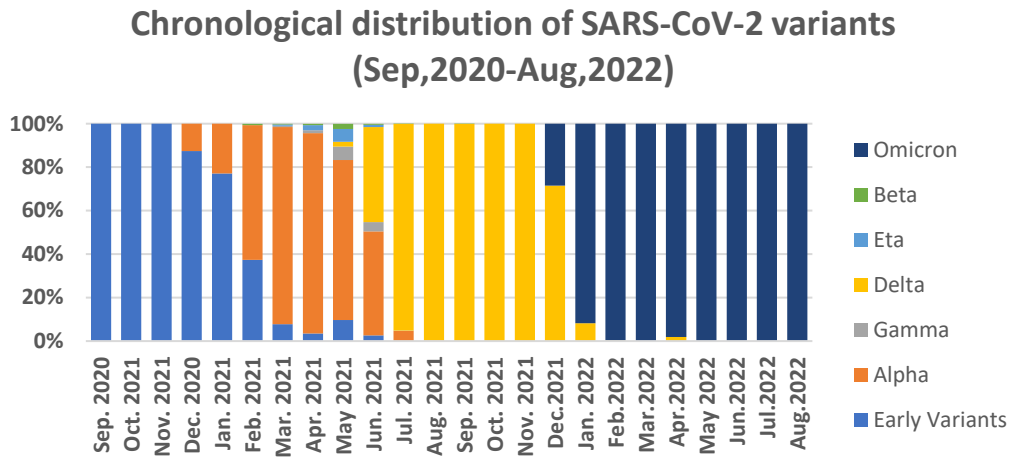

Figure 1. Chronological distribution of SARS-CoV-2 variants during December 2020 to August 2022. "Early variants," as those circulating before the first Alpha identification, are represented by light blue; Alpha cases are represented by orange, Delta in yellow, and Omicron in dark blue. While Alpha, Delta, and Omicron predominated during the study period, Beta, Eta, and Gamma variants (here depicted in green, pink, and grey, respectively) only caused local clusters with few cases.

For the three main variants circulating in Lombardy, Alpha, Delta, and Omicron, the kinetics of frequency circulation were calculated and analyzed with respect to the vaccination campaign. As shown in Fig. 2, the number of Alpha, Delta, and Omicron cases increased when the number of distributed vaccine doses was at its lowest level.

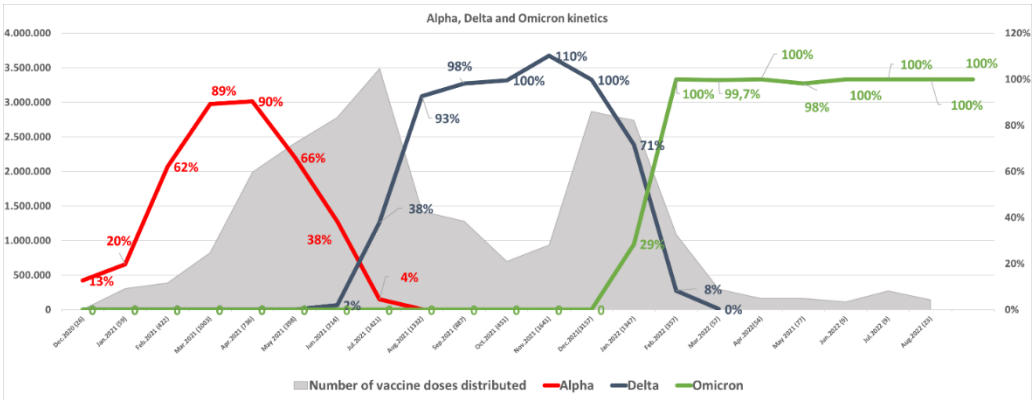

Figure 2. Growth rates and kinetics of Alpha (orange), Delta (yellow), and Omicron (dark blue) cases. The grey area represents the number of distributed vaccine doses. Together with new amino acid changes and competition between variants, the decrease in the number of doses distributed contributed to the circulation of new variants.

3.4 Whole-genome sequencing results

A total of 2613 (16.6% of the total number of samples included in the study) strains were sequenced with next-generation sequencing approach. Of these, 670 were classified as "early variants," 177 as Alpha variant strains, 653 as Delta, and 747 as Omicron strains. The success rates of next-generation sequencing for each of the main variants analysed are presented in Supplementary Table S2. In Alpha strains, the N501Y mutation was present in all sequences, in accordance with the pattern of mutations observed for each Variant of Concern. The most prevalent mutations were A570D and T76I, P681H, D614G, and S982A, which were present in 90% of all Alpha cases. It is noteworthy that 18.6% of all Alpha sequences exhibited the E484K mutation. The Delta variant exhibited the presence of the D614G, P681R, D950N, T478K, T19R, L452R, and G142D mutations in over 75% of the analyzed sequences. It is noteworthy that 42.6% of all Delta sequences harboured the G142D mutation, which was subsequently identified in Omicron strains. Conversely, none of the Delta sequences contained the E484K mutation. Furthermore, approximately 40% of all Delta sequences exhibited EFR156G deletions. A total of 747 strains belonging to the Omicron clade were subjected to sequencing. The initial Omicron lineage identified was B.1.1.529, which was subsequently followed by BA.1, BA.2, BA.4, BA.5, BE.x, and BF.x. Overall, 147 mutations were observed in the Spike protein, among which 30 (20.4%) occurred in the receptor-binding domain. The N501Y mutation was identified in 21.4% of BA.4 sequences and 50.2% of BA.1 sequences. It was observed that none of the Omicron strains harboured the substitution E484K. However, in 37.2% of BA.2 sequences, the glutamic acid residue in position 484 was substituted with alanine. It is noteworthy that the H655Y mutation, which had previously been observed only in Gamma strains, was identified in more than 80% of the sequences. Table S3 provides a comprehensive overview of all nonsynonymous nucleotide changes detected for each Omicron lineage. Furthermore, the amino acid deletions identified are presented in Supplementary Table S4. In both tables, the number of cases is expressed as a percentage value.

4. DISCUSSION

At the beginning of the surveillance period in September 2020, the pandemic in Lombardy was sustained by multiple lineages. This finding was already observed in the initial weeks following the initial detection of SARS-CoV-2 in Italy, as described by Alteri et al. [1]. In particular, at least 25 different lineages were observed to be circulating. This suggests that the restoration of national and international connections after the lockdown period promoted viral evolution and circulation.

In general, the epidemiological situation in Lombardy was similar to that observed nationally. After its first detection, the Alpha variant spread rapidly through the European continent [12, 13]. Indeed, the variant was responsible for less than 15% of cases in December 2020 but was identified in almost all positive cases between January and March 2021, becoming the predominant variant. This implies that all novel mutations present in the Alpha variant, including N501Y, facilitated a more rapid dissemination of this variant. The data presented here are consistent with those reported by Lai et al. [14] regarding other Italian regions. Conversely, other variants, such as Beta and Gamma, did not disseminate as readily as Alpha and resulted in only modest clusters in the Milan and Cremona provinces. In contrast, in other countries, these variants caused extensive clusters [15]. In May 2021, the first cases of the Delta variant were identified. By that time, the majority of the Italian population had received at least one dose of the anti-SARS-CoV-2 vaccination. Nevertheless, the circulation of the Delta variant was rapid and extensive. Accordingly, the hypothesis was that the Delta variant might evade the host's vaccine-induced immune response, as reported by Cassaniti et al. [16]. Since the beginning of 2022, the Omicron variant and its sublineages have constituted the latest variants to be identified. The initial isolation was recorded in December 2021.

As illustrated in Figure 2, the incidence of Alpha, Delta, and Omicron cases exhibits a negative correlation with the number of administered vaccine doses. In Italy, the vaccination campaign commenced on 27 December 2020 for healthcare workers. In early February 2021, vaccination was available for the general populations; the initial phase of distribution commenced with the elderly, with a peak in doses administered between June and July 2021. A further peak in vaccine distribution was recorded at the end of 2021, between November 2021 and January 2022 (regione.lombardia.it/wps/portal/istituzionale/HP/vaccinazionicovid/dashboard-vaccini). In this context, the reduction in vaccine coverage (reported as the grey area) occurred concurrently with other significant factors, including the competition between variants, which contributed to the circulation of the three main variants (Alpha in December 2020, Delta in May 2021, and Omicron in December 2021). To date, all cases of SARS-CoV-2 infection have been attributed to the Omicron variant, particularly the BA.4 and BA.5 sub-lineages, as also reported by other studies [17, 18, 19].

Furthermore, next-generation sequencing of Omicron variants has identified 147 mutations occurring in different sub-lineages with varying combinations and percentages. This study has several limitations. The study was conducted in a retrospective and prospective ways. The patients included in this study were referred to several hospitals in every Lombardy province; thus, it was not possible to retrieve any clinical data, particularly for outpatients. Although they were able to discriminate between the major VOCs, the real-time PCR assays conducted in our laboratory for variant identification were unable to discriminate between variants with similar mutation patterns. Furthermore, only samples with a Ct value lower than 30 were suitable for Spike sequencing. Consequently, 10% of the genotyping results are unavailable. Given

the epidemiological focus of the study, the genetic results obtained by next-generation sequencing were discussed in brief.

5. CONCLUSION

The active surveillance of SARS-CoV-2 variants is of paramount importance for the monitoring of their epidemiology and evolution. and This study provides an insight into the circulation of SARS-CoV-2 variants in the Lombardy region over a two-year period. We observed the circulation of new variants every few months during the study period: each of them contained new mutations that allowed them to become predominant thanks to a higher transmissibility and ability to escape the immune system. Fortunately, not all of them were associated with increased severity of clinical manifestations [20, 21]. From this perspective, the introduction of vaccination has led to a reduction in severe cases. From a future perspective, it would be very important to retrieve as many clinical records as possible from the patients included in this study to try to establish a correlation between the most frequent mutations and the clinical course of the infections.

REFERENCES

1. Alizon S, Haim-Boukobza S, Foulongne V, Verdurme L, Trombert-Paolantoni S, Lecorche E, Roquebert B, Sofonea MT. Rapid spread of the SARS-CoV-2 Delta variant in some French regions, June 2021. Euro Surveill. 2021 Jul;26(28):2100573. doi: 10.2807/1560-7917.ES.2021.26.28.2100573. PMID: 34269174; PMCID: PMC8284044.

2. Alteri C, Cento V, Piralla A, Costabile V, Tallarita M, Colagrossi L, Renica S, Giardina F, Novazzi F, Gaiarsa S, Matarazzo E, Antonello M, Vismara C, Fumagalli R, Epis OM, Puoti M, Perno CF, Baldanti F. Genomic epidemiology of SARS-CoV-2 reveals multiple lineages and early spread of SARS-CoV-2 infections in Lombardy, Italy. Nat Commun. 2021 Jan 19;12(1):434. doi: 10.1038/s41467-020-20688-x. PMID: 33469026; PMCID: PMC7815831.

3. Cassaniti I, Bergami F, Percivalle E, Gabanti E, Sammartino JC, Ferrari A, Adzasehoun KMG, Zavaglio F, Zelini P, Comolli G, Sarasini A, Piralla A, Ricciardi A, Zuccaro V, Maggi F, Novazzi F, Simonelli L, Varani L, Lilleri D, Baldanti F. Humoral and cell-mediated response against SARS-CoV-2 variants elicited by mRNA vaccine BNT162b2 in healthcare workers: a longitudinal observational study. Clinical Microbiology and Infections. 2021 Sep 25:S1198-743X(21)00536-X. doi: 10.1016/j.cmi.2021.09.016. Epub ahead of print. PMID: 34582981; PMCID: PMC8464022.

4. Faria NR, Mellan TA, Whittaker C, Claro IM, Candido DDS, Mishra S, Crispim MAE, Sales FCS, Hawryluk I, McCrone JT, Hulswit RJG, Franco LAM, Ramundo MS,

de Jesus JG, Andrade PS, Coletti TM, Ferreira GM, Silva CAM, Manuli ER, Pereira RHM, Peixoto PS, Kraemer MUG, Gaburo N Jr, Camilo CDC, Hoeltgebaum H, Souza WM, Rocha EC, de Souza LM, de Pinho MC, Araujo LJT, Malta FSV, de Lima AB, Silva JDP, Zauli DAG, Ferreira ACS, Schnekenberg RP, Laydon DJ, Walker PGT, Schlüter HM, Dos Santos ALP, Vidal MS, Del Caro VS, Filho RMF, Dos Santos HM, Aguiar RS, Proença-Modena JL, Nelson B, Hay JA, Monod M, Miscouridou X, Coupland H, Sonabend R, Vollmer M, Gandy A, Prete CA Jr, Nascimento VH, Suchard MA, Bowden TA, Pond SLK, Wu CH, Ratmann O, Ferguson NM, Dye C, Loman NJ, Lemey P, Rambaut A, Fraiji NA, Carvalho MDPSS, Pybus OG, Flaxman S, Bhatt S, Sabino EC. Genomics and epidemiology of the P.1 SARS-CoV-2 lineage in Manaus, Brazil. Science. 2021 May 21;372(6544):815-821. doi: 10.1126/science.abh2644. Epub 2021 Apr 14. PMID: 33853970; PMCID: PMC8139423.

5. Gaiarsa S, Giardina F, Batisti Biffignandi G, Ferrari G, Piazza A, Tallarita M, Novazzi F, Bandi C, Paolucci S, Rovida F, Campanini G, Piralla A, Baldanti F. Comparative analysis of SARS-CoV-2 quasispecies in the upper and lower respiratory tract shows an ongoing evolution in the spike cleavage site. Virus Res. 2022 Jul 2;315:198786. doi: 10.1016/j.virusres.2022.198786. Epub 2022 Apr 14. PMID: 35429618; PMCID: PMC9008095.

6. Hoffmann M, Arora P, Groß R, Seidel A, Hörnich BF, Hahn AS, Krüger N, Graichen L, Hofmann-Winkler H, Kempf A, Winkler MS, Schulz S, Jäck HM, Jahrsdörfer B, Schrezenmeier H, Müller M, Kleger A, Münch J, Pöhlmann S. SARS-CoV-2 variants B.1.351 and P.1 escape from neutralizing antibodies. Cell. 2021 Apr 29;184(9):2384-2393.e12. doi: 10.1016/j.cell.2021.03.036. Epub 2021 Mar 20. PMID: 33794143; PMCID: PMC7980144.

7. Italian Ministry of Health. Aggiornamento sulla diffusione a livello globale delle nuove variant SARS-CoV-2, valutazione del rischio e misure di controllo. 0003787-31/01/2021-DGPRE-DGPRE-P.

8. Korber B, Fischer WM, Gnanakaran S, Yoon H, Theiler J, Abfalterer W, Hengartner N, Giorgi EE, Bhattacharya T, Foley B, Hastie KM, Parker MD, Partridge DG, Evans CM, Freeman TM, de Silva TI; Sheffield COVID-19 Genomics Group, McDanal C, Perez LG, Tang H, Moon-Walker A, Whelan SP, LaBranche CC, Saphire EO, Montefiori DC. Tracking Changes in SARS-CoV-2 Spike: Evidence that D614G Increases Infectivity of the COVID-19 Virus. Cell. 2020 Aug 20;182(4):812-827.e19. doi: 10.1016/j.cell.2020.06.043. Epub 2020 Jul 3. PMID: 32697968; PMCID: PMC7332439.

9. Lai A, Bergna A, Menzo S, Zehender G, Caucci S, Ghisetti V, Rizzo F, Maggi F, Cerutti F, Giurato G, Weisz A, Turchi C, Bruzzone B, Ceccherini Silberstein F, Clementi N, Callegaro A, Sagradi F, Francisci D, Venanzi Rullo E, Vicenti I, Clementi M, Galli M; collaborative group SCIRE SARS-CoV-2 Italian Research Enterprise.

Circulating SARS-CoV-2 variants in Italy, October 2020-March 2021. Virology Journal. 2021 Aug 14;18(1):168. doi: 10.1186/s12985-021-01638-5. PMID: 34391446; PMCID: PMC8364297.

10. Li A, Yung A, Tran C, Boulet M. SARS-CoV-2 Omicron BA.5: Riding the seventh wave in Central Canada. Influenza Other Respir Viruses. 2022 Aug 30. doi: 10.1111/irv.13046. Epub ahead of print. PMID: 36039608.

11. Lindstrøm JC, Engebretsen S, Kristoffersen AB, Rø GØI, Palomares AD, Engø-Monsen K, Madslien EH, Forland F, Nygård KM, Hagen F, Gantzel G, Wiklund O, Frigessi A, de Blasio BF. Increased transmissibility of the alpha SARS-CoV-2 variant: evidence from contact tracing data in Oslo, January to February 2021. Infect Dis (Lond). 2022 Jan;54(1):72-77. doi: 10.1080/23744235.2021.1977382. Epub 2021 Oct 7. PMID: 34618665.

12. Phan T, Boes S, McCullough M, Gribschaw J, Marsh JW, Harrison LH, Wells A. Emergence of SARS-CoV-2 Omicron BA.5 variant of concern in Western Pennsylvania, United States. J Med Virol. 2022 Oct;94(10):4593-4594. doi: 10.1002/jmv.27945. Epub 2022 Jun 23. PMID: 35715381; PMCID: PMC9349382.

13. Rambaut A, Loman N, Pybus O, Barclay W, Barrett J, al. e. Preliminary genomic characterisation of an emergent SARS-CoV-2 lineage in the UK defined by a novel set of spike mutations. https://virologicalorg/t/preliminary-genomic-characterisation-ofan-emergent-sars-cov-2-lineage-in-the-uk-defined-by-a-novelset-of-spike-mutations/563 2020;Accessed May 1, 2020.

14. Singh J, Rahman SA, Ehtesham NZ, Hira S, Hasnain SE. SARS-CoV-2 variants of concern are emerging in India. Nature Medicine. 2021;27(7):1131-1133.

15. Tallarita M, Giardina F, Novazzi F, Gaiarsa S, Batisti Biffignandi G, Paolucci S, Rovida F, Piralla A, Baldanti F. Spread of multiple SARS-CoV-2 lineages April-August 2020 anticipated the second pandemic wave in Lombardy (Italy). Pediatr Allergy Immunol. 2022 Jan;33 Suppl 27(Suppl 27):89-92. doi: 10.1111/pai.13641. PMID: 35080310; PMCID: PMC9542480.

16. Tegally H, Wilkinson E, Giovanetti M, Iranzadeh A, Fonseca V, Giandhari J, Doolabh D, Pillay S, San EJ, Msomi N, Mlisana K, von Gottberg A, Walaza S, Allam M, Ismail A, Mohale T, Glass AJ, Engelbrecht S, Van Zyl G, Preiser W, Petruccione F, Sigal A, Hardie D, Marais G, Hsiao NY, Korsman S, Davies MA, Tyers L, Mudau I, York D, Maslo C, Goedhals D, Abrahams S, Laguda-Akingba O, Alisoltani-Dehkordi A, Godzik A, Wibmer CK, Sewell BT, Lourenço J, Alcantara LCJ, Kosakovsky Pond SL, Weaver S, Martin D, Lessells RJ, Bhiman JN, Williamson C, de Oliveira T. Detection of a SARS-CoV-2 variant of concern in South Africa. Nature. 2021 Apr;592(7854):438-443. doi: 10.1038/s41586-021-03402-9. Epub 2021 Mar 9. PMID: 33690265.

17. Tegally H, Moir M, Everatt J, Giovanetti M, Scheepers C, Wilkinson E, Subramoney K, Makatini Z, Moyo S, Amoako DG, Baxter C, Althaus CL, Anyaneji UJ, Kekana D, Viana R, Giandhari J, Lessells RJ, Maponga T, Maruapula D, Choga W, Matshaba M, Mbulawa MB, Msomi N; NGS-SA consortium, Naidoo Y, Pillay S, Sanko TJ, San JE, Scott L, Singh L, Magini NA, Smith-Lawrence P, Stevens W, Dor G, Tshiabuila D, Wolter N, Preiser W, Treurnicht FK, Venter M, Chiloane G, McIntyre C, O'Toole A, Ruis C, Peacock TP, Roemer C, Kosakovsky Pond SL, Williamson C, Pybus OG, Bhiman JN, Glass A, Martin DP, Jackson B, Rambaut A, Laguda-Akingba O, Gaseitsiwe S, von Gottberg A, de Oliveira T. Emergence of SARS-CoV-2 Omicron lineages BA.4 and BA.5 in South Africa. Nat Med. 2022 Jun 27. doi: 10.1038/s41591-022-01911-2. Epub ahead of print. PMID: 35760080.

18. Tian D, Sun Y, Zhou J, Ye Q. The Global Epidemic of the SARS-CoV-2 Delta Variant, Key Spike Mutations and Immune Escape. Front Immunol. 2021 Nov 30;12:751778. doi: 10.3389/fimmu.2021.751778. PMID: 34917076; PMCID: PMC8669155.

19. Umair M, Ikram A, Salman M, Haider SA, Badar N, Rehman Z, Ammar M, Rana MS, Ali Q. Genomic surveillance reveals the detection of SARS-CoV-2 delta, beta, and gamma VOCs during the third wave in Pakistan. Journal of Medical Virology. 2021 Nov 2. doi: 10.1002/jmv.27429. Epub ahead of print. PMID: 34726786.

Supplementary materials

Table S1. Primers used for *spike* gene sequencing.

Primer name	Sequence (5'-3')
SARS-2-S-F3	TATCTTGGCAACCACGCGACAA
SARS-2-S-F4	CTACTTTAGATTCGAGACCCAGTCC
SARS-2-S-F5	GATGAGTCAGACAAATCGCTCCAGG
SARS-2-S-F6	TCAGGATGTTAACTGCACAGAAGTCC
SARS-2-S-F7	TGCCTTGGTGATATTGCTAGAGACC
SARS-2-S-F8	CAGCACCTCTGGTGTAGTCTTCTTGC
SARS-2-S-R3	ACCCTTGGAGAGTGCTAGTTGCCATCTC
SARS-2-S-R6	TTCTGCACCAAGTCACTGTGTAGGCA
SARS-2-S-R8	GCTGCAGCACCAGCTGTCCAACCTGA

Table S2. Success rate of Next-Generation Sequencing for each of the main variants.

SARS-CoV-2 variant	Number of samples analyzed by NGS	Number of samples sequenced by NGS	Success Rate
Early variants	704	670	95.1%
Alpha	187	177	94.6%
Delta	692	653	94.4%
Omicron	834	747	89.6%

Table S3. Aminoacidic changes of Spike protein identified in all Omicron lineages detected. Values reported are expressed as percentages.

	B.1.1.529 (n=9)	**BA.1 (n=303)**	**BA.2 (n=250)**	**BA.4 (n=14)**	**BA.5 (n=139)**	**BE.x (n=17)**	**BF.x (n=15)**
V3G				14.3			
L5F					7.9		6.7
P9L		0.3					
S13I		0.3					
T19I	55.6	0.3	69.2	85.7	96.4	94.1	86.7
T29I		0.7					
W64R			0.4				
W64S		0.33					
A67V	11.1	82.2					
I68T			0.8				
S71F			0.4				
D80Y		0.3					
P82L			0.4				
T95I	11.1	68.0					
N121D		0.3					
N121S		0.3					
N122D		0.3					
N122S		0.3					
T124A		0.3					
D138N					0.7		
G142D	55.6	0.3	84.0	100	96.4	94.1	93.3
M153I			0.4				
S172F		0.3					
K187R			0.4				

	B.1.1.529 (n=9)	**BA.1 (n=303)**	**BA.2 (n=250)**	**BA.4 (n=14)**	**BA.5 (n=139)**	**BE.x (n=17)**	**BF.x (n=15)**
Y200C					0.7		
P209L		0.3					
P209S						5.9	
V213G	11.1	0.3	40.4	42.9	39.6	35.3	33.3
D215E			1.2				
I231T		0.7					
T24OT		1.0					
L242F		0.3					
H245N			0.4				
S255F		0.3	0.8				
G261D					0.7		
A263E		0.3					
P272L		0.3			0.7		
A288V		0.7					
V289I			0.8		0.7	5.9	
T307S					0.7		
L335S							13.3
G339D	66.7	43.2	52.4	100	93.5	100	73.3
E340G		0.3					
E340K		0.3					
E340Q			0.4				
E340V		0.3					
R346I					0.7		
R346K		23.1					
R346T			0.4		0.7		

	B.1.1.529 (n=9)	**BA.1 (n=303)**	**BA.2 (n=250)**	**BA.4 (n=14)**	**BA.5 (n=139)**	**BE.x (n=17)**	**BF.x (n=15)**
Y369H		0.3					
S371F	33.3		59.2	78.6	66.9	76.5	46.7
S371L	11.1	53.8					
S371P		0.7					
S371Y			0.4				
S373P	44.4	55.1	60.4	78.6	67.6	76.5	46.7
S375F	44.4	54.8	60.4	78.6	67.6	82.4	46.7
S375Y		0.7					
T376A	33.3	0.3	60.4	78.6	67.6	82.4	46.7
R408S	33.3	0.3	51.2	92.9	84.2	94.1	20.0
K417N	22.2	14.5	42.0	92.9	78.4	94.1	20.0
K417T			0.4				
N440K	22.2	30.7	21.6		9.4	17.6	
V445A		0.3					
G446S	11.1	30.0					
Y449R					0.7		
N450K					0.7		
L455V					0.7		
K458L					0.7		
S459C					0.7		
S477N	22.2	53.8	46.4	28.6	37.4	52.9	20.0
T478K	22.2	53.8	46.8	28.6	35.3	52.9	20.0
E484A	22.2	37.0	37.2	28.6	26.6	52.9	13.3
F486V				28.6	26.6	52.9	13.3
Q493R	22.2	36.0	37.6				

	B.1.1.529 (n=9)	**BA.1 (n=303)**	**BA.2 (n=250)**	**BA.4 (n=14)**	**BA.5 (n=139)**	**BE.x (n=17)**	**BF.x (n=15)**
G496S	11.1	49.8					
Q498R	22.2	49.8	41.6	21.4	30.2	47.1	20.0
N501Y	22.2	50.2	42.0	21.4	31.7	47.1	20.0
Y505H	22.2	49.5	41.6	21.4	30.9	47.1	20.0
A520S			0.4				
N532D					0.7		
T547I					0.7		
T547K	11.1	94.1					
R567K		0.3					
Q580R		0.3				5.9	
D614G	66.7	94.1	90.8	92.9	99.3	100	100
H625R		0.3					
Q628K		0.7					
S640F				21.4			
H655Y	77.8	97.4	98.4	100	97.8	100	100
N658S				57.1			
Q677H			0.4				
N679K	88.9	83.5	87.6	100	99.3	100	100
P681H	88.9	61.7	97.2	100	99.3	100	100
A684V					0.7		
A701V		46.5		7.1	0.7		6.7
N703I		0.3					
S704L			2.4		0.7		
S735P		1.0					
T747I							33.3

	B.1.1.529 (n=9)	**BA.1 (n=303)**	**BA.2 (n=250)**	**BA.4 (n=14)**	**BA.5 (n=139)**	**BE.x (n=17)**	**BF.x (n=15)**
N764K	55.6	71.9	76.8	100	95.7	88.2	66.7
R765L					0.7		
T768N		0.3					
G769R		0.3					
G769V			0.4				
Q784L					0.7		
D796Y	44.4	61.7	58.8	92.9	96.4	100	46.7
P809S		0.3	0.4				
D843N		0.3					
A845S			0.4				
A845V					0.7		
A846S					0.7		
N856K	22.2	95.0					
T859I		0.3					
T883I			1.6				
N919T		0.3					
D936A		0.3					
D936Y		0.3					
S940F		0.3					
Q954H	88.9	98.0	98.0	100	100	100	100
L962P		0.3					
N969K	77.8	91.1	84.8	100	95.7	100	93.3
L981F	11.1	90.8					
V987F				7.1	1.4		
A1020S							40.0

	B.1.1.529 (n=9)	**BA.1 (n=303)**	**BA.2 (n=250)**	**BA.4 (n=14)**	**BA.5 (n=139)**	**BE.x (n=17)**	**BF.x (n=15)**
Q1071R					0.7		
I1081V		0.3					
K1086E						5.9	
A1087S					0.7		
F1089L		0.3					
F1103L		0.3					
N1108S		0.3					
G1124V		0.3					
E1144Q					0.7		
S1147L				7.1			
P1162L		1.0		7.1	0.7		
S1175P		0.3					
V1176A		0.3					
V1177A		0.3					
I1183V		0.3					
S1196P		0.3					
L1197P		0.3					
I1198F		0.3					
C1243F			0.4				
G11251V					2.2		
D1259N	11.1						
V1264L			0.8			5.9	
L1265F			0.8				

Table S4. Aminoacidic deletions of Spike protein identified in all Omicron lineages detected. Values reported are expressed as percentages.

	B.1.1.529 (n=9)	**BA.1 (n=303)**	**BA.2 (n=250)**	**BA.4 (n=14)**	**BA.5 (n=139)**	**BE.x (n=17)**	**BF.x (n=15)**
PLVSSQ9Q_del					0.7		
LPPA24S_del	55.5	0.3	53.2	85.7	92.1	94.1	73.3
LPF56F_del		0.3					
IHV68I_del	11.1	89.8	0.4	100	97.0	94.1	93.3
LGVY141Y_del					0.7		
GVYY142D_del	11.1	72.3					
VY143D_del		0.3					
YY144Y_del				7.1	2.2	11.8	
NL211I_del		39.9			0.7		

Chapter 5

SARS-CoV-2 infections in pediatric patients: a comparison of three pandemic waves

Francesca Rovida, Federica A.M. Giardina, Guglielmo Ferrari, Stefania Paolucci, Antonio Piralla, Fausto Baldanti

Role: Second author

Author's contribution: Methodology, writing, review and editing

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SARS-CoV-2 infections in pediatric patients: a comparison of three pandemic waves.

Francesca Rovida[1], Federica A.M. Giardina[1], Guglielmo Ferrari[1], Stefania Paolucci[1], Antonio Piralla[1], Fausto Baldanti[1,2].

[1] *Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy;*

[2] *Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia; Italy.*

ABSTRACT

The pediatric population appears to be at lower risk of developing severe clinical manifestations of COVID-19. However, the clinical and epidemiological characteristics of COVID-19 in children are not fully understood. This retrospective observational study aimed to evaluate the incidence of paediatric laboratory-confirmed COVID-19 from February 2020 to April 2021. A total of 740 (5.1% of the total) pediatric COVID-19 cases were observed during the study period. The peak of pediatric cases was observed in November 2020 with 239 cases. During the first wave of the pandemic, the frequency of pediatric cases was 0.89% (49/5877 cases), ranging from 0.6% in February 2020 to 1.3% in April 2020. In contrast, after the beginning of the second wave, the frequency of pediatric cases increased from 5.3% in September 2020 to 9.4% in February 2021, with an overall frequency of 8.2% (690/8416 cases). A different rate of SARS-CoV-2 circulation in the pediatric population was observed between the pandemic waves. Two peaks of cases were observed during the second wave. The last peak was associated with the spread of a more transmissible SARS-CoV-2 strain (VOC 202012/01).

Keywords: COVID-19; SARS-CoV-2; epidemiology; pandemic; pediatric infection.

1. INTRODUCTION

Since the first report of coronavirus disease 2019 (COVID-19) in China on 30 December 2019, SARS-CoV-2 has spread worldwide [1, 2]. By 12 April 2021, 135 million confirmed infections and nearly 3 million deaths had been reported worldwide [3]. At the beginning of the pandemic, Lombardy was the Italian region most affected by the SARS-CoV-2 virus [4]. In general, viral respiratory infections are mainly concentrated in the paediatric population. However, as observed during the first wave of the pandemic, the pediatric population was not primarily affected, and data on the actual burden of the

pandemic in the pediatric population are very limited. Compared to adults, there are relatively few studies on pediatric COVID-19 [5, 6, 7]. In particular, the clinical and epidemiological characteristics of COVID-19 in children aged 0-16 years are not yet fully understood. In the scenario of rapid global spread of SARS-CoV-2 infection, the increasing number of paediatric cases is expected. This retrospective analysis aimed to evaluate the frequency of paediatric infection among COVID-19 cases diagnosed in our centre since the beginning of the pandemic.

A total of 14353 COVID-19 cases were detected at our regional reference laboratory (Molecular Virology Unit, Fondazione IRCCS Policlinico San Matteo Pavia, Italy) between 20 February 2020 and 12 April 2021. The presence of SARS-CoV-2 RNA was assessed by specific real-time RT-PCR, as previously described [8]. Among the COVID-19 cases, 740 (5.1% of the total) were observed in patients aged <15 years, with 397 (53.6%) males and 343 (46.4%) females. As shown in Figure 1A, the peak of paediatric cases was observed in November 2020 with 239 cases. After December 2020, an overall decrease of cases was observed until a second peak in March 2021 with 138 cases. On the contrary, during the first wave of the pandemic (February - June 2020), the peak of pediatric cases was observed in March 2020 with 28 cases, corresponding to the month with the highest number of cases (n = 3960) observed in our hospital (Figure 1A). Between May 2020 and September 2020, less than 10 cases/month were observed, corresponding to the reduced circulation of SARS-CoV-2 in Lombardy. During the first wave of the pandemic, the frequency of pediatric cases was 0.89% (49/5877 cases), ranging from 0.6% in February 2020 to 1.3% in April 2020 (Figure 1B). In contrast, after the start of the second wave, the frequency of pediatric cases increased from 5.3% in September 2020 to 9.4% in February 2021, with an overall frequency of 8.2% (690/8416 cases) (Figure 1B).

Despite evidence of widespread circulation of other respiratory viruses (e.g. influenza and respiratory syncytial virus) in the pediatric population in our study, only 5% of COVID-19 cases were in patients <15 years of age. In addition, two different circulation rates were observed when comparing the first and second pandemic waves (<1% vs. 8.2%). This difference could be explained by the change in containment measures implemented during the March-May 2020 lockdown compared to the second wave (September 2020 - April 2021). As previously observed, most pediatric patients were asymptomatic and can spread the infection to their family members, some of whom develop severe symptoms [9]. In this context, it is therefore expected that the observed transmission rates would be different.

In our analysis, three peaks of cases were observed. The first, in March 2020, was supported by the uncontrolled circulation of SARS-CoV-2, which is likely to have been present in our region since January 2020 [10]. The second peak was observed during the second wave starting in September 2020, with a global increase in the number of newly reported cases in European and Eastern countries [3]. Finally, following the introduction

of SARS-CoV-2 variant of concern (VOC) 202012/01 (alpha, lineage B.1.1.7) in Italy in January 2020, a third peak of cases was observed in March 2021. The occurrence of a third peak was supported by the circulation of VOC 202012/01 associated with an increased transmission rate [11].

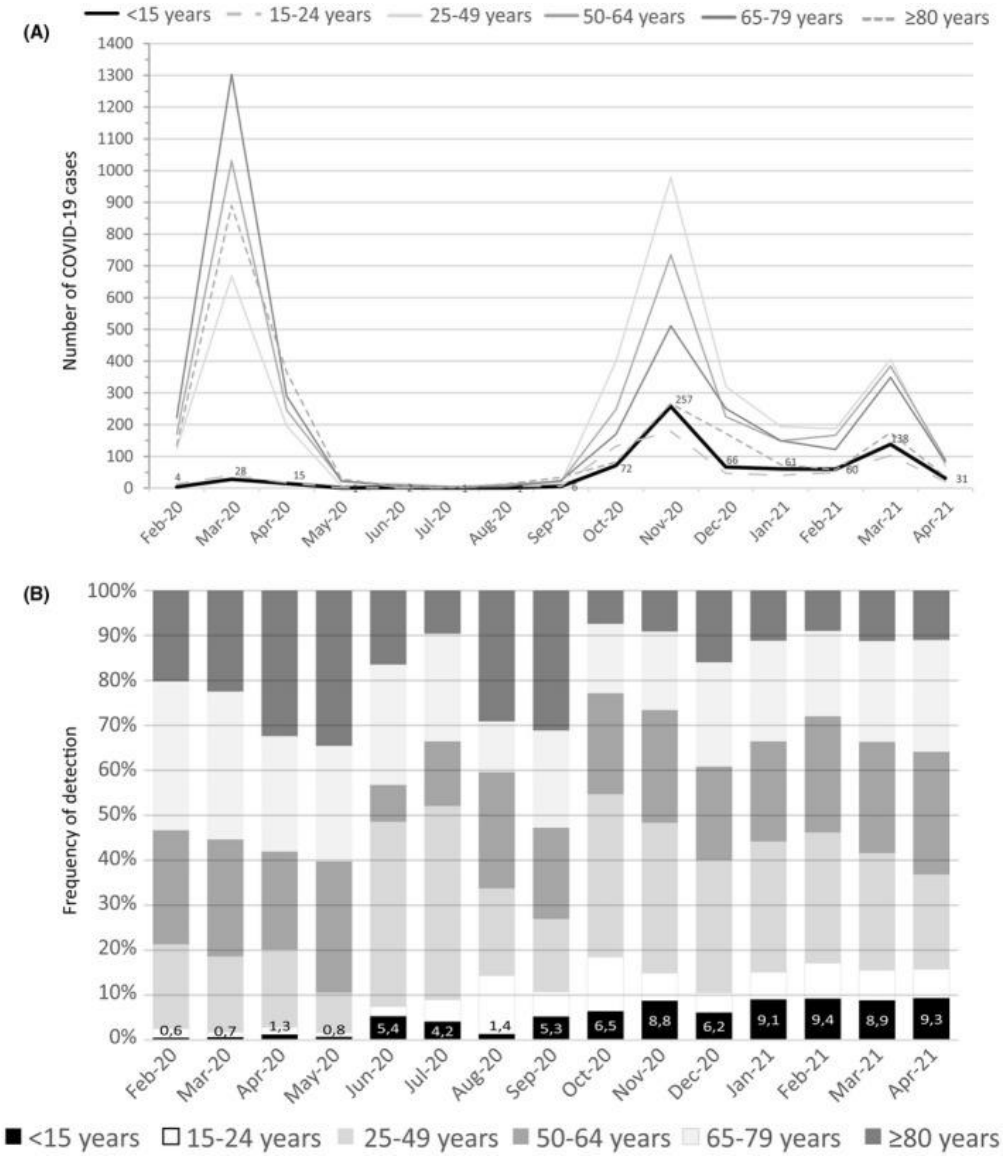

Figure 1. The number of COVID-19 cases according to age category between February 2020 and April 2021 (A). The number of pediatric cases (<15 years) is reported near the peak for each

month. Frequency of SARS-CoV-2 cases detection according to age category (B). The percentage of pediatric cases (<15 years) is reported within the bars for each month.

In conclusion, different rates of SARS-CoV-2 circulation were observed among the pediatric population between the pandemic waves. During the second wave, two peaks of cases were observed. The last peak was associated with the spread of a more transmissive SARS-CoV-2 strain (VOC 202012/01).

REFERENCES

1. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. Lancet Infect Dis. 2020;20:533-534.

2. Wu F, Zhao S, Yu B, et al. A new coronavirus associated with human respiratory disease in China. Nature. 2020;579:265-269.

3. WHO Coronavirus (COVID-19) Dashboard (Accessed 15th April 2021). https://covid19.who.int

4. Riccardo F, Ajelli M, Andrianou XD, et al. Epidemiological characteristics of COVID-19 cases and estimates of the reproductive numbers 1 month into the epidemic, Italy, 28th January to 31st March 2020. Euro Surveill. 2020;25:2000790.

5. Wei M, Yuan J, Liu Y, et al. Novel coronavirus infection in hospitalized infants under 1 year of age in China. JAMA. 2020;323:1313-1314.

6. Dong Y, Mo X, Hu Y, et al. Epidemiology of COVID-19 among children in China. Pediatrics. 2020;145:e20200702.

7. Ma H, Hu J, Tian J, et al. A single-center, retrospective study of COVID-19 features in children: a descriptive investigation. BMC Med. 2020;18:123.

8. Rovida F, Cereda D, Novati S, et al. Low risk for SARS-CoV2 symptomatic infection and early complications in paediatric patients during the ongoing CoVID19 epidemics in Lombardy. Clin Microbiol Infect. 2020;26:1569-1571.

9. Castagnoli R, Votto M, Licari A, et al. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in children and adolescents: a systematic review. JAMA Pediatr. 2020;174:882-889.

10. Alteri C, Cento V, Piralla A, et al. Genomic epidemiology of SARS-CoV-2 reveals multiple lineages and early spread of SARS-CoV-2 infections in Lombardy. Italy. Nat Commun. 2021;12:434.

11. Davies NG, Abbott S, Barnard RC, et al. Estimated transmissibility and impact of SARS-CoV-2 lineage B.1.1.7 in England. Science. 2021;372.

Chapter 6

Comparative analysis of SARS-CoV-2 quasispecies in the upper and lower tract respiratory tract shows an ongoing evolution in the spike cleavage site

Stefano Gaiarsa, Federica A.M. Giardina, Gherard Batisti Biffignandi, Guglielmo Ferrari, Aurora Piazza, Monica Tallarita, Federica Novazzi, Claudio Bandi, Stefania Paolucci, Francesca Rovida, Giulia Campanini, Antonio Piralla, Fausto Baldanti

Role: Co-first author

Author's contribution: Writing – original draft, Formal analysis, Investigation

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Comparative analysis os SARS-CoV-2 quasispecies in the upper and lower tract respiratory tract shows an ongoing evolution in the spike cleavage site.

Stefano Gaiarsa[a,1], Federica Giardina[a,1], Gherard Batisti Biffignandi[b,1], Guglielmo Ferrari[a], Aurora Piazza[b], Monica Tallarita[a], Federica Novazzi[a], Claudio Bandi[c], Stefania Paolucci[a], Francesca Rovida[a,b], Giulia Campanini[a], Antonio Piralla[a], Fausto Baldanti[a,b]

[a]Microbiology and Virology Department, Fondazione IRCCS, Policlinico San Matteo, via Taramelli 5, Pavia 27100, Italy;

[b]Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy;

[c]Department of Biosciences and Pediatric Clinical Research Center "Romeo and Enrica Invernizzi, University of Milan, Mila, Italy.

ABSTRACT

Further studies are required to better understand the genomic evolution of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The objective of this study was to describe the viral quasispecies population of the upper and lower respiratory tract in patients admitted to the intensive care unit. A deep sequencing of the S gene of SARS-CoV-2 was performed on 109 clinical specimens, collected from the upper respiratory tract (URT) and lower respiratory tract (LRT) of 77 patients. A higher incidence of non-synonymous mutations and insertions and deletions (indels) was observed in the lower respiratory tract (LRT) among minority variants. This phenomenon may be explained by the virus's capacity to invade cells without interacting with ACE2 (e.g., by exploiting macrophage phagocytosis). Minority variants are concentrated in the gene portion encoding the spike cleavage site, with a higher incidence in the URT. Four mutations are highly recurring among samples and were found to be associated with the URT. It is noteworthy that 55.8% of minority variants identified in this locus exhibited T>G and G>T transversions. The results of this study indicate the presence of selective pressure and suggest that an evolutionary process is still ongoing in one of the crucial sites of the spike protein associated with the spillover to humans.

Keywords: SARS-CoV-2, Spike protein, Deep sequencing, Minority variants, Deleterious mutations, Spillover.

1. INTRODUCTION

The initial report of the novel coronavirus, SARS-CoV-2, was made in China on 30 December 2019 [1, 2]. Since that time, the virus has spread globally. As of 8 March

2022, there have been 447 million confirmed infections and more than 6 million deaths have been reported worldwide [1] (https://ourworldindata.org/coronavirus). The origin of SARS-CoV-2 remains uncertain, with a hypothesis suggesting the Malayan pangolin (Manis javanica) as a potential intermediate host for the virus. Additionally, recombination signals between pangolin, bat, and human coronavirus sequences have been identified [2, 3, 4, 5]. Indeed, the SARS-CoV-2 genome sequence exhibited a high degree of genomic identity (approximately 96%) with the BatCoV-RaTG13 virus, as well as (approximately 88%) with two additional SARS-like bat viruses (Bat-SL-CoV-ZC45 and Bat-SL-CoV-ZXC21) [6]. In contrast, a comparison of the SARS-CoV genome sequences revealed an overall lower identity, approximately 79.6% [6, 7]. Analyses focusing on the Spike (S) protein yielded similar identity scores (approximately 75%) [6, 7]. The S protein is the primary determinant of viral tropism and is responsible for receptor binding and membrane fusion [8]. Consequently, amino acid alterations to this protein may influence infectivity, viral pathogenesis, and transmissibility. Initially, it was reported that the emergence and subsequent dominance of the mutation D614G in the S protein might have resulted in an enhancement of viral replication and viral fitness [9]. The monitoring of emerging mutations, in particular those in the S protein, has been conducted extensively since the establishment of the Virus Evolution Expert Working Group (VEWG) by the WHO (World Health Organization, 2021). The significant efforts made in this area are also reflected in the large number of SARS-CoV-2 sequences that have been submitted to public repositories, such as GISAID. Initial reports indicated a limited degree of viral diversity for SARS-CoV-2 [10, 11.]However, since December 2020, a process of positive selection has been documented for a series of variants of concern (VOCs), including alpha, delta and omicron, which have been shown to exhibit increased transmission rates [12, 13, 14]. These VOCs have been demonstrated to have a significant impact on public health, with changes in the virus transmissibility and a reduction in the efficacy of vaccines [12, 13]. The concept of quasispecies has been proposed as a strategy of virus evolution [15]. Despite the gradual clarification of the viral kinetics of SARS-CoV-2 infection from the upper respiratory tract (URT) to the lower respiratory tract (LRT), further studies are required to explore the inter-host and intra-host variations of SARS-CoV-2. In general, studies investigating intra-host evolution or the dynamic of SARS-CoV-2 quasispecies have been primarily focused on samples collected from the upper respiratory tract (URT) [16, 17, 18, 19, 20, 21, 22]. Indeed, the dynamics of the SARS-CoV-2 population in the lower respiratory tract (LRT) of patients presenting with severe acute respiratory infections (SARIs) remain poorly investigated. It would be important to elucidate the role of specific mutations in the progression of SARS-CoV-2 from the upper to the lower respiratory tract or to identify specific mutational patterns associated with severe infections. In the present study, high-depth next-generation sequencing (NGS) of the S gene was performed on a set of 109 respiratory samples from the upper respiratory tract (URT; n = 58) and lower respiratory tract (LRT; n = 51) in order to: (i) evaluate the genetic diversity in two different body compartments; and (ii) identify minority variants

potentially associated with progression from the upper to the lower respiratory tract in paired samples from patients admitted to the intensive care unit (ICU) with severe infection.

2. MATERIAL AND METHODS

2.1 Patients and Samples

A total of 109 clinical specimens were collected and analysed from 77 patients diagnosed with COVID-19. These specimens were obtained from the upper respiratory tract (nasopharyngeal swabs; NPS) and the lower respiratory tract (bronchoalveolar lavage; BAL or broncho aspirate; Brasp) (Appendix Table S1). Respiratory samples were collected prospectively from patients admitted to the ICU with severe to critical COVID-19 disease and from patients with mild symptoms who did not require hospitalization, in accordance with the WHO clinical management of COVID-19 guide [23]. Among patients admitted to the ICU with severe to critical COVID-19 infection, whenever feasible, paired URT and LRT samples were collected. All specimens were collected between late February 2020 and January 2021 at the Microbiology and Virology Department of Fondazione IRCCS Policlinico San Matteo in Pavia, which served as the Regional Reference Laboratory for the diagnosis of SARS-CoV-2 infection. The presence of SARS-CoV-2 RNA in respiratory specimens was assessed by a specific real-time reverse transcriptase–polymerase chain reaction (RT-PCR) targeting the RNA-dependent RNA polymerase and E genes, in accordance with the World Health Organization (WHO) guidelines and the protocols proposed by Corman and colleagues [24, 25, 26]. Quantification cycle (Cq) values were employed as a semiquantitative measure of SARS-CoV-2 viral load in accordance with the guidelines set forth by MIQE [27]. The investigation of patient samples at the genetic level was approved by the Ethics Committee of our institution (P_20200085574).

2.2 S gene amplification and sequencing

Total RNA was extracted using the QIAamp Viral RNA Mini Kit in accordance with the manufacturer's instructions, with a starting volume of 400 µL and elution in a final volume of 60 µL. The extracted RNA was subjected to a one-step reverse transcription polymerase chain reaction (RT-PCR) using the SuperScript IV One-Step RT-PCR System (Thermo Fisher Scientific, USA). Two distinct strategies were employed: a "long PCR" approach for the amplification of the entire S gene (approximately 4000 bp) utilising the primer pairs SARS-2-S-F3 (tatcttggcaaaccacgcgaacaa) and SARS-2-S-R3 (acccttggagagtgctagttgccatctc), or alternatively, a semi-nested approach with the following two primer pairs: the initial step involved the use of SARS-2-S-F3 and SARS-2-R6 (ttctgcaccaagtgacatagtgtaggca), followed by a second step utilising SARS-2-F6 (tcaggatgttaactgcacagaagtcc) and SARS-2-S-R3 (see Appendix Table S2 for a complete list of primers and their respective positions). The thermal profile for the retro transcription was 55 °C for 10 min, followed by the "long PCR" with an initial

denaturation/RT inactivation step at 98 °C for 2 min, 42 cycles at 98 °C for 10 sec, the 60 °C for 10 sec and 72 °C for 3 min; final extension was at 72 °C for 5 min. The semi-nested polymerase chain reaction (PCR) was conducted using the Platinum SuperFi DNA Polymerase with 5 µl of the initial DNA sample and the following thermal profile: initial denaturation at 98 °C for 10 sec, followed by annealing at 60 °C for 10 seconds and extension at 72 °C for 2 minutes and 30 seconds. The amplified DNA was purified using AMPure Beads, with elution in TE buffer. The enriched DNA samples were subjected to library preparation using the Nextera XT kit. Sequencing was conducted on an Illumina MiSeq machine, with the objective of obtaining approximately one million 250-base-pair paired-end reads per sample.

2.3 In silico analysis of sequences

The quality of the sequencing reads was assessed using the FastQC program (www.bioinformatics.babraham.ac.uk/projects/fastqc/). The reads in each sample were subjected to quality filtering and trimming using the fastp tool [28]. Furthermore, 28 bases were excised from the 5' and 3' ends of all reads, in order to eliminate sequences derived from the semi-nested PCR primers. The filtered reads were then aligned to the S gene of the Wuhan-hu-1 reference genome (NC_045512.2) [2] using bowtie2 [29]. The haplotype sequences for each sample were obtained from the alignment SAM files using the software CliqueSNV [30]. The hedgehog algorithm was employed to classify the SARS-CoV-2 strains based on the most abundant haplotype of each sample, utilising only the S protein sequences [31]. In parallel, the alignment data was processed with samtools [32] and bam-readcount (https://github.com/genome/bam-readcount) in order to calculate the number of occurrences of each nucleotide and indel in all positions of the reference sequence. Only those nucleotides and indels with a prevalence of at least 1% were considered in the subsequent analyses. A Python and R scripting analysis was employed to extract and classify all mutations, with the following algorithm being used:
(a) for each position in all samples, the nucleotide or indel with the highest prevalence was designated as the "majority variant."
(b) all other bases with at least 1% prevalence were designated as "minority variants" (MVs).
(c) the correlation between the presence of MVs and the respiratory tract district of sampling was tested for all positions of the gene using the Fisher exact test ($p < 0.05$).
(d) the number of MVs in each sample was counted and classified according to mutation type (synonymous, non-synonymous or indel), gene sub-domain [33], and mutation pattern (from which majority base to which low prevalence base). The differential distribution of the number of MVs between URT and LRT samples was tested using the Wilcoxon rank sum test ($p < 0.05$). The test was repeated for each class, with each count weighted by the total number of MVs in the sample. This enabled us to ascertain their association with LRT or URT (Fisher exact test) and to quantify the gene variability sampled in this study. The sequencing reads are accessible via the SRA database under

the BioProject ID PRJNA686083. The scripts generated to perform this project are available on GitHub at https://github.com/SteMIDIfactory/DeepSpike.

2.4 Statistical analysis

Comparisons of Cq, number of minority variants and haplotypes were performed using the Mann-Whitney test for continuous unpaired variables and the Wilcoxon rank sum test for continuous paired variables in respiratory samples. The correlation between two quantitative variables was determined by means of the Spearman correlation test. The Fisher's exact test for categorical variables was employed for the analysis of mutation frequencies between groups of patients. Descriptive statistics and linear regression lines were generated using GraphPad Prism software (version 8.3.0).

3. RESULTS

3.1 Patients population

A total of 77 patients were included in the study. Out of the 77 patients included in the study, 55 (71.4%) presented with severe symptoms and were admitted to the ICU, while 22 (28.6%) had mild infections that did not require hospitalization. Among the 55 patients admitted to the ICU, 28 (50.9%) had paired URT and LRT samples available for analysis. In 19 cases (34.5%), only LRT samples were analysed, with two patients having two serially collected BAL samples and one patient having three serially collected BAL samples. Finally, in 8 cases (14.6%), only URT samples were available. Among the 28 patients with paired URT and LRT samples, the LRT sample was collected at the same time for the majority of paired samples (with a range of -4 to 9 days between samples). Of the 22 patients with mild disease, only URT samples were collected and analysed.

3.2 Database description

A total of 109 samples were included in the NGS analyses, of which 58 (53.2%) were collected from the upper respiratory tract (URT) and 51 (46.8%) from the lower respiratory tract (LRT). The DNA of the S gene was enriched using PCR methods, and deep short-read sequencing was performed. A total of 69,279,628 reads were obtained from sequencing, with an average of 6,355,930 reads for each sample (range 562,140–33,460,360). The reads were then mapped to the SARS-CoV-2 reference strain, with an average depth of 6923x (range 1301x-7954x) being obtained. The mapping of reads enabled the extraction, classification, and enumeration of all genomic variants present in the samples with a prevalence of at least 1% of the sequencing depth (equivalent to approximately 10x depth in the samples with the lowest read yield). The hedgehog algorithm was employed to classify the SARS-CoV-2 strains based on the S protein sequences. The majority of SARS-CoV-2 strains included in this study belonged to the A_1 lineage, which exhibited the D614G mutation in 84 of the 109 strains (77.1%). A total of 13 strains (11.9%) belonged to the B.1.177_1 lineage, which is characterised by

the amino acid substitutions A222V and D614G. Seven strains (6.4%) were classified as alpha VOC, which is a variant of concern. This lineage is defined by the presence of deletion del69-70 and amino acid substitutions N501Y, A570D, D614G, P681H, T716I, S982A, and D1118H. Four strains (3.7%) were classified as B.1_14 (D614G, D839Y) and 1 (0.9%) strains belong to lineage B.1.177.52_1 (A222V, D614G, P1162R), as indicated in Supplementary Material Table S2.

3.3 Viral load and correlation between Cq and intra-host variability

The median viral load, as measured by the Cq value, was observed to be similar in URT samples (23.8; range 13.0–36.0) and LRT samples (22.6; range 12.0–34.9; p = 0.12) (Fig. 1A). Similarly, no difference in the median Cq value was observed among the 28 paired samples (p = 0.83). In order to describe the variability in the viral load on paired samples, a correlation analysis and a plot of the difference in Cq value between paired URT and LRT samples were performed. In 13/28 (46.4%) paired samples, the Ct value was lower in URT (higher viral load) as compared to LRT samples, with a median ΔCq of 2.8 (range 0.04–11.0 ΔCq) (pink circles on Fig. 1. In contrast, in 15/28 (53.6%) paired samples, the Ct value was lower (higher viral load) in LRT samples as compared to URT samples, with a median ΔCq of 4.7 (range 0.1 to 9.3 ΔCq) (light blue circles on Fig. 1B). Furthermore, an overall correlation was observed among the paired samples (rho=0.74, Fig. 1C).

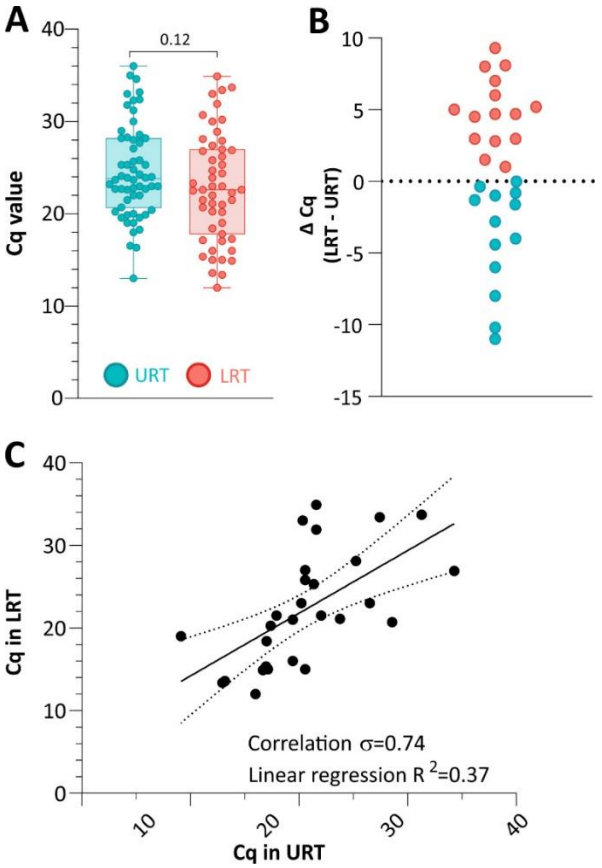

Figure 1. (A) Distribution of Cq in the URT and LRT samples. (B, C) Differences and correlation plots for Cq values in 28 paired URT and LRT samples. The statistic Spearman's correlation coefficient and linear regression R2 value are also reported.

In general, viral replication has been associated with the diversification of viral populations within hosts. For this reason, we compared the Cq values, which represent the viral load, with the number of MVs and haplotypes observed. No evidence of a correlation between Cq and the number of MVs was observed in both URT (σ=0.16) and LRT samples (σ = 0.11) (Fig. 2 A). Similar findings were observed in the comparison of Cq and the number of haplotypes in URT samples (σ = 0.01) as well as in the LRT (σ = -0.003) (Fig. 2B).

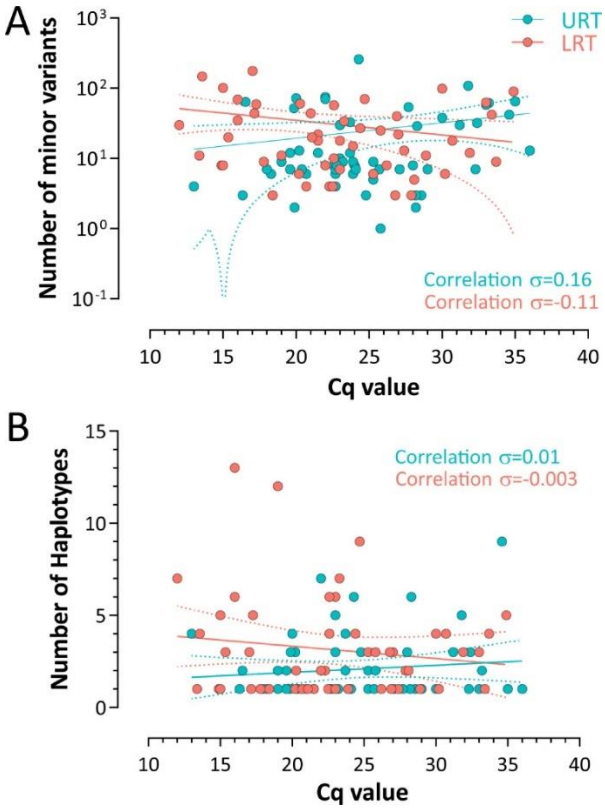

Figure 2. Comparison of number of haplotypes and minority variants in upper vs lower respiratory tract samples. (A) Correlation between the number of minority variants and viral load expressed in cycle of quantification (Cq). (B) Correlation between the number of haplotypes and viral load expressed in Cq.

3.4 Haplotypes and minority variants counts

The number of MVs is marginally higher in LRT samples (median 13.5; range 3–99) than in URT samples (median 8; range 0–263), although this difference is not statistically significant ($p = 0.07$, Fig. 3A). Conversely, the number of haplotypes identified was significantly greater in LRT samples (median 2; range 1–13) than in URT samples (median 1; range 1–9; $p = 0.02$, Fig. 3B). The ratio of non-synonymous (dN) to synonymous (dS) substitutions (dN/dS) was calculated. The median value observed in LRT samples (median 2.65, range 0–11) was greater than those observed in UTR samples (median 1.81, range 0–8; $p = 0.02$), indicating a higher positive selective pressure in the lung environment (Fig. 3C).

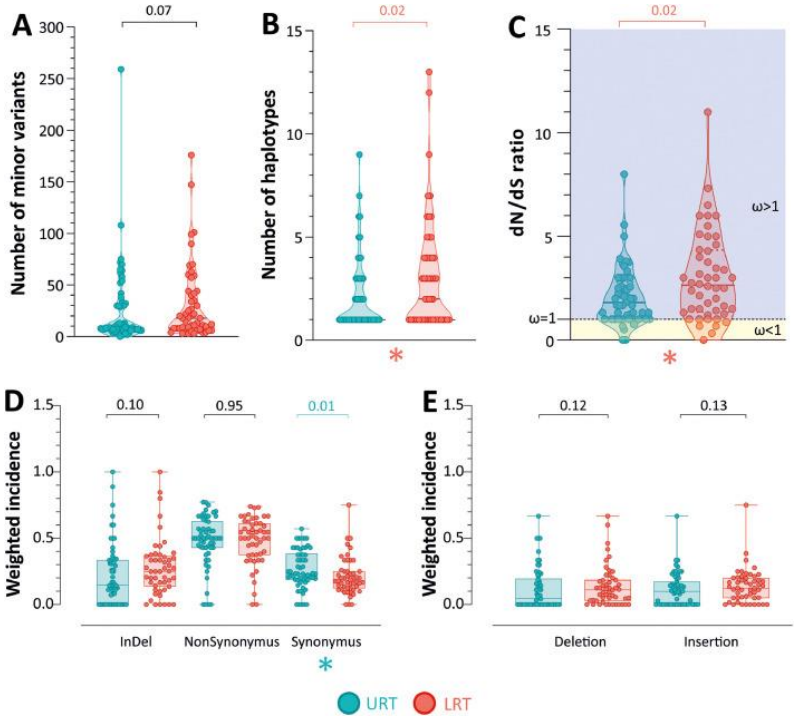

Figure 3. Distribution of the number of (A) minority variants, (B) haplotypes and (C) dN/dS ratio in the URT and LRT samples. Values are represented as a boxplot with all points inscribed. (D) Distribution of the weighted incidence of synonymous, non-synonymous, and insertions and deletions (Indel) in the URT and the LRT samples. (E) Distribution of the weighted incidence of frameshifting insertions and deletions in the URT and the LRT. Values are weighted by dividing them by the total number of minority variants in the sample.

A more detailed analysis revealed that the weighted incidence of synonymous mutations was higher in the URT samples than in the LRT samples ($p = 0.01$, Fig. 3D). However, although not statistically significant, a greater number of indels was observed in the LRT samples than in the URT samples ($p = 0.10$, Fig. 3D). Finally, no significant difference was observed in the number of non-synonymous mutations. The analysis was repeated with only deletions and frameshift insertions, which demonstrated a greater incidence in the LRT, though not to a statistically significant degree ($p = 0.12$ and $p = 0.13$, Fig. 3E). The weighted incidence of MVs was calculated for all regions of the gene corresponding to the functional and structural domains of the protein. The analysis revealed that MVs in the N-terminal Domain (NTD) were more prevalent in the LRT ($p < 0.001$). Conversely, mutations in the fusion peptide (FP, $p = 0.006$) and in subdomain 2 (SD2, $p < 0.001$) are more prevalent in the upper respiratory tract (URT) (Fig. 4A). Furthermore, MVs in the region coding for the protein subunit S1 are more common in the LRT, while in subunit S2 we observed a significantly higher abundance in the URT (Fig. 4B). This result is to be expected, as it reflects the values observed in the functional domains.

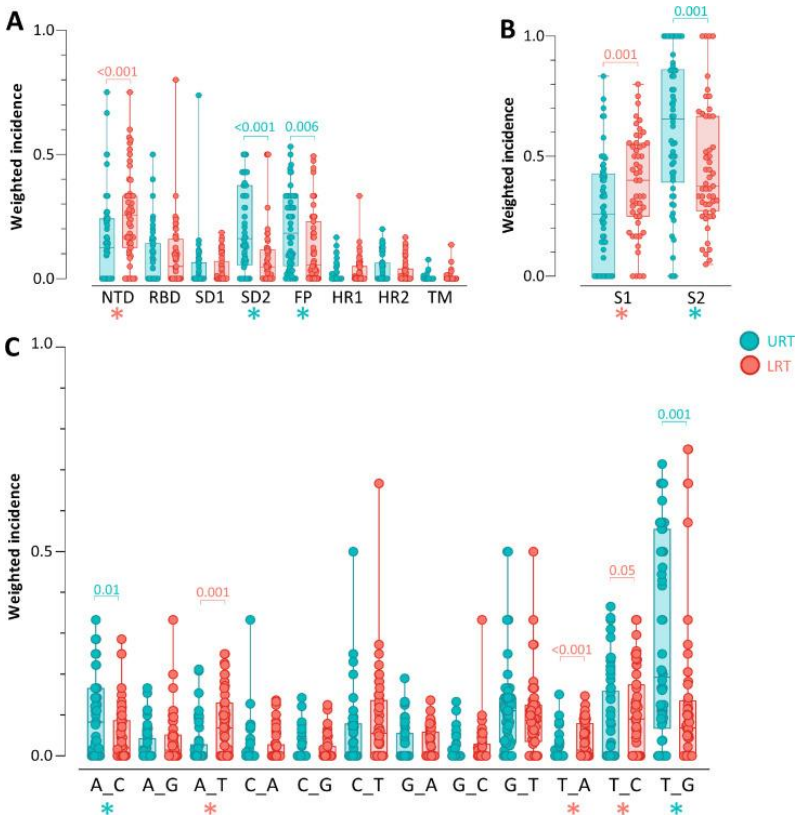

Figure 4. (A) Distribution of the weighted incidence of minority variants in the S gene subdomains (NTD: N-Terminal Domain; RBD: Receptor-Binding Domain; SD1: Structural Domain 1; SD2: Structural Domain 2; FP: Fusion Peptide; HR1: Heptad Repeat 1; HR2: Heptad Repeat 2; TM: TransMembrane domain) in the URT and LRT samples. (B) Distribution of the weighted incidence of minority variants in the two S gene subunits in the URT and the LRT (S1: Subunit 1; S2: Subunit 2). (C) Distribution in the URT and in the LRT of the weighted incidence of minority variants, classified by mutation patterns. Values are weighted by dividing them by the total number of minority variants in the sample.

The incidence of mutation patterns was also evaluated, both in terms of the total number of events and in relation to the total number of MVs observed in each sample. Figure 4C illustrates the weighted incidence of each mutation pattern in both respiratory tract compartments, whereas Appendix Figure S3 depicts the absolute incidence. Both analyses demonstrate a higher prevalence of A>C and T>G mutations in the MVs of URT samples, while A>T, T>A, and T>C have a higher incidence in the LRT. Finally, we examined the correlation between the presence of MVs (binary value) and the two respiratory tract districts (illustrated in Fig. 5A). Figure 5B illustrates the prevalence of MVs along the gene. The distribution of mutations is uniform across the sequence, with the exception of the region surrounding the cleavage site between the two subunits of the gene. The mutation sites are highly concentrated in both the upper and lower

respiratory tract samples, with a greater concentration observed in the former. This is consistent with the findings in Fig. 5A. Furthermore, the presence of mutations is associated with the respiratory district in nine codons, five of which are correlated with URT, and four with LRT (see Table 1). Four of the MVs associated with URT are located in the vicinity of the cleavage site between the two subunits. In this region, a high concentration of MV transversions between T and G was observed, with 248 out of 643 (38.6%) mutations being T>G and 11 (17.3%) being G>T (total = 359; 55.8%).

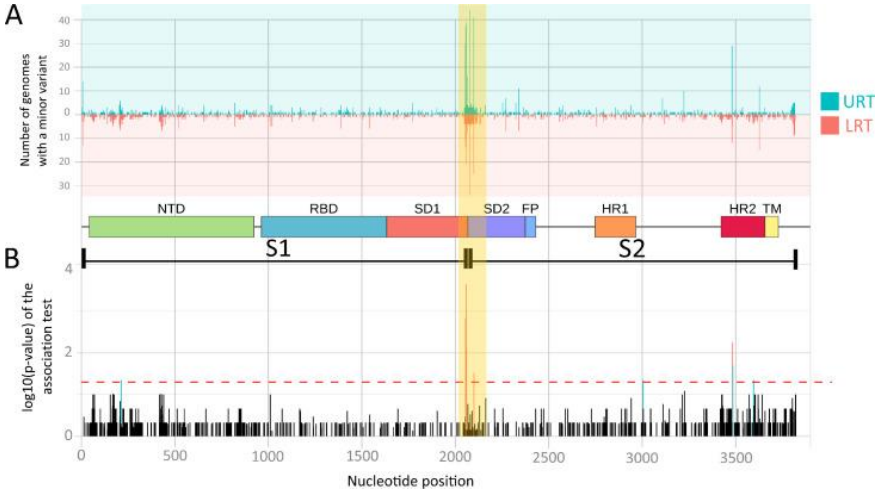

Figure 5. Graphical distribution of changes along S protein gene. (A) Number of samples containing minority variants in each position. Two separate histograms are used for URT and LRT samples, which are indicated upside down for image clarity. (B) Correlation of the presence of minority variants with URT and LRT in each position of the gene. Bar height represents the log10 (p-value) of the Fisher exact test. In the middle, a scheme of the gene subdomains and subunits is used as separator. NTD: N-Terminal Domain; RBD: Receptor-Binding Domain; SD1: Structural Domain 1; SD2: Structural Domain 2; FP: Fusion Peptide; HR1: Heptad Repeat 1; HR2: Heptad Repeat 2; TM: TransMembrane domain; S1: Subunit 1; S2: Subunit 2.

Codon position	Amino acid	Reference	Mutation	Mutation type	Associated with
212	71	C(S)	A(Y)/T(F)	NotSyn	LRT
2055	685	T(R)	G(R)	Syn	URT
2058	686	T(S)	G(R)	NotSyn	URT
2060	687	T(V)	G(G)	NotSyn	URT
2100	700	T(G)	G(G)	Syn	URT
3005	1002	A(Q)	T(L) / -	NotSyn/Del	LRT
3483	1161	A(S)	C(S)	Syn	URT
3485	1162	C(P)	T(L)/G(R)	NotSyn	LRT
3596	1199	A (D)	G(G)/T(G)	NotSyn	LRT

Table 1. Description of the 9 Minority Variants (MVs) positions associated with either URT or LRT compared to the reference sequence (NC_045512.2). Global frequency is referred to the frequency of the mutation in the same amino acid in GISAID global database. Data are accessible at www.cov.lanl.gov.

4. DISCUSSION

The evolution of SARS-CoV-2 was initially relatively slow in comparison to other RNA viruses [34]. Nevertheless, its rapid global dissemination has permitted the documentation of thousands of mutations in public databases. Some of these mutations have been beneficial and have emerged worldwide [35]. The emergence of VOCs was facilitated by over 400 million infections worldwide [36], with a greater number of mutations observed in S sequences in comparison to other genomic regions [37]. In general, mutations in viral structural proteins, such as the S glycoprotein, can play a crucial role in the virulence of the virus. This is because they may determine changes in the virus's cellular tropism and the generation of antibody escape variants. This has been observed in the Delta and Omicron variants [38, 39, 40]. The emergence of these variants has been facilitated by the evolution of the viral quasispecies and the severity of SARS-CoV-2 infection is driven by progression from the upper respiratory tract (URT) to the lower respiratory tract (LRT) [41]. In this context, our study has investigated the genetic diversity in SARS-CoV-2 quasispecies, focusing on structural S protein sequences in two body compartments, with the objective of: (i) to evaluate the genetic diversity in two different body compartments; (ii) to identify minority variants potentially associated with progression from the upper to the lower respiratory tract on paired samples from patients admitted to the intensive care unit with severe infection.

Since April 2020, the G614 variant of the SARS-CoV-2 virus has become the most prevalent worldwide, exhibiting an enhanced fitness advantage [42]. This finding was also confirmed by other studies that compared the D614 and G614 variants and found

that the G614 variant was associated with increased replication in human lung epithelial cells [9]. Conversely, the G614 variant was not found to be associated with an increased disease severity, and its role in pathogenesis remains to be elucidated [9, 35]. More than 60% of patients included in this study had severe infections developing severe pneumonia and requiring oxygen therapy. The presence of mutations associated with these symptoms was investigated by obtaining sequences from the LRT samples. All SARS-CoV-2 sequences generated in this study exhibited the G614 variant, whereas the original D614 variant was not identified among either the majority or minority variants. Consequently, no evidence was found to suggest that the G614 variant is associated with a more severe presentation. Furthermore, our analysis did not reveal any evidence of mutations on the S gene associated with the progression from the upper to the lower respiratory tract. This result was also observed in a series of paired samples and is consistent with the finding previously reported by Rueca et al. in a smaller number of patients [43]. Our data are consistent with the findings of Wylezich and colleagues, who reported no evidence of compartment-specific patterns of mutations between different respiratory compartments [44]. This finding suggests that disease severity may be primarily determined by host factors such as comorbidities, age, and the absence of pre-existing immunity [16]. Furthermore, deletions have been observed in a few samples as MVs, although at a lower frequency than that reported in a recent publication [45].

In general, the S gene sequences obtained in this study exhibited a greater degree of variability (number of haplotypes) in LRT samples when compared to URT samples. This discrepancy was not attributable to differences in viral load, as comparable Ct values were observed in URT and LRT samples. Indeed, no correlation was observed between viral load and viral diversity, which is consistent with the findings previously reported by Siqueira and colleagues, who investigated quasispecies variation in cancer patients [20]. The discrepancy in viral population between URT and LRT can be attributed to the hypothesis of independent replication in the two respiratory districts, which is also supported by the findings of Wölfel et al. (2020) [46]. Furthermore, a higher selective pressure for positive mutations has been observed in the lung environment in comparison to the upper respiratory tract (dN/dS>1). A similar observation was reported by Sun et al., who suggested that the diversification of quasispecies mutants indicated the potential for independent virus replication in different tissues or organs [21]. The considerable variability observed in the LRT samples also resulted in an increased number of frameshifting deletions and insertions. The presence of deleterious mutations could indicate a loss of function of the S protein in a fraction of the viral population. This subpopulation may be maintained through replication and cell invasion events that do not involve the ACE2 receptor (e.g., within syncytia or in macrophages after phagocytosis) [47]. Nevertheless, further studies may be required to elucidate and investigate these theories in greater depth.

Nucleotide changes arise during the replication and persistence of viruses, with A>G being a notable example. These have been shown to be related to host editing mechanisms such as APOBEC and ADARs [48, 49]. The A>G transition is caused by

deamination from adenosine to inosine (A>I), which is generated by ADARs. Consequently, the considerable prevalence of T>C observed in the LRT, as reported by Di Giorgio et al. (2020) and in this study, is consistent with the hypothesis that T>C in SARS-CoV-2 may also be associated with the ADARs mechanism. Although not associated with the host editing mechanism, the T>G pattern in MVs is of particular interest in this study, as they are the most prevalent pattern observed in the entire dataset and were found to be associated with the URT.

Finally, the cleavage site between S1 and S2 of the S protein corresponds to one of the two genetic sites in which Andersen and colleagues identified crucial mutations associated with the spillover of the virus to humans [50]. For this reason, it can be hypothesised that this site is subject to an evolutionary selective pressure, with the driving force of this evolution residing in the URT. Modifications (mutations or deletions) in the S1/S2 junction site have been associated with virus attenuation in hamsters [51]. An alternative explanation for these observations is that there is no negative selection on random mutations. This is because the cleavage site is a highly conserved region of the protein, and therefore SNPs have a minimal effect on the protein structure. This hypothesis provides an explanation for the high density of mutations observed in the cleavage site (S1/S2 junction) in both URT and LRT samples. However, this does not account for the higher prevalence in the URT and the presence of associated mutations. Moreover, other studies have identified specific low-frequency mutations nearby the cleavage site, including deletions that have been associated with milder symptoms [45, 52]. In contrast, the majority of mutations observed in our study were T>G and G>T transversions in this site (359/643 MVs). Given that such mutations are typically rare changes in nucleic acids, this observation serves to reinforce the notion of selective pressure. It is noteworthy that the T>G pattern has also been identified as an inexplicable intra-host mutational signature in HPV [53].

In conclusion, the results of the present study indicate that severe SARS-CoV-2 infections are not associated with a specific mutational pattern. Nevertheless, a considerable degree of variability was observed in the viral population of the lower respiratory tract, which was also associated with a positive selective pressure. The impact of this difference on immune response escape, tissue tropism and pathogenicity remains to be elucidated. Furthermore, evidence of potential ongoing evolution was observed in one of the gene loci that were crucial for the spillover to humans. This underscores the necessity of genomic surveillance to anticipate and forestall the emergence of vaccine-resistant mutants.

REFERENCES

1. Dong E., Du H., Gardner L. An interactive web-based dashboard to track COVID-19 in real time. Lancet. Infect. Dis. 2020;20(5):533–534. doi: 10.1016/S1473-3099(20)30120-1.

2. Wu F., Zhao S., Yu B., Chen Y.M., Wang W., Song Z.G., Hu Y., Tao Z.W., Tian J.H., Pei Y.Y., Yuan M.L., Zhang Y.L., Dai F.H., Liu Y., Wang Q.M., Zheng J.J.,

Xu L., Holmes E.C., Zhang Y.Z. A new coronavirus associated with human respiratory disease in China. Nature. 2020;579(7798):265–269. doi: 10.1038/s41586-020-2008-3.
3. Lam T.T., Jia N., Zhang Y.W., Shum M.H., Jiang J.F., Zhu H.C., Tong Y.G., Shi Y.X., Ni X.B., Liao Y.S., Li W.J., Jiang B.G., Wei W., Yuan T.T., Zheng K., Cui X.M., Li J., Pei G.Q., Qiang X., Cheung W.Y., Li L., Sun F., Qin S., Huang J., Leung G.M., Holmes E.C., Hu Y., Guan Y., Cao W.C. Identifying SARS-CoV-2-related coronaviruses in Malayan pangolins. Nature. 2020;583(7815):282–285. doi: 10.1038/s41586-020-2169-0.
4. Wong M.C., Javornik Cregeen S.J., Ajami N.J., Petrosino J.F. Evidence of recombination in coronaviruses implicating pangolin origins of nCoV-2019. bioRxiv Prepr. Serv. Biol. 2020 doi: 10.1101/2020.02.07.939207. 2020.02.07.939207.
5. Xiao K., Zhai J., Feng Y., Zhou N., Zhang X., Zou J.J., Li N., Guo Y., Li X., Shen X., Zhang Z., Shu F., Huang W., Li Y., Zhang Z., Chen R.A., Wu Y.J., Peng S.M., Huang M., Xie W.J., Cai Q.H., Hou F.H., Chen W., Xiao L., Shen Y. Isolation of SARS-CoV-2-related coronavirus from Malayan pangolins. Nature. 2020;583(7815):286–289. doi: 10.1038/s41586-020-2313-x.
6. Zhou P., Yang X.L., Wang X.G., Hu B., Zhang L., Zhang W., Si H.R., Zhu Y., Li B., Huang C.L., Chen H.D., Chen J., Luo Y., Guo H., Jiang R.D., Liu M.Q., Chen Y., Shen X.R., Wang X., Zheng X.S., Zhao K., Chen Q.J., Deng F., Liu L.L., Yan B., Zhan F.X., Wangm Y.Y., Xiao G.F., Shi Z.L. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature. 2020;579(7798):270–273. doi: 10.1038/s41586-020-2012-7.
7. Gralinski L.E., Menachery V.D. Return of the coronavirus: 2019-nCoV. Viruses. 2020;12(2):135. doi: 10.3390/v12020135.
8. Belouzard S., Millet J.K., Licitra B.N., Whittaker G.R. Mechanisms of coronavirus cell entry mediated by the viral spike protein. Viruses. 2012;4(6):1011–1033. doi: 10.3390/v4061011.
9. Shi P.Y., Plante J., Liu Y., Liu J., Xia H., Johnson B., Lokugamage K., Zhang X., Muruato A., Zou J., Fontes-Garfias C., Mirchandani D., Scharton D., Kalveram B., Bilello J., Ku Z., An Z., Freiberg A., Menachery V., Xie X., Weaver S. Spike mutation D614G alters SARS-CoV-2 fitness and neutralization susceptibility. Res. Sq. 2020:70482. doi: 10.21203/rs.3.rs-70482/v1. rs.3.rs.
10. Karamitros T., Papadopoulou G., Bousali M., Mexias A., Tsiodras S., Mentis A. SARS-CoV-2 exhibits intra-host genomic plasticity and low-frequency polymorphic quasispecies. J. Clin. Virol. 2020;131 doi: 10.1016/j.jcv.2020.104585.
11. Simmonds P. Rampant C→U hypermutation in the genomes of SARS-CoV-2 and other coronaviruses: causes and consequences for their short- and long-term evolutionary trajectories. mSphere. 2020;5(3):e00408–e00420. doi: 10.1128/mSphere.00408-20.
12. Harvey W.T., Carabelli A.M., Jackson B., Gupta R.K., Thomson E.C., Harrison E.M., Ludden C., Reeve R., Rambaut A., COVID-19 Genomics UK (COG-UK) Consortium. Peacock S.J., Robertson D.L. SARS-CoV-2 variants, spike mutations and

immune escape. Nature reviews. Microbiology. 2021;19(7):409–424. doi: 10.1038/s41579-021-00573-0.
13. Tao K., Tzou P.L., Nouhin J., Gupta R.K., de Oliveira T., Kosakovsky Pond S.L., Fera D., Shafer R.W. The biological and clinical significance of emerging SARS-CoV-2 variants. Nat. Rev. Genet. 2021;22(12):757–773. doi: 10.1038/s41576-021-00408-x.
14. Volz E., Mishra S., Chand M., Barrett J.C., Johnson R., Geidelberg L., Hinsley W.R., Laydon D.J., Dabrera G., O'Toole Á., Amato R., Ragonnet-Cronin M., Harrison I., Jackson B., Ariani C.V., Boyd O., Loman N.J., McCrone J.T., Gonçalves S., Jorgensen D., Myers R., Hill V., Jackson D.K., Gaythorpe K., Groves N., Sillitoe J., Kwiatkowski D.P., COVID-19 Genomics UK (COG-UK) consortium. Flaxman S., Ratmann O., Bhatt S., Hopkins S., Gandy A., Rambaut A., Ferguson N.M. Assessing transmissibility of SARS-CoV-2 lineage B.1.1.7 in England. Nature. 2021;593(7858):266–269. doi: 10.1038/s41586-021-03470-x.
15. Domingo E., Perales C. Viral quasispecies. PLoS Genet. 2019;15(10) doi: 10.1371/journal.pgen.1008271.
16. Al Khatib H.A., Benslimane F.M., Elbashir I.E., Coyle P.V., Al Maslamani M.A., Al-Khal A., Al Thani A.A., Yassine H.M. Within-host diversity of SARS-CoV-2 in COVID-19 patients with variable disease severities. Front. Cell. Infect. Microbiol. 2020;10 doi: 10.3389/fcimb.2020.575613.
17. Jary A., Leducq V., Malet I., Marot S., Klement-Frutos E., Teyssou E., Soulié C., Abdi B., Wirden M., Pourcher V., Caumes E., Calvez V., Burrel S., Marcelin A.G., Boutolleau D. Evolution of viral quasispecies during SARS-CoV-2 infection. Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. 2020;26(11):1560.e1–1560.e4. doi: 10.1016/j.cmi.2020.07.032.
18. Pérez-Lago L., Aldámiz-Echevarría T., García-Martínez R., Pérez-Latorre L., Herranz M., Sola-Campoy P.J., Suárez-González J., Martínez-Laperche C., Comas I., González-Candelas F., Catalán P., Muñoz P., García de Viedma D., On Behalf Of Gregorio Marañón Microbiology-Id Covid Study Group . Different within-host viral evolution dynamics in severely immunosuppressed cases with persistent SARS-CoV-2. Biomedicines. 2021;9(7):808. doi: 10.3390/biomedicines9070808.
19. Shen Z., Xiao Y., Kang L., Ma W., Shi L., Zhang L., Zhou Z., Yang J., Zhong J., Yang D., Guo L., Zhang G., Li H., Xu Y., Chen M., Gao Z., Wang J., Ren L., Li M. Genomic diversity of severe acute respiratory syndrome-coronavirus 2 in patients with coronavirus disease 2019. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 2020;71(15):713–720. doi: 10.1093/cid/ciaa203.
20. Siqueira J.D., Goes L.R., Alves B.M., de Carvalho P.S., Cicala C., Arthos J., Viola J., de Melo A.C., Soares M.A. SARS-CoV-2 genomic and quasispecies analyses in cancer patients reveal relaxed intrahost virus evolution. bioRxiv Prepr. Serv. Biol. 2020 doi: 10.1101/2020.08.26.267831. 2020.08.26.267831.
21. Sun F., Wang X., Tan S., Dan Y., Lu Y., Zhang J., Xu J., Tan Z., Xiang X., Zhou Y., He W., Wan X., Zhang W., Chen Y., Tan W., Deng G. SARS-CoV-2 quasispecies

provides an advantage mutation pool for the epidemic variants. Microbiol. Spectr. 2021;9(1) doi: 10.1128/Spectrum.00261-21.
22. To K.K., Tsang O.T., Leung W.S., Tam A.R., Wu T.C., Lung D.C., Yip C.C., Cai J.P., Chan J.M., Chik T.S., Lau D.P., Choi C.Y., Chen L.L., Chan W.M., Chan K.H., Ip J.D., Ng A.C., Poon R.W., Luo C.T., Cheng V.C., Chan J.F., Hung I.F., Chen Z., Chen H., Yuen K.Y. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. Lancet Infect. Dis. 2020;20(5):565–574. doi: 10.1016/S1473-3099(20)30196-1.
23. World Health Organization (WHOa). 2020. Clinical management of severe acute respiratory infection when novel coronavirus (2019-nCoV) infection is suspected: interim guidance. World Health Organization. Available at: https://apps.who.int/iris/handle/10665/330893 [Accessed on 19 October 2020].
24. Corman V.M., Landt O., Kaiser M., Molenkamp R., Meijer A., Chu D.K., Bleicker T., Brünink S., Schneider J., Schmidt M.L., Mulders D.G., Haagmans B.L., van der Veer B., van den Brink S., Wijsman L., Goderski G., Romette J.L., Ellis J., Zambon M., Peiris M., Goossens H., Reusken C., Koopmans M.P., Drosten C. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. Bull. Eur. Mal. Transm. Eur. Commun. Dis. Bull. 2020;25(3) doi: 10.2807/1560-7917.ES.2020.25.3.2000045.
25. World Health Organization (WHOb). 2020. Diagnostic detection of 2019-nCoV by real-time RT-PCR. Available at: https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf. [Accessed 30 January 2020].
26. World Health Organization (WHO). 2021. Terms of Reference for the Technical Advisory Group on SARS-CoV-2 Virus Evolution (TAG-VE). COVID-19: Surveillance, case investigation and epidemiological protocols. Available at: https://www.who.int/publications/m/item/terms-of-reference-for-the-technical-advisory-group-on-sars-cov-2-virus-evolution-(tag-ve) [Accessed 1 December 2021].
27. Bustin S.A., Benes V., Garson J.A., Hellemans J., Huggett J., Kubista M., Mueller R., Nolan T., Pfaffl M.W., Shipley G.L., Vandesompele J., Wittwer C.T. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 2009;55(4):611–622. doi: 10.1373/clinchem.2008.112797.
28. Chen S., Zhou Y., Chen Y., Gu J. Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34(17):i884–i890. doi: 10.1093/bioinformatics/bty560. (Oxford, England).
29. Langmead B., Salzberg S.L. Fast gapped-read alignment with Bowtie 2. Nat. Methods. 2012;9(4):357–359. doi: 10.1038/nmeth.1923.
30. Knyazev S., Tsyvina V., Shankar A., Melnyk A., Artyomenko A., Malygina T., Porozov Y.B., Campbell E.M., Switzer W.M., Skums P., Zelikovsky A., Knyazev S. CliqueSNV: an efficient noise reduction technique for accurate assembly of viral variants from NGS data. biorXiv. 2018 doi: 10.1101/264242.

31. O'Toole Á., Pybus O.G., Abram M.E., Kelly E.J., Rambaut A. Pango lineage designation and assignment using SARS-CoV-2 spike gene nucleotide sequences. BMC Genom. 2022;23(1):121. doi: 10.1186/s12864-022-08358-2.
32. Li H., Handsaker B., Wysoker A., Fennell T., Ruan J., Homer N., Marth G., Abecasis G., Durbin R. & 1000 Genome project data processing subgroup. Bioinformatics. 2009;25(16):2078–2079. The Sequence Alignment/Map format and SAMtools. (Oxford, England)10.1093.
33. Huang Y., Yang C., Xu X.F., Xu W., Liu S.W. Structural and functional properties of SARS-CoV-2 spike protein: potential antivirus drug development for COVID-19. Acta Pharmacol. Sin. 2020;41(9):1141–1149. doi: 10.1038/s41401-020-0485-4.
34. van Dorp L., Richard D., Tan C., Shaw L.P., Acman M., Balloux F. No evidence for increased transmissibility from recurrent mutations in SARS-CoV-2. Nat. Commun. 2020;11(1):5986. doi: 10.1038/s41467-020-19818-2.
35. Long S.W., Olsen R.J., Christensen P.A., Bernard D.W., Davis J.J., Shukla M., Nguyen M., Saavedra M.O., Yerramilli P., Pruitt L., Subedi S., Kuo H.C., Hendrickson H., Eskandari G., Nguyen H., Long J.H., Kumaraswami M., Goike J., Boutz D., Gollihar J., McLellan J.S., Chou C.W., Javanmardi K., Finkelstein I.J., Musser J.M. Molecular architecture of early dissemination and massive second wave of the SARS-CoV-2 virus in a major Metropolitan area. mBio. 2020;11(6):e02707–e02720. doi: 10.1128/mBio.02707-20.
36. Parra-Lucares A., Segura P., Rojas V., Pumarino C., Saint-Pierre G., Toro L. Emergence of SARS-CoV-2 variants in the world: how could this happen? Life. 2022;12(2):194. doi: 10.3390/life12020194. (Basel, Switzerland).
37. Yusof W., Irekeola A.A., Wada Y., Engku Abd Rahman E., Ahmed N., Musa N., Khalid M.F., Rahman Z.A., Hassan R., Yusof N.Y., Yean Yean C. A global mutational profile of SARS-CoV-2: a systematic review and meta-analysis of 368,316 COVID-19 patients. Life. 2021;11(11):1224. doi: 10.3390/life11111224. (Basel, Switzerland).
38. Andrews N., Stowe J., Kirsebom F., Toffa S., Rickeard T., Gallagher E., Gower C., Kall M., Groves N., O'Connell A.M., Simons D., Blomquist P.B., Zaidi A., Nash S., Iwani Binti Abdul Aziz N., Thelwall S., Dabrera G., Myers R., Amirthalingam G., Gharbia S., Barrett J.C., Elson R., Ladhani S.N., Ferguson N., Zambon M., Campbell C.N.J., Brown K., Hopkins S., Chand M., Ramsay M., Lopez Bernal J. Covid-19 vaccine effectiveness against the omicron (B.1.1.529) variant. N. Engl. J. Med. 2022 doi: 10.1056/NEJMoa2119451. 10.1056/NEJMoa2119451. Advance online publication.
39. Dejnirattisai W., Huo J., Zhou D., Zahradník J., Supasa P., Liu C., Duyvesteyn H.M.E., Ginn H.M., Mentzer A.J., Tuekprakhon A., Nutalai R., Wang B., Dijokaite A., Khan S., Avinoam O., Bahar M., Skelly D., Adele S., Johnson S.A., Amini A., Ritter T.G., Mason C., Dold C., Pan D., Assadi S., Bellass A., Omo-Dare N., Koeckerling D., Flaxman A., Jenkin D., Aley P.K., Voysey M., Costa Clemens S.A., Naveca F.G., Nascimento V., Nascimento F., Fernandes da Costa C., Resende P.C., Pauvolid-Correa

A., Siqueira M.M., Baillie V., Serafin N., Kwatra G., Da Silva K., Madhi S.A., Nunes M.C., Malik T., Openshaw P.J.M., Baillie J.K., Semple M.G., Townsend A.R., Huang K.A., Tan T.K., Carroll M.W., Klenerman P., Barnes E., Dunachie S.J., Constantinides B., Webster H., Crook D., Pollard A.J., Lambe T., OPTIC Consortium. ISARIC4C Consortium. Paterson N.G., Williams M.A., Hall D.R., Fry E.E., Mongkolsapaya J., Ren J., Schreiber G., Stuart D.I., Screaton G.R. SARS-CoV-2 omicron-B.1.1.529 leads to widespread escape from neutralizing antibody responses. Cell. 2022;185(3):467–484. doi: 10.1016/j.cell.2021.12.046. e15Epub 2022 Jan 4. PMID: 35081335; PMCID: PMC8723827.

40. Mlcochova P., Kemp S.A., Dhar M.S., Papa G., Meng B., Ferreira I., Datir R., Collier D.A., Albecka A., Singh S., Pandey R., Brown J., Zhou J., Goonawardane N., Mishra S., Whittaker C., Mellan T., Marwal R., Datta M., Sengupta S., Ponnusamy K., Radhakrishnan V.S., Abdullahi A., Charles O., Chattopadhyay P., Devi P., Caputo D., Peacock T., Wattal C., Goel N., Satwik A., Vaishya R., Agarwal M., Indian SARS-CoV-2 Genomics Consortium (INSACOG) Genotype to Phenotype Japan (G2P-Japan) Consortium. CITIID-NIHR BioResource COVID-19 Collaboration. Mavousian A., Lee J.H., Bassi J., Silacci-Fegni C., Saliba C., Pinto D., Irie T., Yoshida I., Hamilton W.L., Sato K., Bhatt S., Flaxman S., James L.C., Corti D., Piccoli L., Barclay W.S., Rakshit P., Agrawal A., Gupta R.K. SARS-CoV-2 B.1.617.2 Delta variant replication and immune evasion. Nature, 2021;599(7883):114–119. doi: 10.1038/s41586-021-03944-y. Epub 2021 Sep 6. PMID: 34488225; PMCID: PMC8566220.

41. Ke R., Zitzmann C., Ribeiro R.M., et al. Kinetics of SARS-CoV-2 infection in the human upper and lower respiratory tracts and their relationship with infectiousness. medRxiv. 2020 doi: 10.1101/2020.09.25.20201772. 2020.09.25.20201772.

42. Korber B., Fischer W.M., Gnanakaran S., Yoon H., Theiler J., Abfalterer W., Hengartner N., Giorgi E.E., Bhattacharya T., Foley B., Hastie K.M., Parker M.D., Partridge D.G., Evans C.M., Freeman T.M., de Silva T.I., Sheffield COVID-19 Genomics Group. McDanal C., Perez L.G., Tang H., Moon-Walker A., Whelan S.P., LaBranche C.C., Saphire E.O., Montefiori D.C. Tracking changes in SARS-CoV-2 Spike: evidence that D614G increases infectivity of the COVID-19 virus. Cell. 2020;182(4):812–827. doi: 10.1016/j.cell.2020.06.043. e19.

43. Rueca M., Bartolini B., Gruber C., Piralla A., Baldanti F., Giombini E., Messina F., Marchioni L., Ippolito G., Di Caro A., Capobianchi M.R. Compartmentalized replication of SARS-Cov-2 in upper vs. lower respiratory tract assessed by whole genome quasispecies analysis. Microorganisms. 2020;8(9):1302. doi: 10.3390/microorganisms8091302.

44. Wylezich C., Schaller T., Claus R., Hirschbühl K., Märkl B., Kling E., Spring O., Höper D., Schlegel J., Beer M., Dintner S. Whole-genome analysis of SARS-CoV-2 samples indicate no tissue specific genetic adaptation of the virus in COVID-19 patients' upper and lower respiratory tract. Diagn. Microbiol. Infect. Dis. 2021;101(4) doi: 10.1016/j.diagmicrobio.2021.115520.

45. Andrés C., Garcia-Cehic D., Gregori J., Piñana M., Rodriguez-Frias F., Guerrero-Murillo M., Esperalba J., Rando A., Goterris L., Codina M.G., Quer S., Martín M.C., Campins M., Ferrer R., Almirante B., Esteban J.I., Pumarola T., Antón A., Quer J. Naturally occurring SARS-CoV-2 gene deletions close to the spike S1/S2 cleavage site in the viral quasispecies of COVID19 patients. Emerg. Microbes Infect. 2020;9(1):1900–1911. doi: 10.1080/22221751.2020.1806735.
46. Wölfel R., Corman V.M., Guggemos W., Seilmaier M., Zange S., Müller M.A., Niemeyer D., Jones T.C., Vollmar P., Rothe C., Hoelscher M., Bleicker T., Brünink S., Schneider J., Ehmann R., Zwirglmaier K., Drosten C., Wendtner C. Virological assessment of hospitalized patients with COVID-2019. Nature. 2020;581(7809):465–469. doi: 10.1038/s41586-020-2196-x.
47. Abassi Z., Knaney Y., Karram T., Heyman S.N. The lung macrophage in SARS-CoV-2 infection: a friend or a foe? Front. Immunol. 2020;11:1312. doi: 10.3389/fimmu.2020.01312.
48. Carpenter J.A., Keegan L.P., Wilfert L., O'Connell M.A., Jiggins F.M. Evidence for ADAR-induced hypermutation of the drosophila sigma virus (Rhabdoviridae) BMC Genet. 2009;10:75. doi: 10.1186/1471-2156-10-75.
49. Di Giorgio S., Martignano F., Torcia M.G., Mattiuz G., Conticello S.G. Evidence for host-dependent RNA editing in the transcriptome of SARS-CoV-2. Science Advances. 2020;6(25):eabb5813. doi: 10.1126/sciadv.abb5813.
50. Andersen K.G., Rambaut A., Lipkin W.I., Holmes E.C., Garry R.F. The proximal origin of SARS-CoV-2. Nat. Med. 2020;26(4):450–452. doi: 10.1038/s41591-020-0820-9.
51. Wang P., Lau S.Y., Deng S., Chen P., Mok B.W., Zhang A.J., Lee A.C., Chan K.H., Tam R.C., Xu H., Zhou R., Song W., Liu L., To K.K., Chan J.F., Chen Z., Yuen K.Y., Chen H. Characterization of an attenuated SARS-CoV-2 variant with a deletion at the S1/S2 junction of the spike protein. Nat. Commun. 2021;12(1):2790. doi: 10.1038/s41467-021-23166-0.
52. Lau S.Y., Wang P., Mok B.W., Zhang A.J., Chu H., Lee A.C., Deng S., Chen P., Chan K.H., Song W., Chen Z., To K.K., Chan J.F., Yuen K.Y., Chen H. Attenuated SARS-CoV-2 variants with deletions at the S1/S2 junction. Emerg. Microbes Infect. 2020;9(1):837–842. doi: 10.1080/22221751.2020.1756700.
53. Zhu B., Xiao Y., Yeager M., Clifford G., Wentzensen N., Cullen M., Boland J.F., Bass S., Steinberg M.K., Raine-Bennett T., Lee D., Burk R.D., Pinheiro M., Song L., Dean M., Nelson C.W., Burdett L., Yu K., Roberson D., Lorey T., Franceschi S., Castle P.E., Walker J., Zuna R., Schiffman M., Mirabello L. Mutations in the HPV16 genome induced by APOBEC3 are associated with viral clearance. Nat. Commun. 2020;11(1):886. doi: 10.1038/s41467-020-14730-1.

Supplementary materials

Table S1. Overall patients and samples informations.

Patient ID	Patient Sex	Patient Age	ID sample	Type	Amplification	Sampling Date	Ct	Variant	Hedgehog lineage
10	M	71	10_BAL	BAL	Onestep	24/02/2021	20.27	D614G	B.1.177_1
10	M	71	10_TNAS	TNAS	Onestep	24/02/2021	20.23	D614G	B.1.177_1
7386	M	82	10068_TNAS	TNAS	Onestep + Nested	26/02/2020	27.7	D614G	A_1
3237	F	24	10069_TNAS	TNAS	Onestep	26/02/2020	22.7	D614G	A_1
2777	F	46	10070_TNAS	TNAS	Onestep	26/02/2020	33.2	D614G	A_1
8428	M	73	10834_BAL	BAL	Onestep + Nested	27/02/2020	26.8	D614G	A_1
3782	M	75	10949_BAL	BAL	Onestep	27/02/2020	23.3	D614G	A_1
12	F	24	12_BAL	BAL	Onestep	29/01/2021	14.91	D614G	B.1.177_1
12	F	24	12_TNAS	TNAS	Onestep	29/01/2021	19.6	D614G	B.1.177_1
8159	M	71	12374_BAL	BAL	Onestep	29/02/2020	21.1	D614G	A_1
4691	M	77	12744_BAL	BAL	Onestep	29/02/2020	22.5	D614G	A_1
927	M	54	13075_TNAS	TNAS	Onestep	01/03/2020	20.7	D614G	A_1
927	M	54	13077_BRASP	BRASP	Onestep	01/03/2020	21.5	D614G	A_1
5917	M	62	13078_BRASP	BRASP	Onestep	01/03/2020	22.6	D614G	A_1
2373	F	60	13355_TNAS	TNAS	Onestep	01/03/2020	25.3	D614G	A_1
6489	F	71	13500_TNAS	TNAS	Onestep	01/03/2020	28.2	D614G	A_1
6489	F	71	13536_BRASP	BRASP	Onestep + Nested	01/03/2020	23	D614G	A_1
6875	M	66	13542_BRASP	BRASP	Onestep	01/03/2020	22	D614G	A_1
14	M	67	14_BAL	BAL	Onestep	04/03/2021	17.28	Alpha	A_9
201	F	41	14370_TNAS	TNAS	Onestep	02/03/2020	18.3	D614G	A_1
6013	M	66	14639_BAL	BAL	Onestep	03/03/2020	21.5	D614G	A_1
6013	M	66	14668_TNAS	TNAS	Onestep	03/03/2020	24.3	D614G	A_1
15	M	73	15_BAL	BAL	Onestep	07/12/2020	12	D614G	B.1.177_1
15	M	73	15_TNAS	TNAS	Onestep	07/12/2020	19	D614G	B.1.177_1
4312	M	86	15805_BRASP	BRASP	Onestep	04/03/2020	20.2	D614G	A_1

Patient ID	Patient sex	Patient Age	ID sample	Type	Amplification	Sampling Date	Ct	Variant	Hedgehog lineage
7010	M	83	15845_BAL	BAL	Onestep	04/03/2020	33	D614G	A_1
16	M	59	16_BAL	BAL	Onestep	03/04/2021	15	Alpha	A_9
16	M	59	16_TNAS_	TNAS	Onestep	02/04/2021	20	Alpha	A_9
4156	M	70	16111_TNAS	TNAS	Onestep + Nested	05/03/2020	33	D614G	A_1
1871	M	68	16464_TNAS	TNAS	Onestep	05/03/2020	19.9	D614G	A_1
1871	M	68	18238_BAL	BAL	Onestep	07/03/2020	18.4	D614G	A_1
442	F	72	18579_TNAS	TNAS	Onestep	07/03/2020	28.2	D614G	A_1
573	F	54	18845_BAL	BAL	Onestep	08/03/2020	16	D614G	A_1
19	M	50	19_BAL	BAL	Onestep	08/11/2020	17.15	D614G	B.1.177.52_1
3370	M	69	19900_TNAS	TNAS	Onestep	09/03/2020	21.5	D614G	A_1
20	M	78	20_BAL	BAL	Onestep	25/02/2021	17.03	D614G	B.1.177_1
7561	M	72	20606_BAL	BAL	Onestep	10/03/2020	17.8	D614G	A_1
4448	F	43	20938_TNAS	TNAS	Onestep	10/03/2020	31.2	D614G	A_1
21	M	54	21_BAL	BAL	Onestep	28/11/2020	21	D614G	B.1.177_1
21	M	54	21_TNAS	TNAS	Onestep	28/11/2020	22	D614G	B.1.177_1
9072	F	36	21386_TNAS	TNAS	Onestep	11/03/2020	25.7	D614G	A_1
22	F	58	22_BAL	BAL	Onestep	05/12/2020	15	D614G	B.1.177_1
22	F	58	22_TNAS	TNAS	Onestep	05/12/2020	23	D614G	B.1.177_1
231	M	64	22427_TNAS	TNAS	Onestep	12/03/2020	28	D614G	A_1
5236	M	78	22594_TNAS	TNAS	Onestep	12/03/2020	30	D614G	A_1
23	M	76	23_BAL	BAL	Onestep	01/03/2021	13.39	D614G	B.1.177_1
23	M	76	23_TNAS	TNAS	Onestep	01/03/2021	16.35	D614G	B.1.177_1
24	M	55	24_BAL	BAL	Onestep	18/11/2020	19	D614G	A_1
24	M	55	24_TNAS	TNAS	Onestep	18/11/2020	13	D614G	A_1
8768	M	50	24321_TNAS	TNAS	Onestep	15/03/2020	24.8	D614G	A_1
4966	M	80	24352_TNAS	TNAS	Onestep	15/03/2020	23.2	D614G	A_1
5236	M	78	24508_BRASP	BRASP	Onestep	15/03/2020	20.7	D614G	A_1
4723	M	69	24874_TNAS	TNAS	Onestep	16/03/2020	23	D614G	A_1
25	M	41	25_BAL	BAL	Onestep	03/12/2020	27	D614G	A_1
25	M	41	25_TNAS	TNAS	Onestep	02/12/2020	23	D614G	A_1
8890	F	78	25059_TNAS	TNAS	Onestep	16/03/2020	25.3	D614G	A_1
153	F	52	25145_TNAS	TNAS	Onestep + Nested	16/03/2020	32.3	D614G	A_1
7640	M	77	25239_TNAS	TNAS	Onestep	16/03/2020	25.3	D614G	A_1

Patient ID	Patient sex	Patient Age	ID sample	Type	Amplification	Sampling Date	Ct	Variant	Hedgehog lineage
1376	M	62	25495_BAL	BAL	Onestep	16/03/2020	23.9	D614G	A_1
3567	M	64	25667_TNAS	TNAS	Onestep	16/03/2020	27.1	D614G	B.1_14
7262	M	55	25759_TNAS	TNAS	Onestep	17/03/2020	23.9	D614G	A_1
4257	M	79	26022_TNAS	TNAS	Onestep + Nested	17/03/2020	36	D614G	A_1
4030	M	47	26214_TNAS	TNAS	Onestep	17/03/2020	23.9	D614G	A_1
27	M	80	27_BAL	BAL	Onestep	14/03/2021	16	Alpha	A_9
27	M	80	27_TNAS	TNAS	Onestep	14/03/2021	22	Alpha	A_9
8639	F	63	27230_BAL	BAL	Onestep	19/03/2020	30.2	D614G	A_1
5174	M	59	27705_TNAS	TNAS	Onestep	19/03/2020	35	D614G	A_1
28	M	44	28_TNAS	TNAS	Onestep	09/01/2021	28.58	D614G	A_1
4723	M	69	28495_BAL	BAL	Onestep + Nested	20/03/2020	25.8	D614G	A_1
7746	M	65	28525_TNAS	TNAS	Onestep	20/03/2020	24.1	D614G	A_1
102	M	59	28677_BAL	BAL	Onestep + Nested	21/03/2020	24.4	D614G	A_1
2533	M	69	28906_BAL	BAL	Onestep + Nested	21/03/2020	27.9	D614G	A_1
3	M	55	3_BAL	BAL	Onestep	30/11/2020	15.36	D614G	A_1
3	M	55	3_TNAS	TNAS	Onestep	30/11/2020	19.86	D614G	A_1
4030	M	47	30051_BAL	BAL	Onestep	23/03/2020	34.9	D614G	A_1
6118	M	64	30531_BAL	BAL	Onestep	24/03/2020	28.9	D614G	A_1
6118	M	64	30532_TNAS	TNAS	Onestep	24/03/2020	29	D614G	A_1
5174	M	59	30606_BAL	BAL	Onestep + Nested	24/03/2020	26.9	D614G	A_1
7262	M	55	30763_BAL	BAL	Onestep + Nested	24/03/2020	31.9	D614G	A_1
102	M	59	30944_BAL	BAL	Onestep + Nested	24/03/2020	30.7	D614G	A_1
3567	M	64	31108_BAL	BAL	Onestep + Nested	25/03/2020	28.1	D614G	B.1_14
1214	M	33	32881_BAL	BAL	Onestep	27/03/2020	22.3	D614G	A_1
1214	M	33	33048_BAL	BAL	Onestep + Nested	28/03/2020	27.4	D614G	A_1
3455	M	72	35202_TNAS	TNAS	Onestep	01/04/2020	32.4	D614G	A_1
3455	M	72	35203_BAL	BAL	Onestep + Nested	01/04/2020	33.7	D614G	A_1
6118	M	64	35214_BAL	BAL	Onestep	01/04/2020	33.4	D614G	A_1

Patient ID	Patient sex	Patient Age	ID sample	Type	Amplification	Sampling Date	Ct	Variant	Hedgehog lineage
1214	M	33	35977_BAL	BAL	Onestep + Nested	02/04/2020	30	D614G	A_1
5	M	64	5_BAL	BAL	Onestep	01/03/2021	13.58	Alpha	A_9
5	M	64	5_TNAS	TNAS	Onestep	28/02/2021	16.55	Alpha	A_9
2611	F	32	5291_BAL	BAL	Onestep + Nested	21/02/2020	25.3	D614G	A_1
349	M	64	6123_TNAS	TNAS	Onestep	23/02/2020	22.6	D614G	A_1
1943	M	36	6491_TNAS	TNAS	Onestep	23/02/2020	23.7	D614G	A_1
6935	F	36	6500_TNAS	TNAS	Onestep	23/02/2020	22.7	D614G	A_1
829	F	37	6565_TNAS	TNAS	Onestep	23/02/2020	24	D614G	A_1
3880	F	57	6616_TNAS	TNAS	Onestep	23/02/2020	18	D614G	A_1
317	F	3	6699_TNAS	TNAS	Onestep + Nested	23/02/2020	34.6	D614G	A_1
5681	F	64	6709_TNAS	TNAS	Onestep	23/02/2020	19.6	D614G	A_1
2447	M	61	7347_TNAS	TNAS	Onestep	24/02/2020	19	D614G	A_1
8159	M	71	8076_TNAS	TNAS	Onestep	25/02/2020	25.8	D614G	A_1
2611	F	32	8078_TNAS	TNAS	Onestep	25/02/2020	23.7	D614G	A_1
4569	M	37	8085_TNAS	TNAS	Onestep + Nested	25/02/2020	28.3	D614G	A_1
3029	M	64	8182_BRASP	BRASP	Onestep	24/02/2020	26.2	D614G	A_1
7010	M	83	8403_TNAS	TNAS	Onestep	25/02/2020	22.8	D614G	A_1
5558	M	73	8519_TNAS	TNAS	Onestep	25/02/2020	22.7	D614G	B.1_14
4852	M	40	9004_TNAS	TNAS	Onestep	25/02/2020	31.8	D614G	A_1
5558	M	73	9127_BAL	BAL	Onestep	26/02/2020	23	D614G	B.1_14
9122	F	73	9225_BAL	BAL	Onestep	26/02/2020	22.6	D614G	A_1
5865	F	59	9675_TNAS	TNAS	Onestep	26/02/2020	20.4	D614G	A_1
1421	M	75	9882_BAL	BAL	Onestep + Nested	26/02/2020	24.7	D614G	A_1

Please note that hedgehog lineages nomenclature does not correspond to the pangolin nomenclature.

B.1.177_1: S:A222V|S:D614G; A_1: S:D614G; A_9: del:21765:6|del:21991:3|S:N501Y|S:A570D|S:D614G|S:P681H|S:T716I|S:S982A|S:D1118H; B.1.177.52_1: S:A222V|S:D614G|S:P1162R; B.1_14: S:D614G|S:D839Y

Table S2. List of primer for amplification and sequencing of S gene.

Primer name	**Sequence 5'-3'**	**Position**
SARS2-S-F3	TATCTTGGCAAACCACGCGAACAA	21289-21312
SARS2-S-R3	ACCCTTGGAGAGTGCTAGTTGCCATCTC	25620-25593
SARS2-S-F4	CTACTTTAGATTCGAAGACCCAGTCC	21885-21910
SARS2-S-F5	GATGAAGTCAGACAAATCGCTCCAGG	22775-22800
SARS2-S-F6	TCAGGATGTTAACTGCACAGAAGTCC	23398-23423
SARS2-S-F7	TGCCTTGGTGATATTGCTGCTAGAGACC	24080-24107
SARS2-S-F8	CAGCACCTCATGGTGTAGTCTTCTTGC	24726-24752
SARS2-S-F9	ATGACCAGTTGCTGTAGTTGTCTCAAGG	25271-25298
SARS2-S-R4	CCTTTGTGTTACAAACCAGTGTGTGCCA	24883-24856
SARS2-S-R5	CAGCCTCAACTTTGTCAAGACGTGAA	24530-24505
SARS2-S-R6	TTCTGCACCAAGTGACATAGTGTAGGCA	23668-23641
SARS2-S-R7	GTCCACAAACAGTTGCTGGTGCATGTA	23141-23115
SARS2-S-R8	GCTGCAGCACCAGCTGTCCAACCTGAA	22353-22327
SARS2-S-R9	TCAAACCTCTTAGTACCATTGGTCCCAG	21801-21774

Chapter 7

Molecular epidemiology of Rhinovirus/Enterovirus and their role on cause severe and prolonged infection in hospitalized patients

Federica A.M. Giardina, Antonio Piralla, Guglielmo Ferrari, Federica Zavaglio, Irene Cassaniti, Fausto Baldanti

Role: First author

Author's contribution: Methodology, Formal analysis, Investigation, Data curation, Writing - original draft preparation.

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Molecular epidemiology of Rhinovirus/Enterovirus and their role on cause severe and prolonged infection in hospitalized patients.

Federica A.M. Giardina[1], Antonio Piralla[1], Guglielmo Ferrari[1], Federica Zavaglio[1], Irene Cassaniti[1], Fausto Baldanti[1,2].

[1] *Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy;*

[2] *Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy.*

ABSTRACT

Rhinoviruses are one of the most prevalent viruses affecting the respiratory tract, causing both upper and lower respiratory tract infections. They primarily affect children and may result in prolonged infections, particularly in individuals with compromised immune systems. The present study reports the results of a 15-month surveillance of rhinovirus seasonality and circulation in the Lombardy Region of Italy. All rhinovirus/enterovirus-positive samples were subjected to reverse transcription polymerase chain reaction (RT-PCR) amplification of the VP4-VP2 region in order to assign the correct genotype. The median age of patients with RV/EV infections was 9 years, with a range of 0–96 years. The majority of cases were found to be positive for hRV-A and hRV-C, with hRV-B accounting for less than 10% of cases. A total of 6.45% of cases were found to be positive for an enterovirus species. A total of 7% of the patients included in this study exhibited a prolonged infection, with a median duration of 62 days. All patients with prolonged infection were immunocompromised, with the majority being pediatric and infected with hRV-A. Two outbreaks were identified, one occurring in the neonatal intensive care unit (NICU) and the other in the Oncohematology Department. These outbreaks were caused by RV A89 and C43, respectively. A total of 4.5% of the patients were admitted to the ICU, requiring mechanical ventilation. All of these patients had preexisting comorbidities. Thus, the clinical impact of RV/EV infections is not limited only to the common cold, and these viruses should be considered as highly significant respiratory pathogens

Keywords: respiratory infection, rhinovirus, enterovirus, prolonged infection.

1. INTRODUCTION

Human rhinoviruses (RVs) are small viruses belonging to the *Enterovirus* genus within the *Picornaviridae* family. At least 100 genotypes have been identified, with a further new species of RV, named RV-C, including more than 50 genotypes, being discovered in 2006 [1]. RV infections are relatively common and appear to occur in two yearly peaks, in the early fall and spring [2]. In particular, RV-A and RV-C represent the species most frequently detected, while RV-B is less frequent in comparison. Although RVs are considered as the etiologic agents of the "common cold", they have been recently associated with a severe acute respiratory infection (SARI) in children, older people, and immunosuppressed subjects [3, 4, 5]. Clinical manifestations of RV-associated SARI are croup, bronchiolitis or community-acquired pneumonia (CAP) which often requires hospitalization and mechanical ventilation [6, 7, 8]. Among RV species, RV-C seems to be more frequently associated with severe infections [9] including asthma exacerbations in children and life-threatening conditions in infants [10, 11]. Other studies suggest that RV-C is more likely to cause lower respiratory tract infections than other types of RVs in the pediatric population rather than in the adult population [12]. Furthermore, RVs are also implicated in nosocomial outbreaks, as observed in neonatal intensive care units [13, 14]. In addition to RVs, enteroviruses (EVs) belonging to the same *Picornaviridae* family have been observed as emerging pathogens causing a wide range of clinical syndromes, ranging from mild to more severe clinical outcomes [15, 16]. RV and EV shedding usually lasts less than 2 weeks in immunocompetent subjects [17], while prolonged RV infections have been mainly observed in those who are immunocompromised, such as patients undergoing chemotherapy or in a post-transplant phase [18]. This study aimed to investigate clinical and virological features of RV/EV infections providing the increasingly recognized role of these viruses as important disease-causing agents in order to describe their impact on short- and long-term morbidity.

2. MATERIALS AND METHODS

2.1 Study population

The study was conducted on a cohort of patients with a respiratory syndrome, including both inpatients and outpatients. All patients were referred to the Fondazione I.R.C.C.S. Policlinico San Matteo hospital in Pavia, Italy. All respiratory samples (nasal swabs and bronchoalveolar lavages) were collected between 1 September 2017 and 31 December 2018, then analysed for the presence of respiratory viruses. Patients presenting with rhinitis, pharyngitis, and laryngitis were considered to have an upper respiratory tract infection (URTI), whereas patients exhibiting bronchitis, bronchiolitis, and pneumonia (characterised by cough, wheezing, and/or dyspnoea) were classified as having a lower respiratory tract infection (LRTI). All cases where the clinical picture was suggestive of pneumonia, were radiologically confirmed. The term "episode" was used to indicate a single respiratory syndrome, with the duration of the episode defined by the presence of

respiratory symptoms. A new respiratory syndrome caused by a distinct type of RV occurring in the same patient at least three weeks following the resolution of the previous respiratory symptoms was defined as a distinct episode and analysed independently from the previous episode. The term "multiple picornavirus detection" indicated the presence of different RV/EV strains or species during the same episode. An episode was defined as prolonged if the same RV/EV type was detected in specimens collected at least 30 days apart. The study was conducted in accordance with the Declaration of Helsinki and the protocol on respiratory virus epidemiology was approved by the Ethics Committee of our hospital (P-20180022616).

2.2 Molecular Analysis

Viral RNA was extracted on the QiaSymphony platform using a Virus Pathogens DSP Midi Kit (Qiagen, Heidelberg, Germany). A panel of tests was employed to identify respiratory viruses in clinical specimens, including those for RV/EV, human influenza virus type A and B (FluA and FluB), human coronaviruses (hCoVs), human metapneumovirus (hMPV), human respiratory syncytial virus (hRSV), human parainfluenza virus (hPiV) type 1–4 and human adenoviruses (hAdV) [19]. Real-time RT-PCR reactions were conducted on the Rotor-Gene Q instrument with a Quantifast® Pathogen PCR+IC Kit (Qiagen, Heidelberg, Germany), in accordance with the manufacturer's instructions. RV/EV-positive samples were subjected to a nested PCR targeting the VP4-VP2 region of the viral genome, as described by Wisdom et al. [20], with a modified protocol. In detail, the first amplification was performed using the AgPath-ID One-Step RT-PCR kit (Ambion, Austin, TX, USA), in accordance with the manufacturer's instructions. The primers employed in the initial amplification were OS458 (5′CCGGCCCCTGAATGYGGCTAA3′) and OAS1125 (5′ACATRTTYTSNCCAAANAYDCCCAT3′). The thermal profile was as follows: retrotranscription was conducted at 50 °C for 30 minutes and initial PCR activation at 95 °C for 10 minutes, followed by 50 cycles at 95 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for one minute. The final step involved incubation at 72 °C for 5 minutes. Nested amplification was conducted using AmpliTaqGold® with GeneAmp® (Life Technologies, Livingston, NJ, USA) in accordance with the manufacturer's instructions, utilising primers IS547 (5′ACCRACTACTTTGGGTGTCCGTG3′) and IAS1087 (5′TCWGGHARYTTCCAMCACCANCC3′). The thermal profile was as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 sec, 58 °C for 30 sec, and 72 °C for one minute. The final step of the reaction was conducted at 72 °C for 5 min. An alternative protocol targeting the EV's VP1 protein was used whenever the direct typing PCR resulted negative as described by Nix and colleagues [21]. The sequencing reaction was performed using internal primers on an ABI Prism Genetic Analyzer and sequences obtained were analyzed on Sequencer software.

2.3 Phylogenetic analysis

The nucleotide sequences were aligned with MEGA software (version 5.05) [22] using the ClustalW method, which was applied to the nucleotide sequences' alignment. A phylogenetic tree was constructed using the neighbour-joining method and the Kimura-2-parameter to simultaneously estimate the distance among the sequences. The bootstrap values were calculated using 1,000 replicates. The RV/EV type was assigned based on the nearest reference strain observed in the phylogenetic tree (similarity >98%). This approach was taken to ensure the most accurate classification.

2.4 Statistical analysis

Comparisons of the continuous unpaired variables were performed with the Mann–Whitney test. Descriptive statistics and statistical comparisons were carried out using the Graph Pad Prism software (version 8.3.0).

3. RESULTS

3.1 Samples

A total of 3,310 respiratory specimens were collected from both inpatients and outpatients during the study period and subsequently analysed. As illustrated by the blue line in Figure 1, the greatest number of specimens was collected in December 2017 and January 2018, at the outset of the influenza virus's circulation. Conversely, the lowest number of specimens was collected and tested during the summer period in 2018. Of the 3310 specimens collected, 257 (7.6%) were positive for RV/EV, with 177 nasal swabs, 45 nasopharyngeal swabs, and 35 bronchoalveolar lavages. A total of 127/201 (63.2%) patients were admitted to different departments of our hospital, including the Infectious Disease Department, the Paediatric Department, the Haematology Department, the Intensive Care Unit (ICU) or Neonatal Intensive Care Unit (NICU) Department. A total of 66/201 (32.8%) were instead outpatients. Regarding the remaining eight cases (4%) of RV/EV positivity, specimens were sent to our hospital from other health institutes in the Lombardy region. The median age of the RV/EV-positive patients was 9 years old (range 10 days–96 years). Of these patients, 117 were male (58.2%) and 84 were female (41.8%). Of the total number of cases, 188 (93.5%) had a single RV/EV episode, 11 (5.5%) had two different RV/EV episodes, and only 2 (1%) had three RV/EV episodes. A total of 216 episodes of RV/EV infection were identified. Of these, 184 (85.2%) were classified as URTI, while 32 (14.8%) were designated as LRTI.

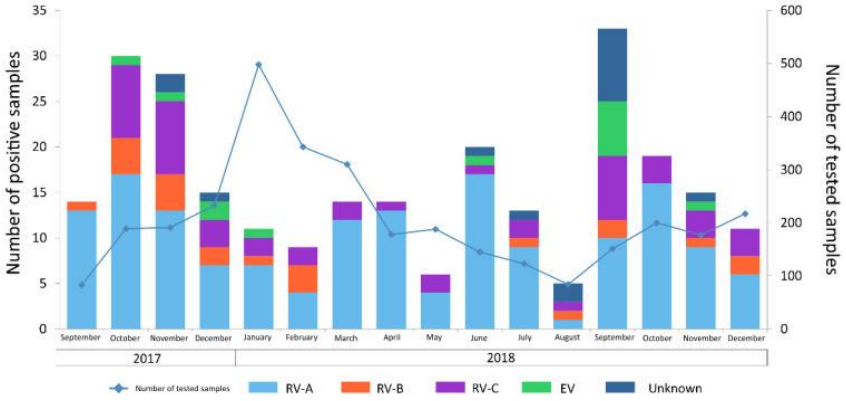

Figure 1. Monthly distribution of cases included in this study. The blue line represents the total number of respiratory specimens collected and tested during the study period. The bars correspond to the RV/EV positive cases with RV-A reported in light blue, RV-B in orange, RV-C in violet and EV cases in green. Blue bars represent those cases for which typing was not possible due to a very low viral load.

Table 1 shows the demographic and virological features of the patients included in this study. In the 3.053 RV/EV negative specimens, hRSV was detected in 274 samples (8.3%), FluB in 126 (3.8%), FluA in 106 (3.2%), hPiV type 1/3 and hAdV in 65 samples each (2.0%), hMPV in 37 (1.1%) and hCoV type OC43/HKU1 in 24 (0.7%). hPiV type 2/4 and hCoV 229E/NL63 represented less than 0.5% of the total number of cases and were detected in 13, 4 and 5 specimens, respectively. Finally, 2334 (73.2%) samples resulted negative for the respiratory viruses included in the panel used.

3.2 The peak of viral load and prolonged infection

The peak of the RV/EV load was between 10^3 and 10^5 in 51.9% of episodes (112/216), $>10^5$ copies/mL in 24.5% (53/216) and $<10^3$ copies/mL in the remaining 23.6% (51/216) of episodes. The median duration of RV/EV episodes was 15 days (range 4–316 days). A total of 11 patients (5.5%) exhibited a prolonged RV/EV infection, with a median duration of 75 days (range 30–316 days). All of these patients were immunocompromised due to their age (less than 30 days old), ongoing chemotherapy, or post-transplant immunosuppressive therapy.

3.3 Typing and coinfections

Out of 216 RV/EV episodes, 127 (58.3%) were caused by RV-A, 44 (19.9%) by RV-C and 20 (9.8%) by RV-B, and the remaining 12 (6.0%) episodes were caused by EV (Figure 2). 40 different genotypes were identified for RV-A, eight for RV-B and 18 for RV-C; the most frequently detected genotypes were A49, B35 and C3, respectively. In regard to the EV-associated episodes, an EV-D68 was identified in seven cases, an EV-C104 was detected in two cases, and a single identification was obtained for EV-C117, CV-A21, and CV-B4. Among the 216 cases of infection, one individual was

simultaneously infected by both RV-A and EV-D68. In 14 RV/EV cases, typing could not be performed due to a very low viral load. The median age of children infected with RV-A was 22 months, 29 months for RV-B, and 7 months for RV-C. The Mann–Whitney test demonstrated a significant difference in the median age between RV-A/B and RV-A/C positive paediatric patients ($p < 0.05$). Additionally, RV-A strains were observed in older individuals (16–65 and >65 years) compared to RV-B and RV-C (Table 1). In 175/216 episodes (81.0%), only RV/EV was detected, whereas in 41/216 (19.0%) cases, at least one additional respiratory virus was also present. In detail, hRSV was detected in 24/41 (58.5%) coinfections, hAdV in 4/41 (9.8%), hPiV3 in 4/41 (9.8%), hMPV in 2/41 (4.9%), hPiV4 in 2/41 (4.97%) and finally, RV/EV was detected with two other viruses (hPiV and hCoV) in only one sample. Given the retrospective nature of this study, it was not possible to retrieve any information about bacterial and fungal coinfections for none of the patients included in the study.

Categories		RV Species				*p*-Value [a]
		RV-A (127)	RV-B (20)	RV-C (44)	EV (12)	
Gender	Male	77 (60.6%)	10 (50.0%)	28 (63.6%)	5 (41.7%)	0.57
	Female	50 (39.4%)	10 (50.0%)	16 (36.4%)	7 (58.3%)	
Age	<1 year	26 (20.5%)	4 (20.0%)	17 (38.6%)	3 (25.0%)	0.02
	1–5 years	24 (18.9%)	7 (35.0%)	13 (29.5%)	2 (16.7%)	
	5–15 years	20 (15.7%)	4 (20.0%)	0	0	
	16–65 years	43 (33.9%)	4 (20.0%)	10 (22.7%)	5 (41.7%)	
	>65 years	14 (11.0%)	1 (5.0%)	4 (9.1%)	2 (16.7%)	
Hospitalization	ICU Dept.	5 (3.9%)	3 (15.0%)	2 (4.5%)	0	0.10
	NICU Dept.	19 (15.0%)	2 (10.0%)	13 (29.5%)	2 (16.7%)	
	Infectious Diseases Dept.	4 (3.1%)	0	2 (2.5%)	2 (16.7%)	
	Other Depts.	96 (75.6%)	14 (70%)	27 (61.4%)	6 (50%)	
	Unknown	3 (2.4%)	1 (5.0%)	0	2 (16.7%)	
Immuno status	Immunocompromised	57 (44.9%)	6 (30.0%)	20 (45.5%)	5 (41.7%)	0.67
	Immunocompetent	50 (39.4%)	11 (55%)	16 (36.4%)	3 (25%)	
	Unknown	20 (15.7%)	3 (15%)	8 (18.2%)	4 (33.3%)	
Viral Load	$<10^3$ copies/ml	25 (19.7%)	5 (25%)	12 (27.3%)	1 (8.3%)	0.46
	10^3–10^5 copies/mL	68 (53.5%)	13 (65.0%)	22 (50.0%)	10 (83.3%)	
	$>10^5$ copies/mL	34 (26.8%)	2 (10.0%)	10 (22.7%)	1 (8.3%)	
Coinfections	No coinfections	106 (83.5%)	12 (60%)	36 (81.8%)	10 (83.3%)	0.17
	Coinfections	21 (16.5%)	8 (40.0%)	8 (18.2%)	2 (16.7%)	
	hADV	0	2 (10%)	2 (4.5%)	0	
	hCMV	6 (4.7%)	1 (5%)	1 (2.3%)	1 (8.3%)	
	hCOVs	1 (0.8%)	0	0	0	
	hMPV	1 (0.8%)	0	1 (2.3%)	0	
	hPIVs	2 (1.6%)	3 (15.0%)	0	0	
	hRSV	11 (8.7%)	2 (10.0%)	4 (9.1%)	1 (8.3%)	

Table 1. Demographic and virological features of all RV/EV episodes with successful typing.

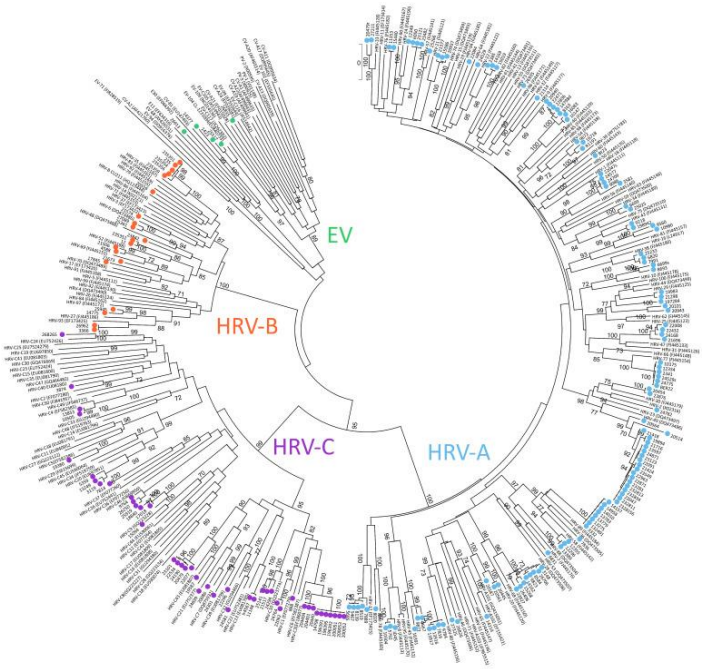

Figure 2. Phylogenetic tree of the RV/EV cases in this study, based on the VP4-VP2 sequences obtained (n = 196). RV-A strains are reported with light blue circles, RV-B with orange circles, RV-C with violet circles and EV cases with green circles. Since a partial VP1 region was analyzed for EV-D68 strains, sequences are not reported here.

3.4 Hospital Outbreak

Sequence analysis of RV/EV strains collected during the study period revealed at least two nosocomial outbreaks occurred in our hospital. The first outbreak occurred in October 2017 in a neonatal intensive care unit (NICU), with four patients infected by RV-C43 in a 30-day period. The other outbreak occurred in the same NICU in June 2018 where four patients were infected by RV-A89 during the same period (12 days).

3.5 EV episodes associated with severe infections

A total of 13 (4.5% of all positive patients) were admitted to the ICU with a severe acute respiratory infection (SARI). Of these, 12 were positive for RV and none of them was EV positive. The median age of the patients admitted to the ICU was 54 years (range 5–66 years), with five patients being less than 11 years of age. In all cases, mechanical ventilation was required. Of the 13 patients, 11 (84.6%) had preexisting comorbidities, including chronic respiratory diseases and haematological malignancies such as acute lymphoblastic leukaemia, lymphoma and myelodysplastic syndrome. RV was identified in bronchoalveolar lavage samples from 8/13 (61.5%) patients, nasal swabs from 3/13 (23%) patients, and in both samples from two patients. In six of the bronchoalveolar lavage samples, the viral load was between 10^4 and 10^5 copies/mL, while in five samples,

the viral load was below 10^4 copies/mL. In one bronchoalveolar lavage sample, the viral load of the RV was found to be greater than 10^6 copies/mL. In seven patients, the only respiratory pathogen detected was RV, including four cases of RV-A, two cases of RV-B, and one case of RV-C. Conversely, in six patients, RV (including three RV-A and three RV-B) was simultaneously detected with CMV (three patients), hAdV (one patient), hPiV3 (one patient), and hPiV4 (one patient). Two patients exhibited evidence of bacterial and fungal coinfections. One patient was positive for *Streptococcus pneumoniae* and also tested positive for cytomegalovirus (CMV), while the other patient was positive for both *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*.

4. DISCUSSION

Influenza viruses and hRSV are well-known respiratory pathogens. Furthermore, RV/EV are increasingly recognised as pathogens that are also responsible for SARI. In our study, 8.9% of all patients presenting to our hospital with acute respiratory syndromes were found to have an RV/EV infection. The majority of cases (nearly 60%) were in children under the age of 16, which is consistent with recent studies investigating the epidemiology of respiratory infections. The aforementioned studies indicate that the majority of RV/EV-positive patients were children under the age of 10 [23]. Additionally, a difference in the median age of children infected by different RV genotypes has been observed [24. 25, 26].

The majority of the observed episodes of RV infections were caused by RV species A and C, while RV-B accounted for approximately 10% of the total episodes. As previously described in other studies, a comparable distribution of RV species was observed worldwide, with a high incidence of RV-A and RV-C often present in equal or similar proportions [27, 28].

The most frequently detected viruses in coinfections were hRSV and AdV, while influenza viruses were never identified in coinfections with RV/EV. This finding is consistent with the results of numerous other studies investigating the epidemiology of respiratory viruses, which have identified hAdV and hBoV as the viruses most frequently detected in coinfections with RV/EV [26, 29]. In our study, the prevalence of coinfection with at least one additional virus was 23.4%. Other studies have reported frequencies of viral coinfection ranging from 9% [30] to 47% [31].

During the course of our study, two instances of intra-hospital transmission were observed, the first in October 2017 and the second in June 2018. The molecular epidemiology of RV/EV has enabled the identification of several outbreaks in neonatal settings, including those requiring mechanical ventilation [14, 32, 33]. Given that RVs are transmitted via aerosolisation or direct contact with an infected individual, it is possible that intra-hospital outbreaks may be caused by contaminated surfaces or staff members during the period of viral shedding following the resolution of symptoms, as has been postulated by Reese and colleagues [13].

In immunosuppressed patients, shedding of RV/EV has been observed for a prolonged period, and this occurrence seems to be correlated with an early phase post-transplant

[17, 34]. This prolonged infection has been sustained by active viral excretion, which can last several months [35]. A total of twelve cases of prolonged RV infection were observed in the course of this study. The majority of cases were observed in patients undergoing chemotherapy or post-transplant therapy, or in newborns. In the majority of cases, the clinical picture associated with this prolonged infection was relatively mild, although a small number of severe cases were also observed. A comparable scenario was also observed in patients with prolonged RV infection following lung transplantation where the majority of them was asymptomatic [36].

Although RV has been considered the causative agent of the common cold, our study found that 4.5% of RV/EV positive patients had pneumonia and were admitted ICU. This finding has been recently investigated in the context of other respiratory viruses. In 2015, Jain and colleagues reported that RV was the virus most frequently detected among ICU adult patients in the U.S., while in paediatric patients, RV was the second most common after hRSV, despite being detected at a similar percentage [37, 38]. Similar results were reported in Europe [39, 40] as well as in Asia [41]. These studies collectively reinforce the necessity to consider RV/EV as the causative agent of severe respiratory infections. Severe RV/EV infections have also been observed in patients with pre-existing comorbidities (17, 38, 42). A significant proportion of severe RV/EV infections were diagnosed based on LRT samples, indicating the importance of adequate sampling collection. Indeed, when both URT and LRT samples from the same patient were analysed for the presence of RV/EV, it was only detected in the LRT samples [19, 43, 44].

This study has several limitations. It is retrospective and the clinical data available for analysis is limited. Information regarding bacterial and fungal coinfection was only available for severe cases and was not investigated in the general population. Moreover, EV cases in our population were limited; hence, the data about them are just preliminary.

5. CONCLUSIONS

RVs/EVs circulate throughout the year, causing upper respiratory tract infections in immunocompetent subjects. However, they do also cause prolonged and severe infections requiring ICU admission in high-risk patients such as the older or immunocompromised populations. For this reason, RV/EV infection should be systematically monitored. The clinical impact of RV/EV infections is not limited only to the common cold, and these viruses should be considered as highly significant respiratory pathogens.

REFERENCES

1. Lamson D., Renwick N., Kapoor V., Liu Z., Palacios G., Ju J., Dean A., St George K., Briese T., Lipkin W.I. MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in

New York State during 2004–2005. J. Infect. Dis. 2006;194:1398–1402. doi: 10.1086/508551.

2. Monto A.S. The seasonality of rhinovirus infections and its implications for clinical recognition. Clin. Ther. 2002;24:1987–1997. doi: 10.1016/S0149-2918(02)80093-5.

3. Zacharie S., Vabret A., Guillois B., Dupont C., Brouard J. Rhinovirus: Des pathogènes sous-estimés chez les patients en période néonatale. Arch. Pediatr. 2017;24:825–832. doi: 10.1016/j.arcped.2017.06.012. (In French)

4. Drysdale S.B., Mejias A., Ramilo O. Rhinovirus—Not just the common cold. J. Infect. 2017;74((Suppl. S1)):S41–S46. doi: 10.1016/S0163-4453(17)30190-1.

5. Aydin Köker S., Demirağ B., Tahta N., Bayram N., Oymak Y., Karapinar T.H., Gözmen S., Düzgöl M., Erçan Bozyer H., Vergin C., et al. A 3-Year Retrospective Study of the Epidemiology of Acute Respiratory Viral Infections in Pediatric Patients with Cancer Undergoing Chemotherapy. J. Pediatr. Hematol. Oncol. 2019;41:e242–e246. doi: 10.1097/MPH.0000000000001418

6. Iwane M.K., Prill M.M., Lu X., Miller E.K., Edwards K.M., Hall C.B., Griffin M.R., Staat M.A., Anderson L.J., Williams J.V., et al. Human rhinovirus species associated with hospitalizations for acute respiratory illness in young US children. J. Infect. Dis. 2011;204:1702–1710. doi: 10.1093/infdis/jir634.

7. Louie J.K., Roy-Burman A., Guardia-Labar L., Boston E.J., Kiang D., Padilla T., Yagi S., Messenger S., Petru A.M., Glaser C.A., et al. Rhinovirus associated with severe lower respiratory tract infections in children. Pediatr. Infect. Dis. J. 2009;28:337–339. doi: 10.1097/INF.0b013e31818ffc1b.

8. Sangil A., Calbo E., Robles A., Benet S., Viladot M.E., Pascual V., Cuchí E., Pérez J., Barreiro B., Sánchez B., et al. Aetiology of community-acquired pneumonia among adults in an H1N1 pandemic year: The role of respiratory viruses. Eur. J. Clin. Microbiol. Infect. Dis. 2012;31:2765–2772. doi: 10.1007/s10096-012-1626-6.

9. Gern J.E. The ABCs of rhinoviruses, wheezing, and asthma. J. Virol. 2010;84:7418–7426. doi: 10.1128/JVI.02290-09.

10. Khetsuriani N., Lu X., Teague W.G., Kazerouni N., Anderson L.J., Erdman D.D. Novel human rhinoviruses and exacerbation of asthma in children. Emerg. Infect. Dis. 2008;14:1793–1796. doi: 10.3201/eid1411.080386.

11. Calvo C., Garcia M.L., Pozo F., Reyes N., Pérez-Breña P., Casas I. Role of rhinovirus C in apparently life-threatening events in infants, Spain. Emerg. Infect. Dis. 2009;15:1506–1508. doi: 10.3201/1509.090493.

12. Piralla A., Rovida F., Campanini G., Rognoni V., Marchi A., Locatelli F., Gerna G. Clinical severity and molecular typing of human rhinovirus C strains during a fall outbreak affecting hospitalized patients. J. Clin. Virol. 2009;45:311–317. doi: 10.1016/j.jcv.2009.04.016.

13. Reese S.M., Thompson M., Price C.S., Young H.L. Evidence of nosocomial transmission of human rhinovirus in a neonatal intensive care unit. Am. J. Infect. Control. 2016;44:355–357. doi: 10.1016/j.ajic.2015.10.018.

14. Marcone D.N., Carballal G., Irañeta M., Rubies Y., Vidaurreta S.M., Echavarría M. Nosocomial Transmission and Genetic Diversity of Rhinovirus in a Neonatal Intensive Care Unit. J. Pediatr. 2018;193:252–255.e1. doi: 10.1016/j.jpeds.2017.09.013.

15. Oberste M.S., Maher K., Schnurr D., Flemister M.R., Lovchik J.C., Peters H., Sessions W., Kirk C., Chatterjee N., Fuller S., et al. Enterovirus 68 is associated with respiratory illness and shares biological features with both the enteroviruses and the rhinoviruses. Pt 9J. Gen. Virol. 2004;85:2577–2584. doi: 10.1099/vir.0.79925-0.

16. Piralla A., Principi N., Ruggiero L., Girello A., Giardina F., De Sando E., Caimmi S., Bianchini S., Marseglia G.L., Lunghi G., et al. Enterovirus-D68 (EV-D68) in pediatric patients with respiratory infection: The circulation of a new B3 clade in Italy. J. Clin. Virol. 2018;99–100:91–96. doi: 10.1016/j.jcv.2018.01.005.

17. Piralla A., Lilleri D., Sarasini A., Marchi A., Zecca M., Stronati M., Baldanti F., Gerna G. Human rhinovirus and human respiratory enterovirus (EV68 and EV104) infections in hospitalized patients in Italy, 2008–2009. Diagn. Microbiol. Infect. Dis. 2012;73:162–167. doi: 10.1016/j.diagmicrobio.2012.02.019.

18. Engelmann I., Dewilde A., Lazrek M., Batteux M., Hamissi A., Yakoub-Agha I., Hober D. In Vivo Persistence of Human Rhinoviruses in Immunosuppressed Patients. PLoS ONE. 2017;12:e0170774. doi: 10.1371/journal.pone.0170774. Erratum in PLoS ONE 2017, 12, e0181296.

19. Piralla A., Pariani E., Rovida F., Campanini G., Muzzi A., Emmi V., Iotti G.A., Pesenti A., Conaldi P.G., Zanetti A., et al. Severe Influenza A Task Force. Segregation of virulent influenza A(H1N1) variants in the lower respiratory tract of critically ill patients during the 2010–2011 seasonal epidemic. PLoS ONE. 2011;6:e28332. doi: 10.1371/journal.pone.0028332.

20. Wisdom A., Leitch E.C., Gaunt E., Harvala H., Simmonds P. Screening respiratory samples for detection of human rhinoviruses (HRVs) and enteroviruses: Comprehensive VP4-VP2 typing reveals high incidence and genetic diversity of HRV species, C. J. Clin. Microbiol. 2009;47:3958–3967. doi: 10.1128/JCM.00993-09. [PMC free article]

21. Nix W.A., Oberste M.S., Pallansch M.A. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original

clinical specimens. J. Clin. Microbiol. 2006;44:2698–2704. doi: 10.1128/JCM.00542-06.

22. Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 2011;28:2731–2739. doi: 10.1093/molbev/msr121.

23. Kenmoe S., Sadeuh-Mba S.A., Vernet M.A., Penlap Beng V., Vabret A., Njouom R. Molecular epidemiology of Enteroviruses and Rhinoviruses in patients with acute respiratory infections in Yaounde, Cameroon. Influenza Other Respir. Viruses. 2021;15:641–650. doi: 10.1111/irv.12851.

24. van der Linden L., Bruning A.H., Thomas X.V., Minnaar R.P., Rebers S.P., Schinkel J., de Jong M.D., Pajkrt D., Wolthers K.C. A molecular epidemiological perspective of rhinovirus types circulating in Amsterdam from 2007 to 2012. Clin. Microbiol. Infect. 2016;22:1002.e9–1002.e14. doi: 10.1016/j.cmi.2016.08.007.

25. Kamau E., Onyango C.O., Otieno G.P., Kiyuka P.K., Agoti C.N., Medley G.F., Cane P.A., Nokes D.J., Munywoki P.K. An Intensive, Active Surveillance Reveals Continuous Invasion and High Diversity of Rhinovirus in Households. J. Infect. Dis. 2019;219:1049–1057. doi: 10.1093/infdis/jiy621.

26. Hung H.M., Yang S.L., Chen C.J., Chiu C.H., Kuo C.Y., Huang K.A., Lin T.Y., Hsieh Y.C., Gong Y.N., Tsao K.C., et al. Molecular epidemiology and clinical features of rhinovirus infections among hospitalized patients in a medical center in Taiwan. J. Microbiol. Immunol. Infect. 2019;52:233–241. doi: 10.1016/j.jmii.2018.08.009.

27. Esposito S., Daleno C., Tagliabue C., Scala A., Tenconi R., Borzani I., Fossali E., Pelucchi C., Piralla A., Principi N. Impact of rhinoviruses on pediatric community-acquired pneumonia. Eur. J. Clin. Microbiol. Infect. Dis. 2012;31:1637–1645. doi: 10.1007/s10096-011-1487-4.

28. Adam D.C., Chen X., Scotch M., MacIntyre C.R., Dwyer D., Kok J. The Molecular Epidemiology and Clinical Phylogenetics of Rhinoviruses Among Paediatric Cases in Sydney, Australia. Int. J. Infect. Dis. 2021;110:69–74. doi: 10.1016/j.ijid.2021.06.046.

29. Bruning A.H.L., Thomas X.V., van der Linden L., Wildenbeest J.G., Minnaar R.P., Jansen R.R., de Jong M.D., Sterk P.J., van der Schee M.P., Wolthers K.C., et al. Clinical, virological and epidemiological characteristics of rhinovirus infections in early childhood: A comparison between non-hospitalised and hospitalised children. J. Clin. Virol. 2015;73:120–126. doi: 10.1016/j.jcv.2015.10.024.

30. Zlateva K.T., van Rijn A.L., Simmonds P., Coenjaerts F.E.J., van Loon A.M., Verheij T.J.M., de Vries J.J.C., Little P., Butler C.C., van Zwet E.W., et al. GRACE Study Group. Molecular epidemiology and clinical impact of rhinovirus infections in

adults during three epidemic seasons in 11 European countries (2007–2010) Thorax. 2020;75:882–890. doi: 10.1136/thoraxjnl-2019-214317.

31. Zhao Y., Shen J., Wu B., Liu G., Lu R., Tan W. Genotypic Diversity and Epidemiology of Human Rhinovirus Among Children with Severe Acute Respiratory Tract Infection in Shanghai, 2013–2015. Front. Microbiol. 2018;9:1836. doi: 10.3389/fmicb.2018.01836.

32. El Idrissi K.R., Isabel S., Carbonneau J., Lafond M., Quach C., Caya C., Fontela P.S., Beltempo M., Boivin G., Lefebvre M.A., et al. Molecular and epidemiologic investigation of a rhinovirus outbreak in a neonatal intensive care unit. Infect. Control Hosp. Epidemiol. 2019;40:245–247. doi: 10.1017/ice.2018.311.

33. Reid A.B., Anderson T.L., Cooley L., Williamson J., Mcgregor A.R. An outbreak of human rhinovirus species C infections in a neonatal intensive care unit. Pediatr. Infect. Dis. J. 2011;30:1095–1096. doi: 10.1097/INF.0b013e31822938d7.

34. Gerna G., Piralla A., Rovida F., Rognoni V., Marchi A., Locatelli F., Meloni F. Correlation of rhinovirus load in the respiratory tract and clinical symptoms in hospitalized immunocompetent and immunocompromised patients. J. Med. Virol. 2009;81:1498–1507. doi: 10.1002/jmv.21548.

35. Tapparel C., Cordey S., Junier T., Farinelli L., Van Belle S., Soccal P.M., Aubert J.D., Zdobnov E., Kaiser L. Rhinovirus genome variation during chronic upper and lower respiratory tract infections. PLoS ONE. 2011;6:e21163. doi: 10.1371/journal.pone.0021163.

36. Ammerman E., Sweet S.C., Storch G.A., Buller R.S., Mason S., Conrad C., Hayes D., Jr., Faro A., Goldfarb S.B., Melicoff E., et al. Epidemiology and persistence of rhinovirus in pediatric lung transplantation. Transpl. Infect. Dis. 2020;22:e13422. doi: 10.1111/tid.13422.

37. Jain S., Self W.H., Wunderink R.G., Fakhran S., Balk R., Bramley A.M., Reed C., Grijalva C.G., Anderson E.J., Courtney D.M., et al. Community-Acquired Pneumonia Requiring Hospitalization among, U.S. Adults. N. Engl. J. Med. 2015;373:415–427. doi: 10.1056/NEJMoa1500245.

38. Jain S., Williams D.J., Arnold S.R., Ampofo K., Bramley A.M., Reed C., Stockmann C., Anderson E.J., Grijalva C.G., Self W.H., et al. Community-acquired pneumonia requiring hospitalization among, U.S. children. N. Engl. J. Med. 2015;372:835–845. doi: 10.1056/NEJMoa1405870.

39. Piralla A., Mariani B., Rovida F., Baldanti F. Frequency of respiratory viruses among patients admitted to 26 Intensive Care Units in seven consecutive winter-spring seasons (2009–2016) in Northern Italy. J. Clin. Virol. 2017;92:48–51. doi: 10.1016/j.jcv.2017.05.004.

40. Visseaux B., Burdet C., Voiriot G., Lescure F.X., Chougar T., Brugière O., Crestani B., Casalino E., Charpentier C., Descamps D., et al. Prevalence of respiratory viruses among adults, by season, age, respiratory tract region and type of medical unit in Paris, France, from 2011 to 2016. PLoS ONE. 2017;12:e0180888. doi: 10.1371/journal.pone.0180888.

41. Choi S.H., Hong S.B., Ko G.B., Lee Y., Park H.J., Park S.Y., Moon S.M., Cho O.H., Park K.H., Chong Y.P., et al. Viral infection in patients with severe pneumonia requiring intensive care unit admission. Am. J. Respir. Crit. Care Med. 2012;186:325–332. doi: 10.1164/rccm.201112-2240OC.

42. Wiemken T., Peyrani P., Bryant K., Kelley R.R., Summersgill J., Arnold F., Carrico R., McKinney W.P., Jonsson C., Carrico K., et al. Incidence of respiratory viruses in patients with community-acquired pneumonia admitted to the intensive care unit: Results from the Severe Influenza Pneumonia Surveillance (SIPS) project. Eur. J. Clin. Microbiol. Infect. Dis. 2013;32:705–710. doi: 10.1007/s10096-012-1802-8.

43. Piralla A., Baldanti F., Gerna G. Phylogenetic patterns of human respiratory picornavirus species, including the newly identified group C rhinoviruses, during a 1-year surveillance of a hospitalized patient population in Italy. J. Clin. Microbiol. 2011;49:373–376. doi: 10.1128/JCM.01814-10.

44. Baldanti F., Campanini G., Piralla A., Rovida F., Braschi A., Mojoli F., Iotti G., Belliato M., Conaldi P.G., Arcadipane A., et al. Severe outcome of influenza A/H1N1/09v infection associated with 222G/N polymorphisms in the haemagglutinin: A multicentre study. Clin. Microbiol. Infect. 2011;17:1166–1169. doi: 10.1111/j.1469-0691.2010.03403.x.

Chapter 8

Molecular characterization of emerging Echovirus 11 (E11) shed light on the recombinant origin of variant associated with severe hepatitis in neonates

Antonio Piralla, Federica Giardina, Guglielmo Ferrari, Stefano Gaiarsa, Greta Romano, Laura Pellegrinelli, Cristina Galli, Arlinda Seiti, Sandro Binda, Antonino Maria Guglielmo Pitrolo, Angelo Genoni, Francesca Drago Ferrante, Federica Novazzi, Nicasio Mancini, Francesca Rovida, Elena Pariani, Fausto Baldanti

Role: Second author

Author's contribution: Experiments performing and data analysis

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Molecular characterization of emerging Echovirus 11 (E11) shed light on the recombinant origin of variant associated with severe hepatitis in neonates

Antonio Piralla[1], Federica Giardina[2], Guglielmo Ferrari[1], Stefano Gaiarsa[1], Greta Romano[1], Laura Pellegrinelli[3], Arlinda Seiti[3], Sandro Binda[3], Antonino Maria Guglielmo Pitrolo[1], Angelo Genoni[4,5], Francesca Drago Ferrante[5], Federica Novazzi[4,5], Nicasio Mancini[4,5], Francesca Rovida[2,1], Elena Pariani[3], Fausto Baldanti[2,1]

[1] Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy;

[2] Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy;

[3] Department of Biomedical Sciences for Health, University of Milan, Milan, Italy;

[4] Laboratory of Medical Microbiology and Virology, Department of Medicine and Technical Innovation, University of Insubria, Varese, Italy;

[5] Laboratory of Medical Microbiology and Virology,University Hospital of Varese, Varese, Italy.

ABSTRACT

Echovirus 11 (E11) has recently gained attention due to its association with severe neonatal infections. Since the few data available, the World Health Organization (WHO) has determined that the public health risk to the general population is low. The present study aimed to investigate the genetic variation and molecular evolution of E11 genomes collected between May and December 2023 in comparison to those strains circulating in the previous years. Whole genome sequencing (WGS) was conducted on 16 E11 strains. A phylogenetic analysis of the WG demonstrated that all Italian strains belonged to genogroup D5, as the other E11 strains recently reported in France and Germany. These strains were found to be aggregated into separate clusters. A cluster-specific recombination pattern was also identified through phylogenetic analysis of different genome regions. Echovirus 6 was identified as the principal recombinant virus in the 3Cpro and 3Dpol regions. The molecular clock analysis indicated that the recombination event is likely to have occurred in June 2018 (95% highest posterior density interval: January 2016–January 2020). A Shannon entropy analysis of the P1 region revealed that 11 amino acids exhibited relatively high entropy. Five of the amino acids were identified in the canyon region, which is responsible for receptor binding with the neonatal Fc receptor. The present study demonstrated that the recombinant origin of a novel lineage of E11 is associated with severe neonatal infections.

Keywords: enteroviruses, neonatal infections, hepatitis, echovirus 11 (E-11), recombinant strains

1. INTRODUCTION

An upsurge in severe neonatal cases and fatalities associated with a novel variant of echovirus 11 (E11) has been documented in France and Italy and China [1-3]. The World Health Organization (WHO) has gained attention due to its association with severe neonatal infections since 2022 [4, 5]. Other cases have been reported in European countries such as Spain, Sweden, and the UK [4, 5]. However, the prevention and control of E11 variants have been hindered by a lack of background data on the virus's circulation and genetic variance. Furthermore, the WHO has evaluated the available data and determined that the risk to the general population is low. Given that non-polio enterovirus (NPEV) infections are not notifiable infectious diseases in Italy, the circulation of E11 in Lombardy (Northern Italy), where the first two cases of E11 neonatal infection were reported, has been implemented in the period April-December 2023. In detail, an event-based surveillance and a hospital-based surveillance considering inpatients exhibiting respiratory or neurological symptoms have shown a total of 20 additional cases [6]. Recently, a wastewater-based surveillance (WBS) performed in Sicily (a region in Southern Italy) between June 2022 and June 2023, showed that the new E11 lineage has circulated in this region since early 2023 [7]. This report, together with European data, showed a silent unrecognized circulation of this new E11 variant. The emergence of the novel E11 lineage has been attributed to recombination events, which appear to have facilitated the successful infectivity of this variant strain [1]. However, a comprehensive analysis exploring the parental genomes, genetic variability, and recombinant origin of this emerging variant is still lacking. Furthermore, the increasing pathogenic role of this variant must be fully elucidated using in vitro models. The present study aimed to investigate the genetic variation and molecular evolution of the E11 complete genomes. To this end, strains collected via our surveillance were sequenced and compared to the sequences obtained from the GenBank database.

2. MATERIALS AND METHODS

2.1 Sample collection

A total of 16 E11 strains identified through event-based and hospital-based surveillance were included in this study. The surveillance period spanned from May to December 2023, and the patients exhibited respiratory or neurological symptoms. The objective of this study is to describe and examine the molecular evolution of E11 strain obtained through WGS of the first two cases of E11 neonatal infection previously reported in Italy [2, 6]. All samples were analysed in two Lombardy regional reference laboratories (Microbiology and Virology department, Fondazione IRCCS Policlinico San Matteo,

Pavia, and Department of Biomedical Sciences for Health, University of Milan, Milan) as previously reported [2, 6]. Additional information on clinical samples is provided in Table S1.

2.2 Next-generation sequencing by metagenomic approach

Total RNA was extracted directly from clinical samples using the QIAsymphony® instrument with the QIAsymphony® DSP Virus/Pathogen Midi Kit (Complex 400 protocol (QIAGEN)) or the QIAamp Viral RNA Mini kit (QIAGEN) by means of an automated extractor (QIAcube, QIAGEN) according to the manufacturer's instructions. The RNA was then treated with TURBO DNase (Thermo Fisher Scientific, Waltham, MA) at 37°C for 20 minutes, after which it was purified using the RNA Clean and Concentrator-5 Kit (Zymo Research). RNA was used for the assessment of the sequencing independent single primer amplification protocol (SISPA), with some modifications as reported by Lorusso et al. [8]. Libraries were prepared using the Nextera DNA Flex Library Prep (Illumina Inc., San Diego, CA) according to the manufacturer's protocol. Sequencing was conducted on the MiSeq (Illumina Inc., San Diego, CA) using the MiSeq Reagent v2 (300-cycle) kit. The FastaQ sequences were analysed using the CZ ID metagenomic pipeline [9]. The E11 virus consensus sequences were obtained by mapping to the reference sequence with the highest coverage breadth and depth, obtained through the metagenomic pipeline. The accession numbers of the sequences generated in this study are as follows: PP498690-PP498703.

2.3 Phylogenetic analysis and recombinant analysis

All available complete genome sequences of E11 (n = 100 strains) were downloaded from GenBank and used in conjunction with the 16 E11 strains originating in this study. The alignment was performed using MAFFT 7.475[10]. Maximum likelihood (ML) trees were constructed in IQ-TREE 5.11 with a substitution model chosen according to BIC within the IQ-TREE [11] internal pipeline with 1,000 bootstrap replicates. Separate DNA similarity searches were conducted on the P1, P2, and P3 coding region sequences using the NCBI WWW-BLAST (basic local alignment search tool) server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) on the GenBank DNA database (BLAST+ v. 2.15.0). Sequences with greater than 85% similarity were considered prospective parental sequences and retrieved from GenBank. A data set of 15 enterovirus strains (listed in Figure 2 and Table S2), selected by the highest similarity score in 3Cpro-3Dpol region, the was employed for recombination signal screening using SimPlot software (version 3.5.1) [12]. A similarity and boot-scanning analysis was conducted using a 400-nucleotide sliding window and a 10-nucleotide shifting interval.

2.4 Phylodynamic analysis of recombinant genome region

The NCBI BLASTn program (https://blast.ncbi.nlm.nih.gov) was employed to perform multiple sequence alignments of the E-11 new lineage within the 3Cpro-3Dpol genome

region. The consensus sequences were examined for nucleotide identity percentages ranging from 85.0% to 100%, with a total of 63 sequences used for phylogenetic analysis. The phylodynamic analyses workflow is illustrated in Figure S1. In detail, MEGA version 11 [13] was employed to perform the alignment utilising the implemented MUSCLE algorithm and to construct the phylogenetic tree based on a Neighbour-joining method with 1,000 bootstrap replicates and a mean nucleotide genetic distance (p-distance). MEGA version 11 and ModelFinder from IQ-TREE v.2.2.2.6 [14] were employed to identify the optimal nucleotide substitution model, which was determined to be GTR + G + I. The temporal signal of the sequences was investigated using TempEst v.1.5.3 [15], which confirmed the presence of sufficient genetic change between sampling times (R2 = 0.82, correlation coefficient = 0.9). A Markov Chain Monte Carlo (MCMC) sampling method, implemented in BEAST v1.10.4 [16], a Bayesian statistical framework, was employed to perform the phylogenetic analysis. The years of sample collection (retrieved from GenBank) were used to calibrate the molecular clock. The analyses were conducted using an uncorrelated lognormal clock with a constant-size model of demographic history. The default priors on the substitution model parameters (GTR + G) were employed in these analyses. The parameter estimates were obtained from a Markov chain Monte Carlo (MCMC) run comprising 2 × 108 generations and a sampling frequency of 10^3. The performance of the transition kernel was evaluated, and the acceptance ratio was found to be greater than 0.234. The posterior distributions for each parameter were visualised with Tracer v.1.7.2 [16], a Markov chain Monte Carlo trace analysis tool which also estimated the effective sample size (ESS) (i.e., the measurement of the number of effectively independent samples in each run) of the parameters sampled from the MCMC. The analysis was deemed to have reached convergence and stability following the burn-in period, with an ESS value exceeding 200. A maximum clade credibility tree was estimated with TreeAnnotator v1.10.4 [16] with the first 10% of trees removed as burn-in. The statistical support for the nodes in the topology was assessed by a posterior probability (PP) value. The resulting tree was visualized using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree).

2.5 Genome mutations analysis

The presence of conserved non-synonymous mutations between the two putative donor genomes E11 (OQ966171) and E6 (OR840838) and the new lineage sequences (n = 37 E11 strains) was assessed using the Snipit pipeline (https://github.com/aineniamh/snipit) [17] which was modified to select non-synonymous mutations and to work with amino acid notation.

2.6 Shannon entropy analysis for measuring diversity

Amino acid variability of the P1 capsid precursor protein (VP4-VP2-VP3-VP1) was assessed using Shannon entropy on the E11 sequences data set (n = 116) used for phylogenetic analysis (described above). Shannon entropy was assessed for all 861

amino acids of P1 using an online analysis tool (available at https://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_one.html) [18] with extremely variable amino acid sites defined as those with entropy values > 0.6. The ChimeraX program [19] was used to visualize the distinct distributions of significant sites based on the 6LA6 model from the Protein Data Bank (PDB) database showing the concealed surface area between the FcRn receptor and capsid proteins (https://www.rcsb.org/structure/6la6).

3. RESULTS

3.1 Whole genome sequence analysis of E11 strains

A total of 16 E11 WGS strains were included in the present study and subjected to analysis. A large open reading frame (ORF) encoding a potential polyprotein precursor of 2195 amino acids (aa) was cleaved into three regions: P1 (VP4, VP2, VP3, and VP1), P2 (2Apro, 2B, and 2C), and P3 (3A, 3B, 3Cpro, and 3Dpol) with 861 aa, 578 aa, and 756 aa, respectively. A data set was constructed for phylogenetic analysis using all available E11 sequences (n = 100) retrieved from GenBank. The phylogenetic tree demonstrated that all 16 strains belonged to genogroup D5, which is similar to other E11 strains that have been recently reported in France and Germany, according to Savolainen-Kopra et al. (2009) [20]. All strains were aggregated into a separate lineage, which included 37 E11 strains collected between 2022 and 2023 (Figure 1A). The average nucleotide genetic identity within this new lineage was 98.7% (range 97.9%–100%). Moreover, nucleotide identity comparisons of WGS demonstrated that the E11 strain, which belonged to a new lineage, had an average of nucleotide genetic identity of 85.1% (range 81.9%–94.2%) compared to other E11 reference strains available in the GenBank database (n = 79 strains). A cluster-specific recombination pattern was also identified through phylogenetic analysis of the P1, P2, and P3 genome regions, which were analysed separately (data not shown). In the P1 and P2 capsid coding regions, the new lineage strains formed a cluster with the strain PMB_230005716902_FRA_2023 (OQ969171, Figure 1B). In the phylogenetic tree based on the P3 sequences, the new lineage strains were found to cluster outside the E11 tree as an outgroup (data not shown). A BLAST analysis of the P3 region sequence revealed a high nucleotide identity of >95% with the Echovirus 6 strain EV6_Fr22_MAR9310 (OR840838, published in GenBank on 18 December 2023). This suggests the occurrence of one putative recombination event.

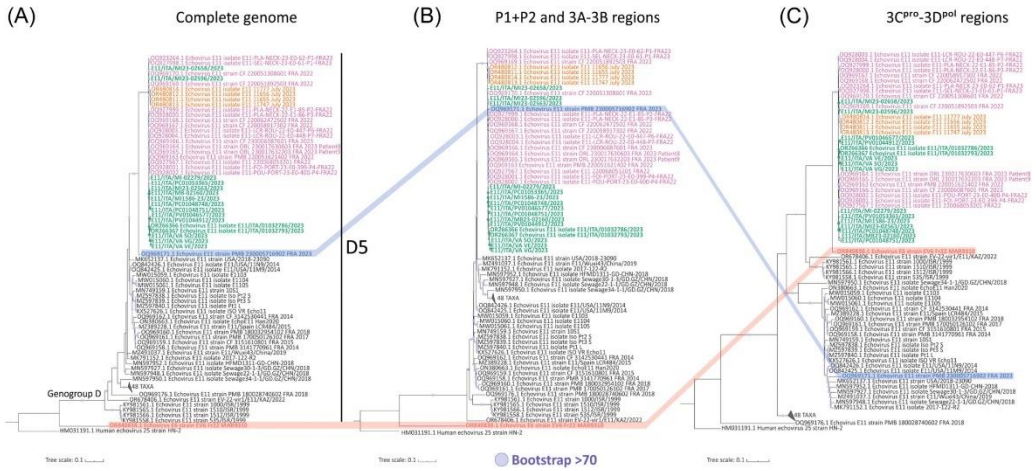


Figura 1. ML Phylogenetic trees constructed based on (A) complete genome (B) P1-P2 and 3A-3B (C) 3Cpro-3Dpol genome regions. Italian E11 strains (n = 16) are colored in green and their most related enterovirus sequences are reported in cerulean (E11, OQ696171) and coral (E6; OR840838), respectively. All available E11strains belonged to new lineage (n = 17 collected in France in 2022-2023 reported in purple and n = 4 collected in Germany in 2023 reported in orange) are included. Scale bars represent the replacement of each site per year.

3.2 Recombination and evolutionary analysis

The BLAST analysis, when combined with the phylogenetic trees presented in Figure 1, indicated that E6 was the primary candidate for the 3Cpro and 3Dpol regions (Figure 1C). The SimPlot software was employed to ascertain the position of the recombination site, utilising all 37 E11 strains, which constituted the query sequences and were assigned to the novel lineage (Figure 2A). The results of the SimPlot analysis demonstrated that the E11 strains exhibited the highest degree of similarity with the E11 (OQ969171) prototype strain in the P1, P2, and 3A-3B regions. In contrast, the highest similarity score was observed in the 3Cpro and 3Dpol regions with the E6 strain (OR840838). The phylogenetic trees constructed with the selected sequences (Figure 1B and C) were in accordance with the findings of SimPlot (Figure 2A) and BootScanning (Figure 2B), which validated the results of the recombination analysis. In order to ascertain the timing of the recombination event, a phylodynamic analysis was conducted utilising an alignment of multiple sequences of the 3Cpro and 3Dpol regions, based on similarities obtained from BLAST analysis. The phylogenetic relationships inferred by the Beast package using the best-fit models were employed to describe the topology of the samples of E11 new lineage strains and E6 as the parental genome of the 3Cpro and 3Dpol regions. A Bayesian molecular clock analysis was conducted to infer the maximum clade credibility (MCC) tree, which is presented in Figure 3. The Bayesian analysis yielded an estimated mean evolutionary substitution rate of 6.32 × 10-3 substitutions per site per year (95% highest posterior density interval: 4.31 × 10-3–8.57 × 10-3). The molecular clock analysis indicated that the recombination event is likely to have occurred in June

2018 (95% HPD interval: January 2016–January 2020; Figure 3). This analysis also assumes that the common ancestor for E11 (new lineage) and E6 strains (OR840838) is located in the 3Cpro and 3Dpol regions.

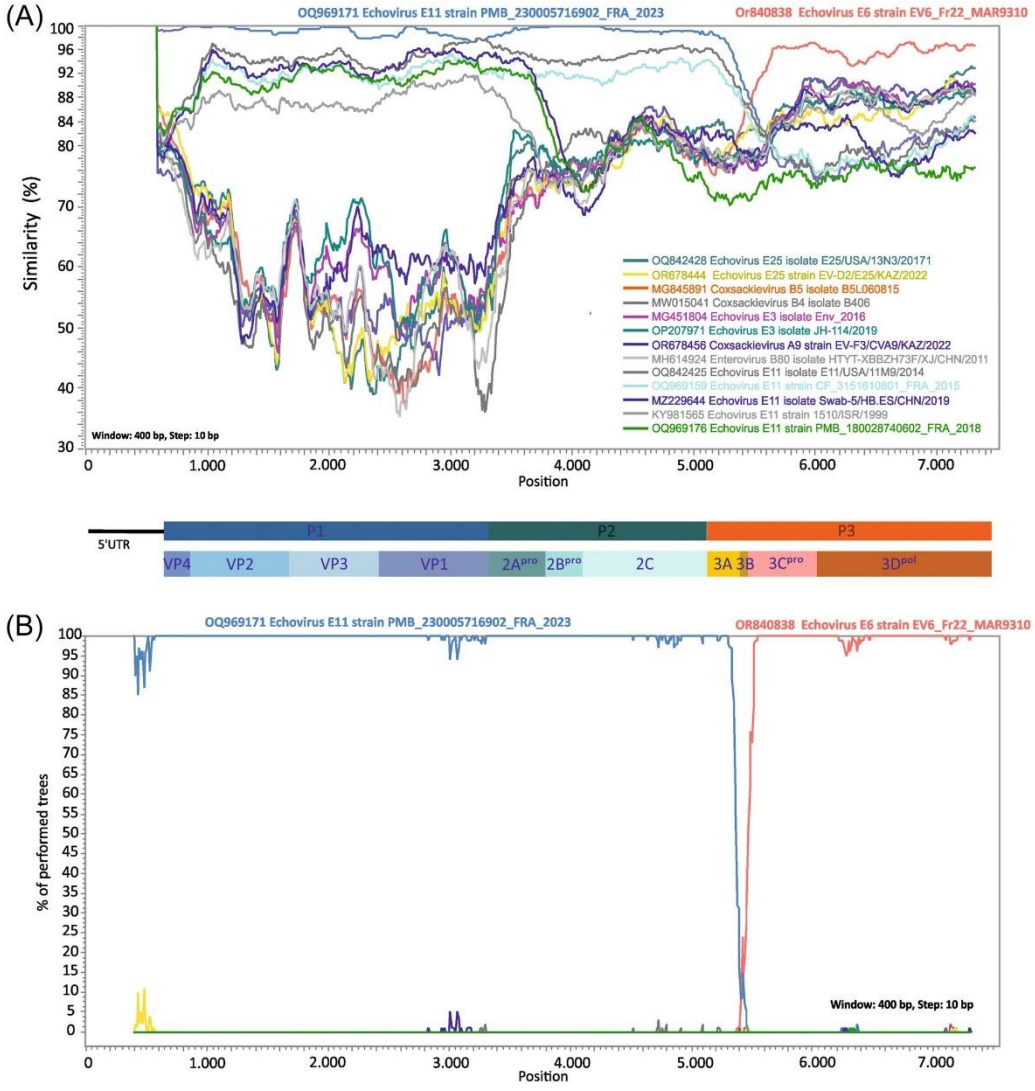


Figure 2. Recombination analyses of the E11 new lineage strains used as query (n = 37; n = 16 Italian, n = 17 from France1 and n = 4 from Germany) with other EV-B strains. (A) Similarity plots and (B) boot scanning analyses of strains with potential parents.

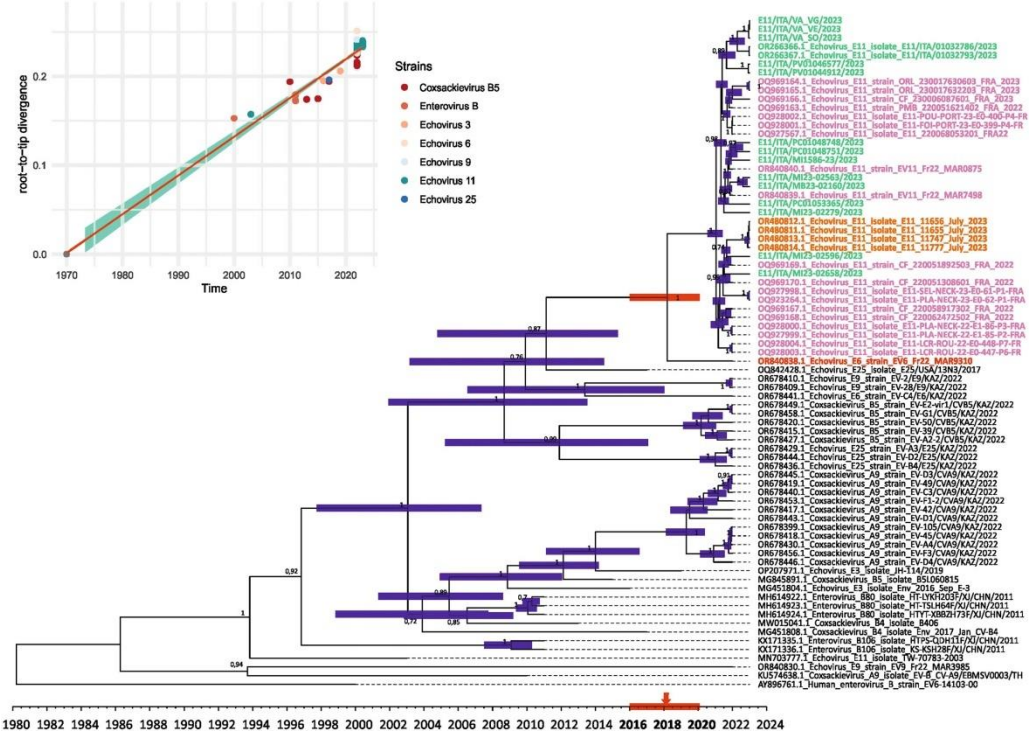


Figure 3. The MCC phylogenetic tree was generated using the MCMC method based on 3Cpro-3Dpol nucleotide sequences of Enterovirus strains with blast nucleotide identity results >85%. The blue bars indicate the 95% highest posterior density (HPD) for ancestor estimates. The HPD estimation of recombinant event is reported with a red bar. The x-axis is the time scale (years).

3.3 Genetic variance of genome

To identify crucial amino acid mutation sites that may affect the virulence of the variants, the modified Snipit script (https://github.com/aineniamh/snipit) was employed to illustrate the relative changes of each amino acid site in comparison to the most related strains. A total of 102/2195 (4.6%) amino acid positions were observed to have at least one change in the coding sequences alignment as compared to the parental genomes (E11, OQ696171 and E6, OR840838) (Figure 4). Of the observed changes, 40 (39.2%) occurred within the P1 region, which encompasses structural proteins. A further 28 (57.5%) occurred within the P2 region, 8 (7.8%) within the 3A-3B region, and 26 (25.5%) within the recombinant 3Cpro and 3Dpol regions. Among the changes observed in the P1 region, 21/40 (52.5%) were found to be located within the VP1 protein. However, none of these changes were observed in the BC- and DE-loop regions. A total of 11 amino acid changes were fixed in all the E11 strains belonging to the novel lineage.

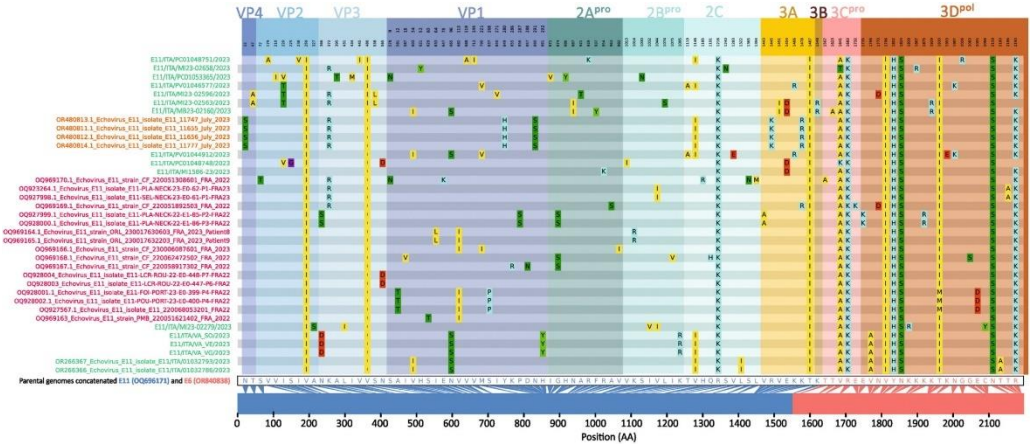


Figure 4. The figure was rendered using the Snipit tool and modified pipeline for amino acid visualization. (https://github.com/aineniamh/snipit). Italian E11 strains are colored in sea green. All available E11 strains belonged to new lineage (n = 17 from France1 reported in purple and n = 4 from Germany reported in orange) are included.

Furthermore, an alignment of P1 sequences was examined using the Shannon entropy online analysis tool. A total of 11 amino acids (VP2, 136 and 138; VP3, 35, 64, 135, and 234; VP1, 92, 144, 235, 262 and 268) exhibited relatively high entropy values (greater than 0.60), indicating a notable degree of amino acid diversity (Figure 5A). The cryo-electron microscopic structure of E11 and FcRn was available and used to illustrate the results of the entropy analysis (Figure 5B). Seven amino acids were selected for analysis, with the assumption that they would be exposed on the surface of the capsid in a canyon region responsible for receptor binding. This assumption was confirmed by the results of the entropy analysis, which indicated that these amino acids were indeed exposed on the surface of the capsid (Figure 5B).

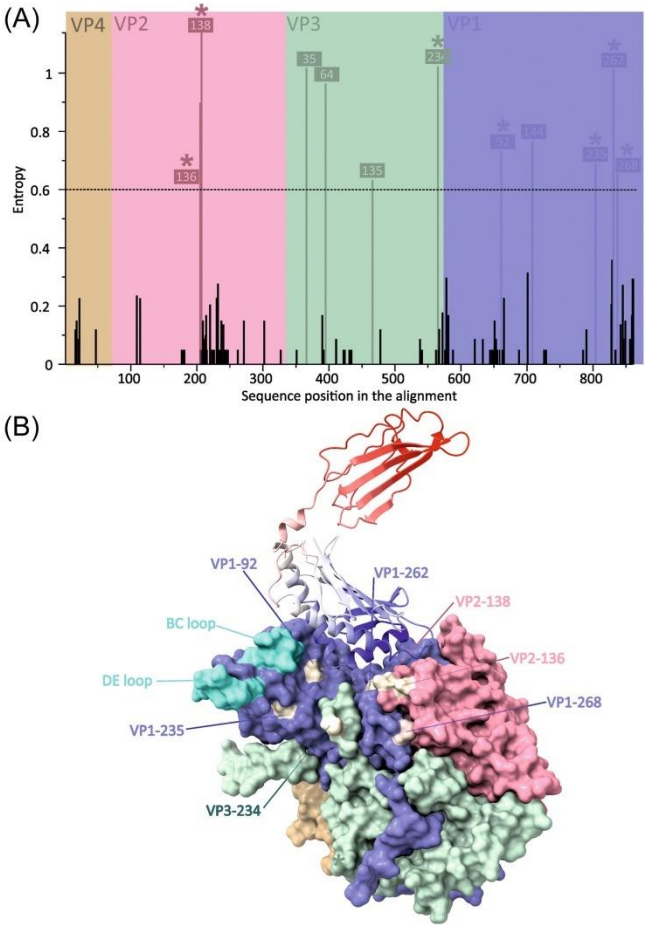


Figure 5. Analysis of the genetic variance of all available E11 sequences (n = 116). (A) Entropy value analysis of all 861 amino acids of the P1 region with a threshold value of 0.6. Position of amino acids with value > 0.6 are reported with a box. (B) 6LA6 as a model to illustrate the distribution of each amino acid site in E11 virus particles. The proteins are colored by chains: VP1 (blue), VP2 (rose gold), VP3 (green), VP4 (yellow). The potential interaction between amino acid sites and the FcRn receptor is also showed in the model. BC and DE loops are highlighted in cyan. Amino acids selected by entropy analysis and exposed at the surface of the 3D model and colored in white and reported with an asterisk in the panel A.

4. DISCUSSION

The evolutionary history of E11 has been well characterised since 2004, with the phylogenetic analysis of E-11 isolates identifying several genogroups, including A, B, C, and D1–D5 [21]. The monitoring of E11 evolution has confirmed the prevalence of genogroup D5, with distinct strains emerging over the past 15 years [20, 22]. Since the summer of 2022, a divergent lineage of E11 belonging to genogroup D5 has been

associated with an increased number of hepatitis episodes in neonates in Europe [4]. Since 2018, severe neonatal infections and mortality associated with genogroup D5 of E11 have been reported in Taiwan, and in the Hubei and Guangdong provinces of China [23, 24]. Furthermore, this scenario has been described in a retrospective study in China reporting data from an E11 outbreak that occurred in 2019 [3].

In this report, severe infections, defined as hepatic dysfunction or liver failure, were observed in 30 out of 105 (28.6%) neonates [3]. However, a recently published commentary of this study raises concerns about the results of previous studies and potential new studies that use genomic data to draw conclusions on the association of E11 and the unexpected clinical picture [25]. The primary medical concerns that have emerged from a general standpoint are the lack of notifiable status for enterovirus infections in numerous EU countries. Consequently, there may be more cases than are currently known. This observation is directly linked to the unrecognized clinical presentations of the majority of enterovirus infections.

Previous studies have indicated that more than 90% of patients with E11 infection are asymptomatic or present with mild fever [26]. Additionally, some enteroviruses may remain undetected for years before suddenly reappearing [27]. The lack of a notifiable disease status has resulted in low awareness among healthcare providers, which may contribute to an underdiagnosis of emerging enterovirus variants. An additional value could be obtained through wastewater surveillance, which can provide an early warning of viral spread in communities and offer crucial information about virus circulation and prevalence, as currently utilized for poliovirus [28].

In this setting, an increased detection rate of E11 belonging to the new lineage in wastewater samples since August 2022 in the Sicily region (Southern Italy) has recently been reported [7]. These findings suggest that WBS is an important tool for enterovirus surveillance to promptly detect the emergence or re-emergence of variants that warrant public health control measures. Phylogenetic analysis of WGS revealed the dissemination of a monophyletic lineage encompassing E11 strains identified in Italy, France, and Germany [1, 2, 4]. However, numerous other EU countries, including Croatia, Spain, Sweden, and the UK, have reported E11 cases during the 2022-2023 period, yet no sequences are currently available [4]. Despite the limited public health impact assigned by the WHO to this new E11 lineage, the implementation of a consolidated surveillance system is needed [4]. A phylogenetic incongruence between structural and nonstructural genes was observed, suggesting that recombination events occurred. However, the similarity as well as the bootscan analyses presented in the French study [1] were performed without the parental genome. In our study, the recombinant origin proposed by Grapin et al. [1] was proved because of the deposition on 18 December 2023 of a WGS of the E6 strain (OR840838, Echovirus E6 strain EV6_Fr22_MAR9310) in the public GenBank database. This strain was identified in France in 2022. Consequently, further analyses utilising diverse methodologies, including phylogenetic analysis and algorithms for sequence similarity and recombination detection (bootscanning), demonstrated that E6 served as the parental

donor of the 3Cpro and 3Dpol genome regions for the novel E11 lineage. Recombination is a well-known mechanism for enterovirus evolution, as previously observed for E11 [29, 30, 31]. However, none of these reports showed recombination in the 3Cpro and 3Dpol regions.

The precise identification of individual recombination events in E11 was achieved through the use of time-correlated tree analysis and the superimposition of branching points in the 3Cpro and 3Dpol tree, which had previously been employed for EV-71 [32]. The tMRCA of the new lineage of E11 and E6 viruses was dated between 2016 and 2020, suggesting a silent circulation of E11 until its emergence in 2022. This finding is also supported by the evolutionary rate calculated for E11, which is congruent with the estimated substitution rates for NPEV viruses as determined by others [32, 33]. Amino acid alterations in structural proteins have been demonstrated to influence viral virulence, as evidenced by the observations made in the context of NPEV [34].

In the present study, a sequence comparison of the P1 region identified several positions exhibiting a significant Shannon entropy, not only in the VP1 region. A series of seven amino acids (VP2, 136 and 138; VP3, 234; VP1, 92, 235, 262 and 268) with increasing entropy are located in close proximity to the "canyon" of the receptor-binding regions within the monomeric structure of the capsid. This is a key area governing the binding of the FcRn receptor [35]. Mutations occurring at this specific site may affect the binding and uncoating process of E11, thereby increasing its transmission ability. None of the selected positions were situated in the critical binding regions for neutralising antibodies that correspond to the BC or DE loop within the VP1 protein. Mutations in these epitopes have been associated with the virus's ability to evade the immune system [36]. While several specific amino acid mutations were observed in the present study, their significance requires further investigation. The impact of recombination on the virulence or pathogenesis of the new E11 lineage remains to be defined and requires further experimental investigation. In many cases, other factors, in addition to the genetic backbone of E11, may have contributed to the severity of these infections, including premature birth, lack of maternal immunity, and the young age of the infants.

In conclusion, the present study demonstrated that the recombinant origin of a novel lineage of E11 is associated with severe neonatal infections. Further studies are required to elucidate the increased pathogenicity of the E11 variant and to better correlate genetic information with unexpected clinical presentations. Whole genome sequencing (WGS) of enteroviruses is required to ascertain the prevalence of recombinant strains and to more accurately evaluate the phylodynamic and phylogeographic characteristics in the context of the molecular epidemiology of emerging enterovirus variants.

REFERENCES

1. Grapin M, Mirand A, Pinquier D, et al. Severe and fatal neonatal infections linked to a new variant of echovirus 11, France, July 2022 to April 2023. Euro Surveill. 2023; 28(22):2300253.
2. Piralla A, Borghesi A, Di Comite A, et al. Fulminant Echovirus 11 hepatitis in male non-identical twins in northern Italy, April 2023. Euro Surveill. 2023; 28(24):2300289.
3. Wang P, Xu Y, Liu M, et al. Risk factors and early markers for echovirus type 11 associated haemorrhage-hepatitis syndrome in neonates, a retrospective cohort study. Front Pediatr. 2023; 11:1063558.
4. World Health Organization. Enterovirus-Echovirus 11 Infection - the European Region. WHO. Accessed February 27, 2024. https://www.who.int/emergencies/disease-outbreak-news/item/2023-DON474
5. Word Health Organization. Enterovirus Infection - France. WHO, 2024. Accessed February 27, 2024. https://www.who.int/emergencies/disease-outbreak-news/item/2023-DON469
6. Pellegrinelli L, Galli C, Giardina F, et al. Increased circulation of echovirus 11 in the general population and hospital patients as elicited by the non-polio enterovirus laboratory-based sentinel surveillance in northern Italy, 2023. Int J Infect Dis. 2024; 142:106998. doi:10.1016/j.ijid.2024.106998
7. Giammanco GM, Filizzolo C, Pizzo M, et al. Detection of Echovirus 11 lineage 1 in wastewater samples in Sicily. Sci Total Environ. 2024; 918:170519. doi:10.1016/j.scitotenv.2024.170519
8. Lorusso A, Calistri P, Mercante MT, et al. A “One-Health” approach for diagnosis and molecular characterization of SARS-CoV-2 in Italy. One Health. 2020; 10:100135.
9. Kalantar KL, Carvalho T, de Bourcy CFA, et al. IDseq-An open source cloud-based pipeline and analysis service for metagenomic pathogen detection and monitoring. Gigascience. 2020; 9(10):giaa111.
10. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013; 30(4): 772-780. doi:10.1093/molbev/mst010
11. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol. 2015; 32(1): 268-274.
12. Lole KS, Bollinger RC, Paranjape RS, et al. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol. 1999; 73: 152-160.
13. Tamura K, Stecher G, Kumar S. MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol. 2021; 38: 3022-3027.

14. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. ModelFinder: fast model selection for accurate phylogenetic estimates. Nature Methods. 2017; 14(6): 587-589.
15. Rambaut A, Lam TT, Max Carvalho L, Pybus OG. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). Virus Evol. 2016; 2(1):vew007.
16. Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. Virus Evol. 2018; 4(1):vey016.
17. O'Toole Á: Aineniamh/Snipit. 2021. https://github.com/aineniamh/snipit
18. Ming D, Cohn JD, Wall ME. Fast dynamics perturbation analysis for prediction of protein functional sites. BMC Struct Biol. 2008; 8:5. doi:10.1186/1472-6807-8-5
19. Meng EC, Goddard TD, Pettersen EF, et al. UCSF ChimeraX: tools for structure building and analysis. Prot Sci. 2023; 32:e4792.
20. Savolainen-Kopra C, Al-Hello H, Paananen A, et al. Molecular epidemiology and dual serotype specificity detection of echovirus 11 strains in Finland. Virus Res. 2009; 139(1): 32-38. doi:10.1016/j.virusres.2008.10.003
21. Oberste MS, Maher K, Pallansch MA. Evidence for frequent recombination within species human enterovirus B based on complete genomic sequences of all thirty-seven serotypes. J Virol. 2004; 78(2): 855-867. doi:10.1128/jvi.78.2.855-867.2004
22. Al-Hello H, Paananen A, Eskelinen M, et al. An enterovirus strain isolated from diabetic child belongs to a genetic subcluster of echovirus 11, but is also neutralised with monotypic antisera to coxsackievirus A9. J Gen Virol. 2008; 89(Pt 8): 1949-1959. doi:10.1099/vir.0.83474-0
23. Ho S-Y, Chiu C-H, Huang Y-C, et al. Investigation and successful control of an echovirus 11 outbreak in neonatal intensive care units. Pediatr Neonatol. 2020; 61: 180-187.
24. Lu J, Kang M, Zeng H, et al. Tracking echovirus eleven outbreaks in Guangdong, China: a metatranscriptomic, phylogenetic, and epidemiological study. Virus Evol. 2020; 6:veaa029.
25. Al-Hello H, Blomqvist S, Savolainen-Kopra C. Commentary: risk factors and early markers for echovirus type 11 associated haemorrhage-hepatitis syndrome in neonates, a retrospective cohort study. Front Pediatr. 2024; 12:1338097. doi:10.3389/fped.2024.1338097
26. Chuang YY, Huang YC. Enteroviral infection in neonates. J Microbiol Immunol Infect. 2019; 52(6): 851-857. doi:10.1016/j.jmii.2019.08.018
27. Forero EL, Knoester M, Gard L, et al. Changes in enterovirus epidemiology after easing of lockdown measures. J Clin Virol. 2023; 169:105617.
28. World Health Organization. Global Polio Eradication Initiative—Guidelines on Environmental Surveillance for Detection of Polioviruses. 2015a. Accessed February 12, 2024. http://polioeradication.org/wp-content/uploads/2016/07/GPLN_GuidelinesES_April2015.pdf

29. Chevaliez S, Szendröi A, Caro V, et al. Molecular comparison of echovirus 11 strains circulating in Europe during an epidemic of multisystem hemorrhagic disease of infants indicates that evolution generally occurs by recombination. Virology. 2004; 325(1): 56-70.
30. Gong YN, Yang SL, Chen YC, Liu YC, Huang YC, Tsao KC. Novel intertypic recombination of Echovirus 11 in the Enterovirus species B. J Med Virol. 2024; 96(1):e29323. doi:10.1002/jmv.29323
31. Rueca M, Lanini S, Giombini E, et al. Detection of recombinant breakpoint in the genome of human enterovirus E11 strain associated with a fatal nosocomial outbreak. Virol J. 2022; 19(1): 97. doi:10.1186/s12985-022-01821-2
32. McWilliam Leitch EC, Cabrerizo M, Cardosa J, et al. The association of recombination events in the founding and emergence of subgenogroup evolutionary lineages of human enterovirus 71. J Virol. 2012; 86(5): 2676-2685. doi:10.1128/JVI.06065-11
33. Lukashev AN, Vakulenko YA. Molecular evolution of types in non-polio enteroviruses. J Gen Virol. 2017; 98(12): 2968-2981. doi:10.1099/jgv.0.000966
34. Bek EJ, McMinn PC. Recent advances in research on human enterovirus 71. Future Virol. 2010; 5: 453-468.
35. Molecular and structural basis of Echovirus 11 infection by using the dual-receptor system of CD55 and FcRn. CSB, 65(1): 67-79.
36. Zhang Y, Li J, Li Q. Immune evasion of enteroviruses under innate immune monitoring. Front Microbiol. 2018; 9:1866. doi:10.3389/fmicb.2018.01866.

Supplementary materials

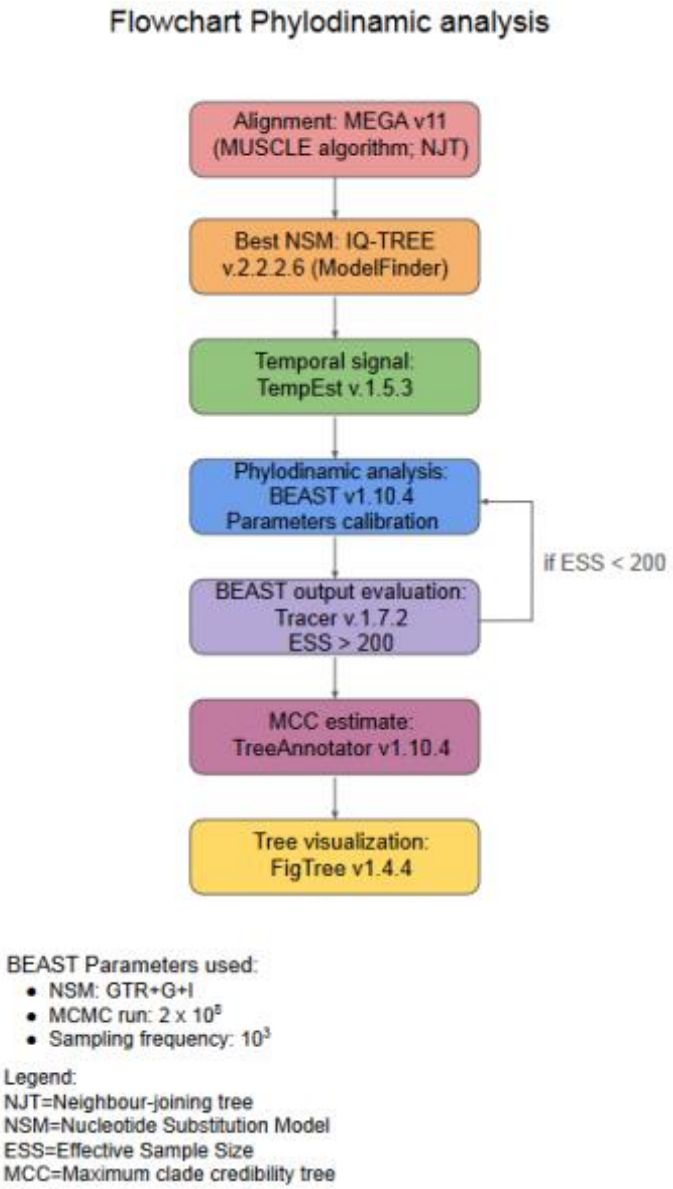
Table S1. E11 strains originated in the present study.

Strain name	Accession number	Type of sample	date
E11/ITA/VA-VG/2023	PP498690	pharyngeal swab	Dec-2023
E11/ITA/VA-VE/2023	PP498691	nasal swab	Dec-2023
E11/ITA/VA-SO/2023	PP498692	vagino-rectal swab	Dec-2023
E11/ITA/PV01044912/2023	PP498693	blood	Jun-2023
E11/ITA/MB23-02160/2023	PP498694	nasopharyngeal aspirate	May-2023
E11/ITA/MI23-02563/2023	PP498695	nasopharyngeal aspirate	Apr-2023
E11/ITA/MI23-02506/2023	PP498696	nasopharyngeal aspirate	Jun-202
E11/ITA/MI23-02279/2023	PP498697	nasopharyngeal aspirate	May-2023
E11/ITA/MI23-01586/2023	PP498698	nasopharyngeal aspirate	Apr-2023
E11/ITA/PC01053365/2023	PP498699	nasopharyngeal aspirate	Jul-2023
E11/ITA/PV01046448/2023	PP498700	bronchoalveolar lavage	Jun-2023
E11/ITA/MI23-02658/2023	PP498701	nasopharyngeal aspirate	Jul-2023
E11/ITA/PC01048751/2023	PP498702	nasopharyngeal aspirate	Jun-2023
E11/ITA/PC01048748/2023	PP498703	nasopharyngeal aspirate	Jun-2023
E11/ITA/01032786/2023	OR266366	urine	Apr-2023
E11/ITA/01032793/2023	OR266367	plasma	Apr-2023

Table S2. Enterovirus species B strains included in the recombinant analysis.

Accession number	**Strain name**
OQ969171	Echovirus E11 strain PMB_230005716902_FRA_2023
OR840838	Echovirus E6 strain EV6_Fr22_MAR9310
OQ842428	Echovirus E25 isolate E25/USA/13N3/20171
OR678444	Echovirus E25 strain EV-D2/E25/KAZ/2022
MG845891	Coxsackievirus B5 isolate B5L060815
MW015041	Coxsackievirus B4 isolate B406
MG451804	Echovirus E3 isolate Env_2016
OP207971	Echovirus E3 isolate JH-114/2019
OR678456	Coxsackievirus A9 strain EV-F3/CVA9/KAZ/2022
MH614924	Enterovirus B80 isolate HTYT-XBBZH73F/XJ/CHN/2011
OQ842425	Echovirus E11 isolate E11/USA/11M9/2014
OQ969159	Echovirus E11 strain CF_3151610801_FRA_2015
MZ229644	Echovirus E11 isolate Swab-5/HB.ES/CHN/ 2019
KY981565	Echovirus E11 strain 1510/ISR/1999
OQ969176	Echovirus E11 strain PMB_180028740602_FRA_2018

Figure S1. Flow chart of steps included in the phylodynamic analysis.



Chapter 9

On the lookout for influenza viruses in Italy during the 2021-2022 season: along came A(H3N2) viruses with a new phylogenetic makeup of their hemagglutinin

Cristina Galli, Laura Pellegrinelli, Federica Giardina, Guglielmo Ferrari, Sara Colonia Uceda Renteria, Federica Novazzi, Elisa Masi, Elisabetta Pagani, Giulia Piccirilli, Maria Vittoria Mauro, Sandro Binda, Benedetta Corvaro, Claudia Tiberio, Eleonora Lalle, Fabrizio Maggi, Cristina Russo, Stefania Ranno, Elisa Vian, Elena Pariani, Fausto Baldanti, Antonio Piralla, on behalf of the AMCLI-GLIVIRE working group

Role: Second author

Author's contribution: Methodology, Writing – review and editing

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On the lookout for influenza viruses in Italy during the 2021-2022 season: along came A(H3N2) viruses with a new phylogenetic makeup of their hemagglutinin

Cristin Galli[a,1], Laura Pellegrinelli[a,1], Federica Giardina[b], Guglielmo Ferrari[b], Sara Colonia Uceda Renteria[c], Federica Novazzi[d,e], Elisa Masi[f], Elisabetta Pagani[f], Giulia Piccirilli[g], Maria Vittoria Mauro[h], Sandro Binda[a], Benedetta Corvaro[i], Claudia Tiberio[j], Eleonora Lalle[k], Fabrizio Maggi[k], Cristina Russo[l], Stefania Ranno[l], Elisa Vian[m], Elena Pariani[a,2], Fausto Baldanti[b,n,2], Antonio Piralla[b,2], on behalf of the AMCLI-GLIVIRE working group

[a]*Department of Biomedical Sciences for Health, University of Milan, Milan, Italy;*

[b]*Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy;*

[c]*Virology Unit, Clinical Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy;*

[d]*Ospedale di Circolo e Fondazione Macchi, ASST Sette Laghi, Varese, Italy;*

[e]*University of Insubria, Varese, Italy;*

[f]*Laboratorio aziendale di Microbiologia e Virologia, Hospital of Bolzano, SABES-ASDAA), Bolzano-Bozen, Italy,*

[g]*Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy;*

[h]*Microbiology & Virology Unit, Annunziata Hub Hospital, Azienda Ospedaliera di Cosenza, Cosenza, Italy;*

[i]*Virology Laboratory, Azienda Ospedaliera Ospedali Riuniti di Ancona, Ancona, Italy;*

[j]*Microbiology and Virology, Cotugno Hospital AORN dei Colli, Naples, Italy;*

[k]*Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani, Rome, Italy;*

[l]*Department of Diagnostic and Laboratory Medicine, Unit of Microbiology and Diagnostic Immunology, Bambino Gesù Children Hospital IRCCS, Rome, Italy;*

[m]*Microbiology Unit, Azienda ULSS2 Marca Trevigiana, Treviso, Italy;*

[n]*Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy.*

ABSTRACT

The objective of this study is to assess the circulation of influenza viruses (IVs) and to evaluate the molecular evolution of A(H3N2) during the 2021-2022 season in Italy. Materials and Methods: A total of 12,393 respiratory specimens (nasopharyngeal swabs or broncho-alveolar lavages) were collected from in/outpatients with influenza illness during the period spanning from 1 January 2022 (week 2022-01) to 31 May 2022 (week 2022-22). These specimens were analysed to identify IV genome sequences and were molecularly characterised by 12 laboratories throughout Italy. A comprehensive phylogenetic analysis of the hemagglutinin (HA) gene sequences was conducted to investigate the evolution of A(H3N2). The predicted vaccine efficacy (pVE) of the vaccine strain against circulating A(H3N2) viruses was estimated using the sequence-based $P_{epitope}$ model. Results: the overall IV-positive rate was 7.2% (894/12,393), with all cases being type A IVs. The majority of influenza A viruses (846/894; 94.6%) were H3N2, with a clear epidemic trend observed in Italy. This was evidenced by a 10% positivity rate threshold being crossed for six consecutive weeks from week 2022-11 to week 2022-16. A phylogenetic analysis of a subset of A(H3N2) strains (n=161) revealed that the study HA sequences were distributed into five different genetic clusters, all of which belonged to the clade 3C.2a, sub-clade 3C.2a1 and the genetic subgroup 3C.2a1b.2a.2. The selective pressure analysis of A(H3N2) sequences demonstrated evidence of diversifying selection, particularly in the amino acid position 156. A comparison between the predicted amino acid sequence of the 2021-2022 vaccine strain (A/Cambodia/e0826360/2020) and the study strains revealed 65 mutations in 59 HA amino acid positions, including the substitution H156S and Y159N in antigenic site B, within major antigenic sites adjacent to the receptor-binding site, suggests the presence of drifted strains. The sequence-based $P_{epitope}$ model indicates that antigenic site B is the dominant antigenic site. Furthermore, the p(VE) against circulating A(H3N2) viruses was estimated to be -28.9%. Discussion and Conclusion: following a prolonged period of minimal IV activity in the wake of the introduction of public health control measures to address the COVID-19 pandemic, A(H3N2) emerged with a novel phylogenetic composition. Although the delayed 2021-2022 influenza season in Italy was characterised by a significant reduction in the width of the epidemic curve and in the intensity of the influenza activity compared to historical data, a marked genetic diversity of the haemagglutinin (HA) of circulating A(H3N2) strains was observed. The identification of the H156S and Y159N substitutions within the main antigenic sites of most HA sequences also indicated the circulation of drifted variants with respect to the 2021-2022 vaccine strain. Molecular surveillance plays a pivotal role in the influenza surveillance architecture and must be reinforced at the local level to enable timely assessment of vaccine effectiveness and detection of novel strains with the potential to impact public health.

Keywords: Influenza, A(H3N2), phylogenetic analysis, preicted vaccine analysis, selective pressure analysis.

1. INTRODUCTION

Seasonal influenza viruses evolve in order to evade pre-existing immunity and gain a competitive advantage through surface protein mutations, which yield new antigenic variants [1]. These new variants cause annual epidemics, accounting for infections in 5–15% of the global population and up to 650,000 deaths a year [2]. Three main features contribute to the rapid evolution of influenza viruses: large populations, short generation times, and high mutation rates [3]. The antigenic drift of influenza viruses is a consequence of mutation in the hemagglutinin (HA) gene, which encodes the main protein target for immune responses. The accumulation of these mutations can result in the emergence of antigenically distinct groups if certain amino acid substitutions are introduced into the HA glycoprotein [1, 4]. The globular head of HA includes the receptor binding site (RBS) [4], which, although usually conserved, may be exposed to mutations that evade antibody recognition [5, 6]. The pace of antigenic selection varies over time for influenza A virus (IAV) subtypes and influenza B virus (IBV) lineages, primarily due to population-level fluctuations in immune pressure. This confounds vaccine strain selection, which relies on the anticipation of antigenic evolution [7]. Among human IAVs, H3N2 subtypes are those with the highest mutation rate. Following their introduction into the human population in 1968, they began circulating and displayed a rapid turnover of the viral population, with the appearance of new antigenic variants every 2-5 years. This resulted in the generation of epidemics that were characterised by high morbidity and mortality, and reduced influenza vaccine efficacy [8]. The constant evolution to evade host immune pressure is achieved through the addition of N-glycosylation sites, antigenic drift, and charged amino acid substitutions near the RBS [1, 8]. In particular, the emergence of novel H3N2 variants has been linked to the accumulation of amino acid substitutions at five antigenic sites (designated as A-E and encompassing over 100 amino acid positions) on the globular head of H3. The substitution of a single amino acid in only one of seven specific amino acid positions adjacent to the RBS may result in significant antigenic changes during the evolution of IAVs [9]. The density of the population and the interconnectedness of regions play a significant role in maintaining viral populations [10, 11, 12]. However, the genetic and antigenic diversity of seasonal influenza has been significantly reduced since the onset of the COVID-19 pandemic in March 2020. Since that time, the majority of countries have observed historically low levels of seasonal influenza virus circulation [13, 14, 15]. This decline can be attributed to non-pharmaceutical interventions (NPIs), including travel restrictions, social distancing, school and workplace closures, mask wearing, and enhanced hygiene. NPIs have similarly disrupted the circulation of other common respiratory viruses, such as respiratory syncytial virus and human metapneumovirus, by limiting opportunities for reintroduction and local transmission [16, 17, 18, 19, 20]. As the use of non-pharmaceutical interventions (NPIs) to limit the spread of the novel coronavirus disease (COVID-19) has been gradually declining and international travel has returned to pre-pandemic levels, a resurgence of influenza virus circulation with an increased severity is expected. This is due to a reduction in population immunity over

the last couple of years. The objectives of this study were i) to describe IVs detection and distribution during the 2021-2022 season in Italy, and ii) to conduct an in-depth phylogenetic analysis of the HA gene of influenza A(H3N2) viruses identified in Italy during the 2021-2022 influenza season in order to evaluate the evolution of these viruses after a long period of very low activity.

2. MATERIALS AND METHODS

2.1 Clinical samples and IAVs/IBVs detection and subtyping methods

A total of 12 laboratories located in 8 Italian regions belonging to 4 macro-areas (according to NUTS classification [21]) analysed respiratory specimens (nasopharyngeal swabs or broncho-alveolar lavages) collected from in/outpatients with influenza illness in the period spanning from January 1, 2022 (week 2022-01) to May 31, 2022 (week 2022-22). The objective was to detect the IV genome. The regions included in the study were the North-West (Lombardy), the North-East (Emilia Romagna, Trentino Alto-Adige, Veneto), the Centre (Lazio, Marche), and the South (Campania, Calabria). Table 1 below provides a detailed overview of the laboratory names and their respective locations within each region and macro-area.

	Laboratory	Region (Macro-area)	IAVs/IBVs RNA detection method	IAVs subtyping method
1	Department of Biomedical Sciences for Health, University of Milan, Milan, Italy	Lombardy (North-West)	Home-made (Word Health Organization (WHO) 2011)	Home-made [(Word Health Organization (WHO) 2011, Centers for Disease Control and Prevention (CDC) 2022)]
2	Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy	Lombardy (North-West)	Home-made (Centers for Disease Control and Prevention (CDC) 2022)	Home-made (Word Health Organization 2022)
3	Virology Unit, Clinical Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy	Lombardy (North-West)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene); Alinity mResp-4-Plex assay (Abbott)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)
4	Ospedale di Circolo e Fondazione Macchi, ASST Sette Laghi, Varese, Italy	Lombardy (North-West)	Alinity M Resp-4-Plex AMP Kit (Abbott)	Home-made [(Word Health Organization (WHO) 2011, Centers for Disease Control and Prevention (CDC) 2022)]
5	Laboratorio Aziendale di Microbiologia e Virologia, Hospital of Bolzano (SABES-ASDAA), Bolzano-Bozen, Italy	Trentino Alto-Adige (North-East)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)
6	Microbiology Unit, Azienda ULSS2 Marca Trevigiana, Treviso, Italy	Veneto (North-East)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)
7	Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy	Emilia Romagna (North-East)	Simplexa Flu A/B & RSV Direct Kit (Diasorin)	Home-made (The European Centre for Disease Prevention and Control (ECDC) 2022)
8	Virology Laboratory, Azienda Ospedaliera Ospedali Riuniti di Ancona, Ancona, Italy	Marche (Centre)	Alinity M-Resp-4-Plex AMP Kit (Abbott)	Home-made [(Word Health Organization (WHO) 2011, Centers for Disease Control and Prevention (CDC) 2022)]
9	Department of Diagnostic and Laboratory Medicine, Unit of Microbiology and Diagnostic Immunology, Bambino Gesù Children Hospital IRCCS, Rome, Italy	Lazio (Centre)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)
10	Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani, Rome, Italy	Lazio (Centre)	Home-made (Centers for Disease Control and Prevention (CDC) 2022)	Home-made (Word Health Organization 2022)
11	Microbiology and Virology, Cotugno Hospital AORN dei Colli, Naples, Italy	Campania (South)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)
12	Microbiology & Virology Unit, Annunziata Hub Hospital, Azienda Ospedaliera di Cosenza, Cosenza, Italy.	Calabria (South)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)

Table 1. Methods used for molecular detection of IVs by each GLIViRe (Study Group on respiratory infections of the Italian Association of Clinical Microbiology) center.

Respiratory samples were collected from outpatients with the symptoms of influenza-like illness (ILI) or from hospitalised patients with symptoms ranging from mild to severe respiratory syndromes such as acute respiratory infection (ARI), severe acute respiratory infection (SARI) and acute respiratory distress syndrome (ARDS). Clinical samples were analysed by means of specific real-time PCR assays according to protocols of each participating laboratory. The methods used by each laboratory are detailed in Table 1.

2.2 A(H3N2) Influenza viruses sequencing and phylogenetic analysis

A representative subgroup of influenza A(H3N2) positive samples were subjected to molecular characterisation by means of the sequence analysis of the complete HA gene (nt. 1-1663). A one-step reverse transcription polymerase chain reaction (RT-PCR) was conducted using the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Thermofisher) on 15 µl of extracted RNA in a final reaction volume of 60 µl. This was done in order to obtain the entire influenza A virus genome. The amplification of the complete HA gene was conducted using an in-house nested-PCR, comprising two reaction mixtures, to amplify two overlapping fragments of 970 nt (nt. 1-969) and 813 nt (nt. 851-1663) [22]. Purification and sequencing of the HA amplicons were conducted using the Sanger method, with both forward and reverse primers employed. All HA nucleotide sequences were obtained directly from clinical specimens and submitted to the GISAID database [23] under the accession numbers provided in Supplementary Table 1. The sequences were aligned with reference sequences retrieved from the online repository GISAID [23] using the ClustalW program implemented in BioEdit software [24]. The alignment was employed to construct the phylogenetic tree via the Neighbor-Joining method and the Kimura 2-parameter model, utilising the bioinformatics programme MEGA6 [25]. A bootstrap analysis with 1000 replicates was conducted, with bootstrap values of ≥70% considered significant. The mean nucleotide identities and mean amino acid similarities were calculated using the Sequence Identity Matrix tool of BioEdit software [24] for intra-group sequence analysis and between study and reference sequences. These included the vaccine reference strain of the Northern hemisphere for the 2021-2022 influenza season (A/Cambodia/e0826360/2020_egg-derived; EPI_ISL_806547). The mean values are expressed as a crude rate with the respective range. The genetic distance between sequences within the same genetic group was calculated using the p-distance model in the MEGA6 program [25], with the results expressed as a mean value and the respective standard deviation (DS). The predicted amino acid sequences were obtained using the Toggle Translation tool implemented in BioEdit [24], with the amino acid residues numbered according to the H3 numbering [26]. The predicted amino acid sequences of the study strains were compared with that of the vaccine strain of the Northern hemisphere for the 2021-2022 influenza season (A/Cambodia/e0826360/2020; EPI_ISL_806547) in order to identify amino acid changes, focusing on mutations within the five HA antigenic sites of A(H3N2) strains [27], particularly at major antigenic sites within the RBS [28]. The predicted efficacy of the vaccine (pVE) against circulating A(H3N2) viruses was estimated by comparing the predicted amino acid sequences of the study and vaccine strains using a sequence-based model, the $P_{epitope}$ model, as previously described by others [29, 30, 31]. The $P_{epitope}$ model is a mathematical framework that enables the estimation of antigenic distance between prevalent circulating strains and the vaccine virus, taking into account the observed amino acid substitutions within the five antigenic sites of the A(H3N2) virus. The antigenic site with the highest $P_{epitope}$ value is

considered the dominant antigenic site and is employed to estimate the pVE by means of the following formula: $(-3.32 \times P_{epitope}(\text{dominant antigenic site}) + 0.66) \times 100\%$. In the event of a perfect match between the circulating strain and the vaccine strain, the Pepitope is null and the pVE is 66% (the maximum pVE that can be estimated by this sequence-based model) [29]. A negative value of pVE indicates that the vaccine efficacy against the circulating strains is suboptimal.

2.3 Selective pressure analysis

In order to evaluate the evolution of HA due to immunological pressure, a series of probabilistic models of codon substitution were employed. In detail, tests for positive selection were conducted using single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), the mixed-effects model of evolution (MEME), fast unconstrained Bayesian approximation (FUBAR), adaptive Branch-Site Random Effects Likelihood (aBSREL), and Branch-site Unrestricted Statistical Test for Episodic Diversification (BUSTED) methods on the Datamonkey 2.0 server [32]. In order to minimise the occurrence of false positives, sites with SLAC, FEL, MEME and aBSREL p-values below 0.05 and a FUBAR posterior probability above 0.95 were accepted as candidates for selection.

2.4 Statistical analysis

A statistical analysis was conducted utilising the Open Source Epidemiologic Statistics for Public Health OpenEpi software, version 3.03 [33]. The frequency of positive samples was expressed as a crude proportion, with the corresponding 95% confidence interval (95% CI) calculated by the Mid-P exact test, assuming a normal distribution. The interquartile range (IQR) was calculated as the difference between the first and third quartiles of the age distribution. The positivity rate was calculated as the number of laboratory-confirmed infections out of the total number of samples. The statistical significance of the observed differences between the proportions of individuals in the various groups was evaluated using the Mid-P exact test based on the binomial distribution. For continuous variables, the paired t-test was employed. The season onset (or start), duration, peak and offset (or end) of influenza were estimated using the RS10 method [34], which defines the start of the epidemic season as the first two consecutive weeks when virus detection exceeds 10% of the virus-positivity rate. A p-value of less than 0.05 was considered to be statistically significant (two-tailed test).

3. RESULTS

3.1 IVs detection and distribution during 2021-2022 season in Italy

A total of 12,393 respiratory specimens were subjected to testing for the presence of IVs. Of the total number of specimens tested, 894 were found to be positive for IVs, resulting in an overall positivity rate of 7.2%. The IV positivity rate by centre exhibited considerable variation, ranging from 1.1% to 14.6% in samples collected in a hospital

setting and 17.3% in an outpatient care setting. Table 2 presents a summary of the IV positivity rates by macro-area. The IVs positivity rate was 7.8% in the centres of north-west Italy, 7.7% in those of north-east Italy, and 1.6% in the participating centres of central and southern Italy.

Macro-area	IVs positivity rate %	IAVs positivity rate %	IBVs positivity rate %	A(H3N2) positivity rate %	A(H1N1) positivity rate %
North-West Italy	7.8%	7.8%	0%	99.6%	0.4%
North-East Italy	7.7%	7.7%	0%	99.5%	0.5%
Central Italy	1.6%	1.6%	0%	97.9%	2.1%
Southern Italy	1.6%	1.6%	0%	100%	0%
Total	7.2%	7.2%	0%	94.6%	5.4%

Table 2. Influenza virus positivity rate by type/subtype and by macro-area.

All the 894 IV-positive samples were IAVs: 94.6% (846/894) of those were H3N2 and 5.4% (48/894) belonged to the H1N1 subtype (Table 2). During the study period (from week 2022-01 to week 2022-22), A(H3N2) detection in respiratory samples had a clear epidemic trend, crossing the 10% positivity rate threshold for six consecutive weeks (from week 2022-11 to week 2022-16). In fact, A(H3N2) epidemic wave started in week 2022-11, peaked in week 2022-13 and ended in week 2022-16 (Fig. 1). During the peak, the overall positivity rate reached 20% (117/578). Temporal distribution showed a geographical pattern from North-West to North-East Italy. Considering results by center in each Italian macro-area, A(H3N2) epidemic was evident in centers of both North-West and North-East Italy, whereas A(H3N2) started to be detected from week 2022-08 and 2022-09 in centers from Southern and Central Italy, respectively, never crossing the 10% positivity rate threshold (Fig. 2).

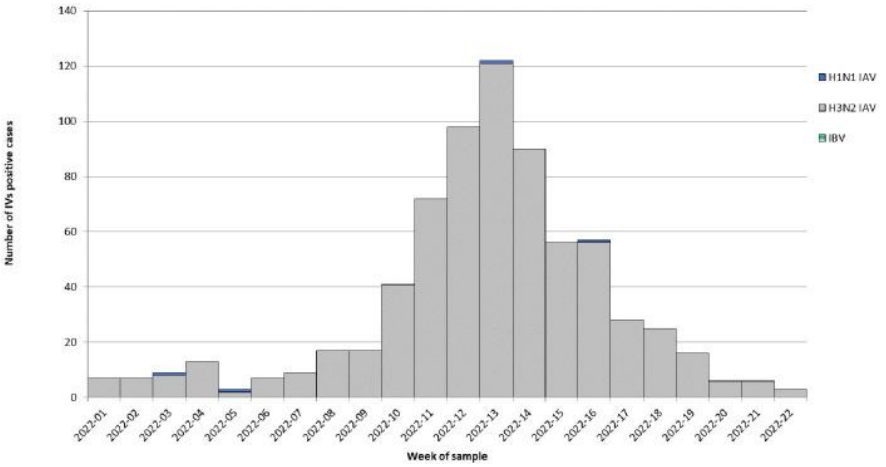

Figure 1. Influenza viruses positive samples by type/subtype and by week of sample collection.

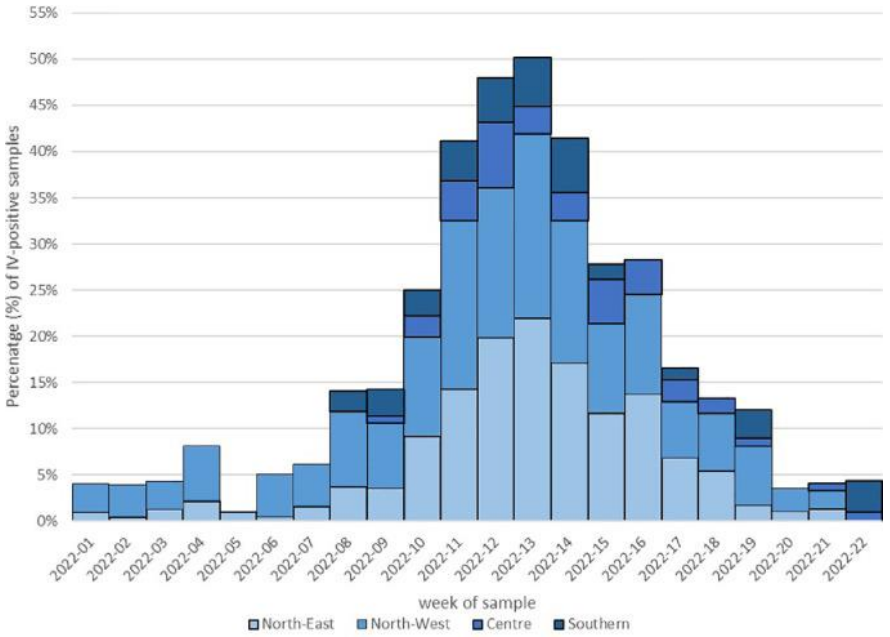

Figure 2. Temporal distribution of IV-positivity rate by week (from week 2022-01 to week 2022-22) and by macro-area.

3.2 Phylogenetic analysis of A(H3N2) IVs

A total of 161 A(H3N2) strains were molecularly characterised by sequencing and their HA sequences were phylogenetically analysed. The H3N2 viruses considered in this study were identified in 161 individuals. The median age of the individuals was 9 years, with an interquartile range (IQR) of 29 years and a range of 0 to 93 years. Of the individuals, 52% were male. Of the 161 individuals, 110 (68%) were inpatients from hospital settings, and 51 (32%) were outpatients from ambulatory care settings. The nucleotide identity of the study strains was found to be 99.3% (range: 98.2%-100%), while the amino acid similarity was 99.2% (range: 97.5%-100%). As illustrated in Fig. 3, all A(H3N2) strains included in this study were found to belong to the 3C.2a clade, sub-clade 3C.2a1, with a mean nucleotide identity of 97% (range: 96.6%-97.3%) and a mean amino acid similarity of 95.7% (range: 95.3%-96.1%) when compared to the reference strain A/Singapore/INFIMH-16-0019/2016.

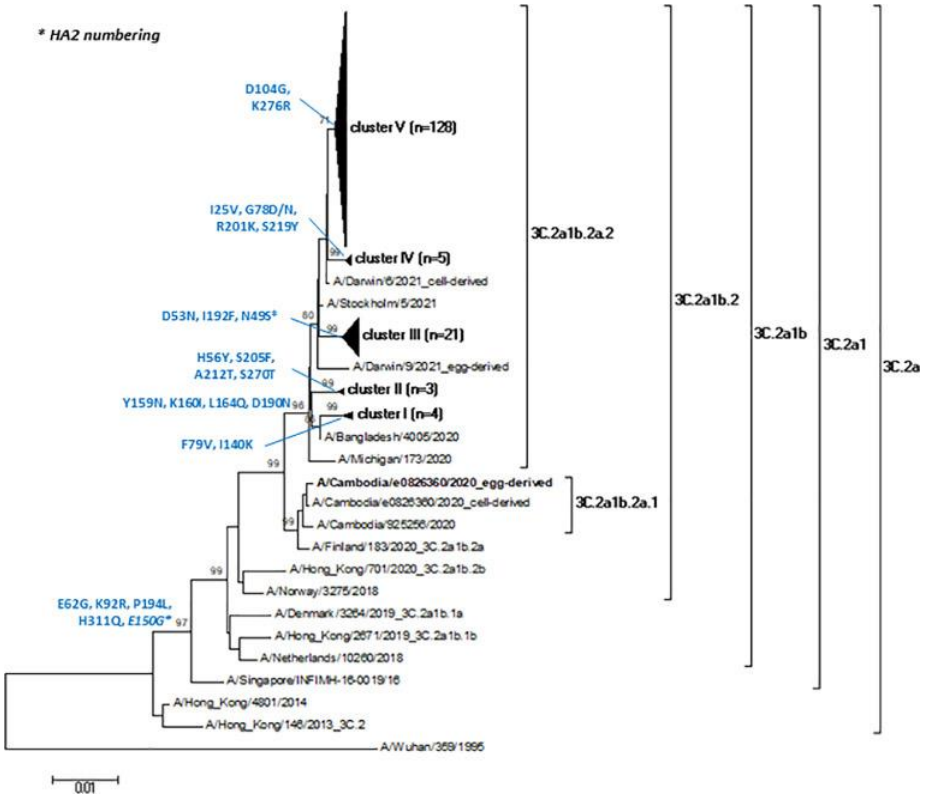

Figure 3. Phylogenetic tree of the 161 HA nucleotide sequences (1479 nt.) of A(H3N2) strains identified in this study. The vaccine A(H3N2) strain of the Northern hemisphere for 2021-2022 influenza season (A/Cambodia/e0826360/2020) is in bold. For reasons of clarity, interior branches representing the study sequence clusters are compressed into elongated triangles, whose height is proportional to the number of taxa condensed and whose width is proportional to the maximum distance between taxa. Amino acid substitutions characterising the main branches are detailed close to each node. Only bootstrap values >70% are displayed.

The amino acid substitutions E62G (in antigenic site E), K92R (in antigenic site E), P194L (in antigenic site B) and H311Q (in antigenic site C) in HA1 and E150G in HA2 were identified in the study sequences, defining the genetic group 3C.2. A1b (bootstrap 99%) exhibited a mean nucleotide identity of 97.5% (range: 97.2%-97.9%) and a mean amino acid similarity of 95.9% (range: 95.5%-96.3%) to the reference strain A/Netherlands/10260/2018. The 3C.2a1b genetic group also includes the 2021-2022 vaccine reference strain A/Cambodia/e0826360/2020. However, while the vaccine strain belonged to the genetic subgroup 2a.1, the A(H3N2) strains of this study were characterised by the amino acid mutations Y159N, K160I, L164Q and D190N (all in the antigenic site B of HA1), and segregated into the genetic subgroup 2a.2. The mean nucleotide identity was 99.1% (range: 98.7%-99.5%), while the mean amino acid similarity was 98.9% (range: 98.5%-99.3%) in comparison to the reference strain

A/Bangladesh/4005/2020. In more detail, the study sequences exhibited a mean intra-group p-distance of 0.006 (standard deviation, SD=0) and were further distributed in five different clusters. A comparison of the study sequences with the A/Bangladesh/4005/2020 reference strain revealed that the sequences segregated into different clusters, each of which was characterised by specific amino acid mutations. Cluster I, comprising 4/161 sequences (2.5%), exhibited amino acid substitutions F79V and I140K (in antigenic site A) in HA1. The mean intra-group nucleotide identity was 99.7% (range: 99.5% - 99.9%), while the mean intra-group amino acid similarity was 99.6% (range: 99.3% - 100%). Cluster II (3/161 sequences; 1.9%) was characterised by the amino acid mutations H56Y, S205F, A212T (in antigenic site D) and S270T in HA1. It exhibited a mean intra-group nucleotide identity of 99.8% (range: 99.7% -99.9%) and a mean intra-group amino acid similarity of 99.8% (range: 99.7%-100%). Cluster III (21/161 sequences; 13%) was characterised by the amino acid substitutions D53N (in antigenic site C) and I192F (in antigenic site B) in HA1 and N49S in HA2. The mean intra-group nucleotide identity was 99.6% (range: 99.2%-100%), while the mean intra-group amino acid similarity was 99.6% (range: 98.9%-100%). Cluster IV (5/161 sequences; 3.1%) was characterised by the amino acid mutations I25V, G78D/N, R201K (in antigenic site D) and S219Y (in antigenic site D) in HA1. It exhibited a mean intra-group nucleotide identity of 99.8% (range: 99.7%-100%) and a mean intra-group amino acid similarity of 99.8% (range: 99.5%-100%). Cluster V (128/161 sequences; 79.5%) was characterised by the amino acid substitutions D104G and K276R (in antigenic site C) in HA1. The mean intra-group nucleotide identity and amino acid similarity was 99.7% (range: 98.9%-100%). Finally, no significant difference was observed in the distribution of HA sequences by type of setting (ambulatory or hospital) or geographical area.

3.3 Comparison between study and vaccine strains and predicted vaccine efficacy

The 161 A(H3N2) HA sequences of this study exhibited a mean nucleotide identity of 98.4% (range: 97.9%-98.7%) and a mean amino acid similarity of 97.5% (range: 97.1%-97.9%) to the A(H3N2) vaccine strain of the Northern hemisphere for the 2021-2022 influenza season, A/Cambodia/e0826360/2020(H3N2). A comparison between the predicted amino acid sequences of A(H3N2) study viruses and the 2021-2022 vaccine reference strain A/Cambodia/e0826360/2020 revealed 65 mutations in 59 HA amino acid positions. Of these, 9/65 (14%) amino acid substitutions were observed in >80% of study sequences, and all of them were within an antigenic site. These sites were antigenic site B (7/9, 78%), antigenic site C (1/9, 11%), and antigenic site D (1/9, 11%). The list of mutations by amino acid position is presented in Supplementary Table 2. In total, 54% (35/65) of amino acid mutations were observed within an antigenic site (designated as A-E). All five antigenic sites exhibited at least one mutated amino acid position. In particular, 55% of the substitutions are located in the antigenic sites D (29%, 10/35) and B (26%, 9/35). A total of 17% (6/35) of the mutations were observed in antigenic site A,

17% (6/35) in antigenic site C, and 11% (4/35) in antigenic site E. Among the seven major antigenic sites (amino acid positions: 145, 155, 156, 158, 159, 189 and 193) adjacent to the RBS, two amino acid positions were characterised by a substitution. The amino acid substitution H156S was observed in 96% of the sequences belonging to cluster III-V, while the amino acid substitution Y159N was observed in all the study strains (161/161; 100%). A comparison between the predicted amino acid sequence of the 2021-2022 vaccine reference strain A/Cambodia/e0826360/2020 (genetic subgroup 3C.2a1b.2a.1) and the circulating A(H3N2) strains of this study (genetic subgroup 3C.2a1b.2a.2) revealed the following changes in 100% HA study sequences: The antigenic site D is occupied by N171K, while the antigenic site B is populated by Y159N, K160I, L164Q, R186D, D190N and P198S. These data are presented in Supplementary Table 2. The $P_{epitope}$ model indicates that antigenic site B is the dominant antigenic site, with a $P_{epitope}$ value of 0.286. The vaccine strain's pVE against the A(H3N2) viruses that were circulating during the 2021-2022 influenza season was estimated to be -28.9%.

3.4 Selective pressure analysis

A total of 156 sites were identified as being under diversifying selection by site-specific analyses in the HA of study A(H3N2) IVs alignment by at least three of the methods used (SLAC, FEL, REL, FUBAR and MEME) (Table 3). The aBSREL analyses indicated the presence of episodic diversifying selection for a branch of the phylogenetic tree, comprising six strains, with the Y159N, T160I, L164Q, N171K, S186D, D190N, and P198S mutations (A/Bolzano/24/2022, A/Varese/04/2022, A/PoliclinicoMilano/22/2022, A/Milano/04/2022, A/Milano/21/2022, and A/Milano/63/2022) in comparison to the vaccine strain. In three of the sequences (A/Bolzano/24/2022, A/Varese/04/2022, A/Policlinico_Milano/22/2022), additional changes were observed (E50K, F79V and I140), while A/Milano/04/2022, A/Milano/21/2022, and A/Milano/63/2022 were characterised by the presence of other additional changes (H56Y, S205F, A212T and S270T). BUSTED analyses revealed that at least one site on at least one test branch exhibited diversifying selection (LRT, p-value=0.033; p-value $\leq$0.05).

Methods	H3 codons or tree branch	
	Diversifying selection	Negative selection
SLAC	None	10, 24, 239
FEL	53, **156**, 192, 201, 219, 378	None
FUBAR	none	10, 24, 35, 56, 66, 65. 90, 108, 142, 214, 239, 321, 403, 436, 470, 496
MEME	**156**	None
aBSREL	Branch with 6 strains[a] (**H156**, D186, D225).	None

Positions selected by at least 3 methods are reported in bold.

[a]A/Bolzano/24/2022, A/Varese/04/2022, A/Policlinico_Milano/22/2022, A/Milano/04/2022, A/Milano/21/2022, and A/Milano/63/2022

Table 3. Selected sites of HA for A(H3N2) IV strains identified in this study.

4 DISCUSSION

Following the worldwide abrupt halt of influenza circulation caused by the emergence and widespread of SARS-CoV-2, scientists have been worrying about increased IVs activity and new viral phylogenetic makeup. In fact, COVID-19 pandemic restrictions such as lockdowns, school closure, facemask use and social distancing helped keep respiratory viruses at bay so much that influenza largely disappeared until early 2022 [15, 35, 36]. Data from the Southern Hemisphere and in particular from the Australia's Department of Health and Aged Care have showed an unusual influenza activity in 2022, spiking and dropping earlier than usual with the laboratory-confirmed influenza rate higher than the five-year average [37]. The objective of our study was to describe the distribution of influenza virus during the 2021-2022 influenza season in Italy. To achieve this, we conducted an in-depth phylogenetic analysis of the haemagglutinin (HA) gene of A(H3N2) influenza viruses.

Our findings indicate that, despite evidence of an influenza epidemic in Italy in 2022, with the epidemic threshold of 10% positivity crossed for six consecutive weeks, the overall influenza positivity rate was 7.2%, a figure significantly lower than that observed during the pre-COVID-19 seasons. These findings are consistent with those of the Istituto Superiore di Sanità (ISS) (2022), the European Centre for Disease Prevention and Control (ECDC) (2022), and Pellegrinelli et al. (2022) [15, 38, 39, 40]. The percentage of respiratory samples positive for influenza exceeded the 10% threshold, indicating the onset of the epidemic, in week 2022-11, a later occurrence than observed in previous seasons when the epidemic typically commenced between weeks 48 and 52. This observation is consistent with the findings of Istituto Superiore di Sanità (ISS) (2022), The European Centre for Disease Prevention and Control (ECDC) (2022), and Pellegrinelli et al. (2022) [15, 38, 39, 40, 41]. In 2022, the European Centre for Disease Prevention and Control (ECDC) published a report on the subject [41]. The percentage

of influenza virus positive specimens at its peak (week 2022-13) was 20%, much lower than what observed during the 2018-2019 season when the percentage of influenza viruses positive samples reached up to 62% [38]. Indeed, the ECDC annual reports indicate that a percentage of influenza virus-positive specimens ranged between 40% (2016-2017) and 49% (2017-2018) in the context of ILI and ARI sentinel consultations from 2016–2017 to 2018-2019 influenza season [38, 39, 41]. In Italy, the temporal distribution did not show a clear geographical pattern, probably in consideration to the low number of influenza viruses detected in Central and Southern Italian macro-area (influenza positive rate of 1.6%).

Overall, our data underlined that the 2021-2022 influenza season in Italy was characterized by a significant reduction of the width of the epidemic curve (6 weeks versus 12-19 weeks observed between 2014-2015 to 2018-2019 season [15, 40] and in the intensity of the influenza activity compared to previous seasons that can be undoubtedly related to the emergence of SARS-CoV-2, the actions introduced to control the COVID-19 pandemic and the increased number of testing. As observed in Europe and in Southern hemisphere countries [37, 42], no influenza B viruses were identified in Italy and among type A influenza viruses, H3N2 subtype largely dominated over H1N1.

To investigate the molecular and evolutionary characteristics of the influenza A(H3N2) viruses circulating during the 2021-2022 season, a phylogenetic analysis of the HA gene sequences of nearly 20% of A(H3N2) viruses detected during the study was conducted. All A(H3N2) study strains belonged to the clade 3C.2a, sub-clade 3C.2a1 and the genetic group 3C.2a1b, sharing a high mean nucleotide identity (99.3%) and a high mean amino acid similarity (99.2%). Our results mirror the European data of influenza virus molecular characterisation during the 2021-2022 season [43, 44]. Despite belonging to the same phylogenetic branch, the HA sequence analysis of study A(H3N2) strains pointed out the constant tendency of influenza viruses to evolve. In fact, A(H3N2) viruses in the clade 3C.2a were dominant since the 2014-2015 influenza season with the 3C.2a1b viruses predominating over the course of the 2019-2020 season [45]. However, the A(H3N2) strains observed in this study, as well as the majority of strains observed in other Northern Hemisphere countries during the 2021-2022 season [43, 44], segregated into the genetic subgroup 3C.2a1b.2a.2. This subgroup has been circulating since October 2020 and was named during the 2021-2022 season [45]. The 3C.2a1b.2a.2 viruses result in the loss of the glycosylation site at residues 158-160 in HA1, which had been a defining feature of clade 3C.2a viruses (e.g. A/Hong Kong/4801/2014). Furthermore, the 161 HA study sequences were distributed into five distinct genetic clusters, each characterized by specific amino acid substitutions. This genetic diversity serves to confirm the influenza viruses' continuous ability to mutate. This observation was also reinforced by the selective pressure analysis, which demonstrated that certain amino acid positions (notably amino acid 156) are subject to diversifying selection, thereby rendering them more susceptible to evolution.

Finally, a comparison between the HA sequences of the A(H3N2) strains of this study and the vaccine strain of the Northern hemisphere for the 2021-2022 season (A/Cambodia/e0826360/2020) revealed a mean nucleotide identity of 98.4% and a mean amino acid similarity of 97.5%. Although the vaccine strain A/Cambodia/e0826360/2020 belongs to the same genetic group (3C.2a1b) as the study sequences, it differs from them in terms of the genetic subgroup (2a.1 for the vaccine strain vs. 2a.2 for the study sequences). A comparison of the predicted amino acid sequences of the HA gene of study A(H3N2) strains with the HA sequence of the 2021-2022 vaccine strain revealed numerous amino acid substitutions (65 mutations in 59 sites). In particular, the majority of the study sequences exhibited the H156S (96%) and Y159N (100%) mutations, which are located within the major antigenic sites of the receptor binding site. The receptor binding site is generally conserved, but it may also be exposed to selective pressure, which determines the introduction of new mutations in order to evade antibody recognition [8]. The amino acid substitutions in the major antigenic sites (in particular positions 145, 155, 156, 158, 159, 189 and 193) are mutations that, more than others, lead to antigenic changes [9]. Consequently, the presence of the H156S and Y159N mutations in the study sequences indicates the circulation of HA drifted A(H3N2) strains compared to the 2021-2022 vaccine strain. This suggests that circulating strains may be able to evade the recognition of vaccine-induced antibodies.

In total, 54% of the HA amino acid substitutions were observed within an antigenic site. Among these, 55% were located in antigenic site D (29%) and B (26%). The $P_{epitope}$ model [29] predicts that the 2021-2022 vaccine strain will have an efficacy of -28.9% against the circulating A(H3N2) viruses. The $P_{epitope}$ model is a mathematical model that accounts for immunological diversity, modularity, and hierarchy during human antibody recognition of influenza antigens, previously described by Bonomo et al. [31]. This is a sequence-based model that enables the estimation of antigenic distance between the predominant circulating strains of A(H3N2) and the vaccine virus. It is important to note that this model can only be used to estimate vaccine efficacy and not vaccine effectiveness. Consequently, the $P_{epitope}$ model cannot supplant the test-negative design studies, which remain the gold standard for estimating vaccine effectiveness [46]. The pVE estimated in our study by the $P_{epitope}$ model indicates that the vaccine's efficacy against A(H3N2) strains circulating during the 2021-2022 influenza season was suboptimal. Despite the fact that the pVE calculated in this study is the result of a sequence-based analysis only, suboptimal vaccine effectiveness against the A(H3N2) strain has also been reported by interim analyses of the 2021-2022 seasonal influenza vaccine effectiveness through observational studies conducted in the US [47], in Denmark [48, 49] and in Canada [48, 49]. The results of these studies have indicated that the observed low vaccine effectiveness may be attributed to the circulation of A(H3N2) drifted variants. A limitation of this study is that no data on the antigenic characteristics of the circulating viruses were available. As a result, the interpretation of the results is

limited to the genotypic characteristics of the viruses, and does not consider any phenotypic alterations. However, in light of the antigenic analyses of circulating influenza viruses conducted by the WHO [45], it can be posited that the strains identified in this study exhibit antigenic differences from the A(H3N2) vaccine strain. Consequently, the vaccine composition was updated for the following influenza season, with the A(H3N2) vaccine strain being replaced. This study is subject to certain limitations, including heterogeneity in the sampled populations among the centres and, to a certain extent, differences in the methods used to detect influenza viruses in respiratory samples.

5 CONCLUSIONS

As influenza viruses have the potential to emerge with new phylogenetic makeup and in consideration that this study has uncovered the introduction of A(H3N2) HA drifted variants after a long period of very low influenza activity in Italy, it is critical to further strengthen molecular surveillance at local level to promptly assess vaccine effectiveness and detect any novel strains with potential impact on public health.

REFERENCES

1. Petrova VN, Russell CA. The evolution of seasonal influenza viruses. Nat. Rev. Microbiol. 2018 Jan;16(1):47–60.
2. Word Health Organization (OMS). Influenza seasonal. https://www.who.int/health-topics/influenza-seasonal#tab=tab_1. Last Accessed: 29/11/2022.
3. Shao W, Li X, Goraya MU, et al. Evolution of influenza A virus by mutation and Re-assortment. Int. J. Mol. Sci. 2017 Aug;18(8):E1650. Weis W, Brown JH, Cusack S, et al. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature. 1988 Jun;333(6172):426–431. [PubMed] [Google Scholar]
4. Thyagarajan B, Bloom JD. The inherent mutational tolerance and antigenic evolvability of influenza hemagglutinin. Elife. 2014 Jul;3:e03300.
5. Lee PS, Wilson IA. Structural characterization of viral epitopes recognized by broadly cross-reactive antibodies. Curr. Top. Microbiol. Immunol. 2015;386:323–341.
6. Barrat-Charlaix P, Huddleston J, Bedford T, et al. Limited predictability of amino acid substitutions in seasonal influenza viruses. Mol. Biol. Evol. 2021 06 25;38(7):2767–2777.
7. Allen JD, Ross TM. H3N2 influenza viruses in humans: viral mechanisms, evolution, and evaluation. Hum. Vaccin. Immunother. 2018;14(8):1840–1847.

8. Koel BF, Burke DF, Bestebroer TM, et al. Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. Science. 2013 Nov;342(6161):976–979.
9. Ebranati E, Pariani E, Piralla A, et al. Reconstruction of the evolutionary dynamics of A(H3N2) influenza viruses circulating in Italy from 2004 to 2012. PLoS One. 2015;10(9).
10. Russell CA, Jones TC, Barr IG, et al. The global circulation of seasonal influenza A (H3N2) viruses. Science. 2008 Apr 18;320(5874):340–346.
11. Bahl J, Nelson MI, Chan KH, et al. Temporally structured metapopulation dynamics and persistence of influenza A H3N2 virus in humans. Proc. Natl. Acad. Sci. U.S.A. 2011 Nov 29;108(48):19359–19364.
12. Sullivan SG, Carlson S, Cheng AC, et al. Where has all the influenza gone? The impact of COVID-19 on the circulation of influenza and other respiratory viruses, Australia, March to September 2020. Euro Surveill. 2020;25(47) 11.
13. Olsen SJ, Azziz-Baumgartner E, Budd AP, et al. Decreased influenza activity during the COVID-19 pandemic - United States, Australia, Chile, and South Africa, 2020. MMWR Morb. Mortal. Wkly. Rep. 2020 Sep 18;69(37):1305–1309.
14. Istituto Superiore di Sanità (ISS) Epicentro. Influnet. 2022 https://www.epicentro.iss.it/influenza/influnet Available at. Last access: 29/11/
15. Baker RE, Park SW, Yang W, et al. The impact of COVID-19 nonpharmaceutical interventions on the future dynamics of endemic infections. Proc. Natl. Acad. Sci. U. S. A. 2020;117(48):30547–30553. 12 01.
16. Adenaiye OO, Lai J, Bueno de Mesquita PJ, et al. Infectious severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) in exhaled aerosols and efficacy of masks during early mild infection. Clin. Infect. Dis. 2022 Aug 24;75(1):e241–e248.
17. Leung NHL, Chu DKW, Shiu EYC, et al. Respiratory virus shedding in exhaled breath and efficacy of face masks. Nat. Med. 2020;26(5):676–680. 05.
18. Tang JW, Bialasiewicz S, Dwyer DE, et al. Where have all the viruses gone? Disappearance of seasonal respiratory viruses during the COVID-19 pandemic. J. Med. Virol. 2021 Jul;93(7):4099–4101.
19. Gomez GB, Mahé C, Chaves SS. Uncertain effects of the pandemic on respiratory viruses. Science. 2021;372(6546):1043–1044. 06 04. [PubMed]
20. NUTS - Nomenclature of territorial units for statistics. Availabe at: https://ec.europa.eu/eurostat/web/nuts/background. Last accessed: 29/11/2022.
21. Galli C, Orsi A, Pariani E, et al. In-depth phylogenetic analysis of the hemagglutinin gene of influenza A(H3N2) viruses circulating during the 2016-2017 season revealed egg-adaptive mutations of vaccine strains. Expert Rev. Vaccines. 2020;19(1):115–122. 01.
22. GISAID. Global initiative on sharing all influenza data. Available at https://www.gisaid.org/. Last accessed: 29/11/2022.

23. Hall T. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 1999:95–98.
24. Tamura K, Stecher G, Peterson D, et al. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 2013 Dec;30(12):2725–2729.
25. Lindstrom S, Sugita S, Endo A, et al. Evolutionary characterization of recent human H3N2 influenza A isolates from Japan and China: novel changes in the receptor binding domain. Arch. Virol. 1996;141(7):1349–1355.
26. Wiley DC, Skehel JJ. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annual Review of Biochemistry. 1987;56:p365–p394.
27. Yang H, Carney PJ, Chang JC, et al. Structure and receptor binding preferences of recombinant human A(H3N2) virus hemagglutinins. Virology. 2015 Mar;477:18–31.
28. Bonomo ME, Deem MW. Predicting Influenza H3N2 vaccine efficacy from evolution of the dominant epitope. Clin. Infect. Dis. 2018 Sep;67(7):1129–1131. Gupta V, Earl DJ, Deem MW. Quantifying influenza vaccine efficacy and antigenic distance. Vaccine. 2006 May;24(18):3881–3888.
29. Bonomo ME, Kim RY, Deem MW. Modular epitope binding predicts influenza quasispecies dominance and vaccine effectiveness: application to 2018/19 season. Vaccine. 2019 May 27;37(24):3154–3158.
30. Weaver S, Shank SD, Spielman SJ, et al. Datamonkey 2.0: a modern web application for characterizing selective and other evolutionary processes. Mol. Biol. Evol. 2018 Mar 01;35(3):773–777.
31. Dean AG, Sullivan KM, Soe MM. OpenEpi: open source epidemiologic statistics for Public Health, Versione. Available at: www.OpenEpi.com. Last access: 29/11/2022.
32. Midgley CM, Haynes AK, Baumgardner JL, et al. Determining the seasonality of respiratory syncytial virus in the United States: the impact of increased molecular testing. J. Infect. Dis. 2017;216(3):345–355. 08 01.
33. The European Centre for Disease Prevention and Control (ECDC) FluNews Europe. 2022 https://flunewseurope.org/ Available at: Last access: 29/11/
34. Word Health Organization (WHO) Global influenza program. Influenza updates. 2022 https://www.who.int/teams/global-influenza-programme/surveillance-and-monitoring/influenza-updates/influenza-updates-archive Available at: Last access: 29/11/
35. Australian Influenza Surveillance Report and Activity Updates –2022. Available at: https://www1.health.gov.au/internet/main/publishing.nsf/Content/cda-ozflu-2022.htm. Last Accessed: 29/11/2022.
36. The European Centre for Disease Prevention and Control (ECDC). Seasonal influenza 2018–2019. Annual Epidemiological Report. Available at:

https://www.ecdc.europa.eu/sites/default/files/documents/AER_for_2018_seasonal-influenza-corrected.pdf. Last Accessed: 29/11/2022.
37. The European Centre for Disease Prevention and Control (ECDC). Seasonal influenza 2017–2018. Annual Epidemiological Report. Available at: https://www.ecdc.europa.eu/sites/default/files/documents/AER_for_2018_seasonal-influenza-corrected.pdf. Last Accessed: 29/11/2022.
38. Pellegrinelli L, Galli C, Bubba L, et al. Respiratory syncytial virus in pediatric influenza-like illness cases in Lombardy, Northern Italy, during seven consecutive winter seasons (from 2014-2015 to 2020-2021) Influenza Other. Respir. Viruses. 2022;16(3):481–491. 05.
39. The European Centre for Disease Prevention and Control (ECDC). Seasonal influenza. Annual Epidemiological Report for 2016. Available at: https://www.ecdc.europa.eu/sites/default/files/documents/AER_for_2016-influenza-seasonal.pdf. Last Accessed: 29/11/2022.
40. The European Centre for Disease Prevention and Control (ECDC) Influenza virus characterisation - Summary Europe. March 2022 https://www.ecdc.europa.eu/sites/default/files/documents/Influenza-characterisation-report-march-2022.pdf Available at: Last Accessed: 29/11/2022. [Google Scholar]
41. The European Centre for Disease Prevention and Control (ECDC). Influenza Virus Characterisation Reports, summary Europe. Available at: https://www.ecdc.europa.eu/en/seasonal-influenza/surveillance-and-disease-data/influenza-virus-characterisation. Last accessed: 29/11/2022.
42. Istituto Superiore di Sanità. ISS: Sorveglianza virologica dell'influenza (rete InfluNet). Available at: https://www.iss.it/sorveglianza-virologica-dellinfluenza-influnet. Last access: 29/11/2022.
43. The Francis Crick Institute - Worldwide Influenza Centre lab. Annual and interim reports. Available at: https://www.crick.ac.uk/research/platforms-and-facilities/worldwide-influenza-centre/annual-and-interim-reports.Last access: 23/11/2022.
44. Chua H, Feng S, Lewnard JA, et al. The use of test-negative controls to monitor vaccine effectiveness: a systematic review of methodology. Epidemiology. 2020 Jan;31(1):43–64.
45. Chung JR, Kim SS, Kondor RJ, et al. Interim estimates of 2021-22 Seasonal influenza vaccine effectiveness - United States, February 2022. MMWR Morb. Mortal. Wkly. Rep. 2022 Mar 11;71(10):365–370.
46. Emborg HD, Vestergaard LS, Botnen AB, et al. A late sharp increase in influenza detections and low interim vaccine effectiveness against the circulating A(H3N2) strain, Denmark, 2021/22 influenza season up to 25 March 2022. Euro Surveill. 2022;27(15) 04.

47. Kim S, Chuang ES, Sabaiduc S, et al. Influenza vaccine effectiveness against A(H3N2) during the delayed 2021/22 epidemic in Canada. Euro Surveill. 2022;27(38) 09.
48. Word Health Organization (WHO). Global influenza surveillance network. Manual for the laboratory diagnosis and virological surveillance of influenza. Available at: http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf. 2011. Last accessed: 29/11/2022.
49. Centers for Disease Control and Prevention (CDC). National Center for Immunization and Respiratory Diseases (NCIRD). Virology, surveillance, and diagnosis branch. Available at: https://www.cdc.gov/ncird/flu.html. Last accessed: 29/11/2022.

Supplementary materials

Table S1. GISAID accession numbers of the 161 study sequences and clusters**.**

Strain ID	HA Segment ID	Strain name	Cluster
EPI_ISL_14871068	EPI2160573	A/Pavia/1048144/2022	III
EPI_ISL_14871069	EPI2160574	A/Pavia/1054072/2022	III
EPI_ISL_14871070	EPI2160575	A/Pavia/1054404/2022	V
EPI_ISL_14871071	EPI2160577	A/Pavia/1055653/2022	V
EPI_ISL_14871072	EPI2160578	A/Pavia/1058057/2022	V
EPI_ISL_14871073	EPI2160579	A/Pavia/1060820/2022	V
EPI_ISL_14871074	EPI2160580	A/Pavia/1061840/2022	V
EPI_ISL_14871075	EPI2160582	A/Pavia/1062489/2022	V
EPI_ISL_14871076	EPI2160583	A/Pavia/1063723/2022	V
EPI_ISL_14871077	EPI2160584	A/Pavia/1064777/2022	III
EPI_ISL_14871078	EPI2160586	A/Pavia/1065173/2022	V
EPI_ISL_14871079	EPI2160587	A/Pavia/1047836/2022	III
EPI_ISL_14871080	EPI2160588	A/Bolzano/26/2022	V
EPI_ISL_14871081	EPI2160589	A/Bolzano/27/2022	V
EPI_ISL_14871082	EPI2160591	A/Bolzano/28/2022	V
EPI_ISL_14871099	EPI2160612	A/Cosenza/03/2022	V
EPI_ISL_14871084	EPI2160593	A/Bolzano/05/2022	V
EPI_ISL_14871085	EPI2160594	A/Bolzano/06/2022	V
EPI_ISL_14871086	EPI2160596	A/Bolzano/07/2022	V
EPI_ISL_14871087	EPI2160597	A/Bolzano/08/2022	V
EPI_ISL_14871088	EPI2160599	A/Bolzano/09/2022	V
EPI_ISL_14871089	EPI2160600	A/Bolzano/10/2022	V
EPI_ISL_14871090	EPI2160601	A/Bolzano/12/2022	V
EPI_ISL_14871091	EPI2160602	A/Bolzano/13/2022	III
EPI_ISL_14871092	EPI2160604	A/Bolzano/17/2022	V
EPI_ISL_14871094	EPI2160605	A/Bolzano/21/2022	V
EPI_ISL_14871095	EPI2160606	A/Bolzano/22/2022	V
EPI_ISL_14871096	EPI2160607	A/Bolzano/23/2022	I
EPI_ISL_14871097	EPI2160609	A/Bolzano/24/2022	I
EPI_ISL_14871098	EPI2160610	A/Cosenza/01/2022	V
EPI_ISL_14871099	EPI2160612	A/Cosenza/03/2022	V

Strain ID	HA Segment ID	Strain name	Cluster
EPI_ISL_14871100	EPI2160613	A/Cosenza/07/2022	V
EPI_ISL_14871101	EPI2160614	A/Cosenza/08/2022	V
EPI_ISL_14871102	EPI2160616	A/Policlinico-Milano/01/2022	V
EPI_ISL_14871103	EPI2160617	A/Policlinico-Milano/02/2022	V
EPI_ISL_14871236	EPI2160831	A/Policlinico-Milano/03/2022	IV
EPI_ISL_14871104	EPI2160619	A/Policlinico-Milano/04/2022	V
EPI_ISL_14871105	EPI2160620	A/Policlinico-Milano/05/2022	V
EPI_ISL_14871106	EPI2160622	A/Policlinico-Milano/06/2022	V
EPI_ISL_14871107	EPI2160623	A/Policlinico-Milano/07/2022	III
EPI_ISL_14871237	EPI2160833	A/Policlinico-Milano/08/2022	V
EPI_ISL_14871239	EPI2160834	A/Policlinico-Milano/09/2022	V
EPI_ISL_14871108	EPI2160624	A/Policlinico-Milano/10/2022	V
EPI_ISL_14871109	EPI2160626	A/Policlinico-Milano/11/2022	V
EPI_ISL_14871110	EPI2160627	A/Policlinico-Milano/12/2022	V
EPI_ISL_14871111	EPI2160628	A/Policlinico-Milano/13/2022	V
EPI_ISL_14871112	EPI2160630	A/Policlinico-Milano/14/2022	V
EPI_ISL_14871113	EPI2160631	A/Policlinico-Milano/15/2022	V
EPI_ISL_14871240	EPI2160835	A/Policlinico-Milano/16/2022	V
EPI_ISL_14871115	EPI2160633	A/Policlinico-Milano/17/2022	III
EPI_ISL_14871116	EPI2160634	A/Policlinico-Milano/18/2022	V

Strain ID	**HA Segment ID**	**Strain name**	**Cluster**
EPI_ISL_14871117	EPI2160636	A/Policlinico-Milano/19/2022	V
EPI_ISL_14871118	EPI2160637	A/Policlinico-Milano/20/2022	V
EPI_ISL_14871119	EPI2160638	A/Policlinico-Milano/21/2022	V
EPI_ISL_14871120	EPI2160640	A/Policlinico-Milano/22/2022	I
EPI_ISL_14871121	EPI2160641	A/Policlinico-Milano/23/2022	V
EPI_ISL_14871122	EPI2160642	A/Policlinico-Milano/24/2022	V
EPI_ISL_14871123	EPI2160644	A/Policlinico-Milano/25/2022	V
EPI_ISL_14871124	EPI2160645	A/Policlinico-Milano/26/2022	III
EPI_ISL_14871125	EPI2160647	A/Policlinico-Milano/27/2022	V
EPI_ISL_14871126	EPI2160648	A/Policlinico-Milano/28/2022	III
EPI_ISL_14871127	EPI2160649	A/Policlinico-Milano/29/2022	V
EPI_ISL_14871128	EPI2160651	A/Policlinico-Milano/30/2022	V
EPI_ISL_14871129	EPI2160653	A/Policlinico-Milano/31/2022	V
EPI_ISL_14871241	EPI2160837	A/Policlinico-Milano/32/2022	V
EPI_ISL_14871130	EPI2160654	A/Varese/01/2022	V
EPI_ISL_14871131	EPI2160655	A/Varese/02/2022	V
EPI_ISL_14871132	EPI2160657	A/Varese/03/2022	III
EPI_ISL_14871133	EPI2160658	A/Varese/04/2022	I
EPI_ISL_14871134	EPI2160660	A/Varese/05/2022	V
EPI_ISL_14871136	EPI2160661	A/Varese/07/2022	V
EPI_ISL_14871137	EPI2160662	A/Varese/08/2022	V
EPI_ISL_14871138	EPI2160664	A/Varese/09/2022	V

Strain ID	HA Segment ID	Strain name	Cluster
EPI_ISL_14871139	EPI2160665	A/Varese/10/2022	V
EPI_ISL_14871140	EPI2160666	A/Varese/11/2022	V
EPI_ISL_14871141	EPI2160668	A/Varese/12/2022	V
EPI_ISL_14871142	EPI2160669	A/Varese/13/2022	V
EPI_ISL_14871143	EPI2160671	A/Varese/14/2022	III
EPI_ISL_14871144	EPI2160672	A/Varese/15/2022	V
EPI_ISL_14871145	EPI2160674	A/Varese/16/2022	V
EPI_ISL_14871146	EPI2160675	A/Varese/17/2022	V
EPI_ISL_14871147	EPI2160676	A/Varese/18/2022	V
EPI_ISL_14871148	EPI2160678	A/Varese/19/2022	V
EPI_ISL_14871149	EPI2160679	A/Varese/20/2022	V
EPI_ISL_14871150	EPI2160681	A/Varese/21/2022	V
EPI_ISL_14871151	EPI2160682	A/Varese/22/2022	V
EPI_ISL_14871152	EPI2160684	A/Bologna/01/2022	V
EPI_ISL_14871153	EPI2160685	A/Bologna/02/2022	V
EPI_ISL_14871154	EPI2160687	A/Bologna/03/2022	V
EPI_ISL_14871156	EPI2160688	A/Bologna/04/2022	V
EPI_ISL_14871157	EPI2160689	A/Bologna/05/2022	V
EPI_ISL_14871158	EPI2160691	A/Bologna/06/2022	V
EPI_ISL_14871159	EPI2160692	A/Bologna/07/2022	V
EPI_ISL_14871160	EPI2160694	A/Bologna/08/2022	V
EPI_ISL_14871161	EPI2160695	A/Bologna/09/2022	V
EPI_ISL_14871162	EPI2160696	A/Bologna/10/2022	V
EPI_ISL_14871163	EPI2160698	A/Bologna/11/2022	V
EPI_ISL_14871164	EPI2160699	A/Bologna/12/2022	V
EPI_ISL_14871165	EPI2160700	A/Bologna/13/2022	V
EPI_ISL_14871166	EPI2160702	A/Bologna/14/2022	V
EPI_ISL_14871167	EPI2160703	A/Bologna/15/2022	V
EPI_ISL_14871168	EPI2160704	A/Bologna/16/2022	V
EPI_ISL_14871169	EPI2160705	A/Bologna/17/2022	V
EPI_ISL_14871170	EPI2160707	A/Bologna/18/2022	V
EPI_ISL_14871171	EPI2160708	A/Bologna/19/2022	V
EPI_ISL_14871172	EPI2160710	A/Bologna/20/2022	V
EPI_ISL_14871173	EPI2160711	A/Bologna/21/2022	V

Strain ID	HA Segment ID	Strain name	Cluster
EPI_ISL_14871174	EPI2160712	A/Bologna/22/2022	V
EPI_ISL_14871175	EPI2160714	A/Bologna/23/2022	V
EPI_ISL_14871176	EPI2160715	A/Bologna/24/2022	V
EPI_ISL_14871177	EPI2160716	A/Milano/01/2022	III
EPI_ISL_14871178	EPI2160718	A/Milano/02/2022	V
EPI_ISL_14871180	EPI2160719	A/Milano/03/2022	III
EPI_ISL_14871181	EPI2160721	A/Milano/04/2022	II
EPI_ISL_14871182	EPI2160722	A/Milano/05/2022	V
EPI_ISL_14871183	EPI2160723	A/Milano/06/2022	V
EPI_ISL_14871184	EPI2160725	A/Milano/07/2022	III
EPI_ISL_14871185	EPI2160726	A/Milano/08/2022	III
EPI_ISL_14871186	EPI2160728	A/Milano/09/2022	III
EPI_ISL_14871187	EPI2160729	A/Milano/10/2022	III
EPI_ISL_14871188	EPI2160730	A/Milano/11/2022	III
EPI_ISL_14871189	EPI2160732	A/Milano/13/2022	III
EPI_ISL_14871190	EPI2160733	A/Milano/14/2022	IV
EPI_ISL_14871191	EPI2160735	A/Milano/15/2022	V
EPI_ISL_14871192	EPI2160736	A/Milano/16/2022	V
EPI_ISL_14871193	EPI2160738	A/Milano/17/2022	V
EPI_ISL_14871194	EPI2160739	A/Milano/19/2022	IV
EPI_ISL_14871195	EPI2160741	A/Milano/21/2022	II
EPI_ISL_14871196	EPI2160742	A/Milano/23/2022	IV
EPI_ISL_14871197	EPI2160743	A/Milano/27/2022	V
EPI_ISL_14871198	EPI2160745	A/Milano/29/2022	V
EPI_ISL_14871200	EPI2160746	A/Milano/31/2022	V
EPI_ISL_14871201	EPI2160747	A/Milano/35/2022	V
EPI_ISL_14871202	EPI2160749	A/Milano/38/2022	V
EPI_ISL_14871203	EPI2160750	A/Milano/39/2022	III
EPI_ISL_14871204	EPI2160751	A/Milano/40/2022	V
EPI_ISL_14871205	EPI2160753	A/Milano/42/2022	V
EPI_ISL_14871206	EPI2160754	A/Milano/48/2022	V
EPI_ISL_14871207	EPI2160755	A/Milano/49/2022	V
EPI_ISL_14871208	EPI2160757	A/Milano/55/2022	V
EPI_ISL_14871209	EPI2160758	A/Milano/63/2022	II

Strain ID	HA Segment ID	Strain name	Cluster
EPI_ISL_14871210	EPI2160760	A/Milano/68/2022	V
EPI_ISL_14871211	EPI2160761	A/Milano/70/2022	IV
EPI_ISL_14871212	EPI2160762	A/Milano/73/2022	V
EPI_ISL_14871213	EPI2160764	A/Milano/76/2022	V
EPI_ISL_14871214	EPI2160765	A/Milano/78/2022	III
EPI_ISL_14871215	EPI2160766	A/Milano/81/2022	V
EPI_ISL_14871216	EPI2160768	A/Milano/85/2022	V
EPI_ISL_14871217	EPI2160769	A/Milano/91/2022	V
EPI_ISL_14871218	EPI2160770	A/Milano/93/2022	V
EPI_ISL_14871219	EPI2160772	A/Milano/95/2022	V
EPI_ISL_14871220	EPI2160773	A/Milano/97/2022	V
EPI_ISL_14871221	EPI2160775	A/Milano/101/2022	V
EPI_ISL_14871223	EPI2160776	A/Milano/102/2022	V
EPI_ISL_14871224	EPI2160778	A/Milano/103/2022	V
EPI_ISL_14871225	EPI2160779	A/Milano/104/2022	V
EPI_ISL_14871226	EPI2160781	A/Milano/112/2022	V
EPI_ISL_14871227	EPI2160782	A/Milano/142/2022	V
EPI_ISL_14871228	EPI2160783	A/Milano/143/2022	V
EPI_ISL_14871229	EPI2160785	A/Milano/144/2022	V
EPI_ISL_14871230	EPI2160787	A/Milano/145/2022	V

Table S2. Substitutions by amino acid position and relative antigenic site and frequency detected by the comparison between the predicted amino acid HA sequences of the 2021-2022 vaccine reference strain (A/Cambodia/e0826360/2020) and the 161 A(H3N2) strains of this study. The amino acid residues responsible for major antigenic changes are in bold.

Amino acid position	Amino acid substitution	Antigenic site	N. of sequences with mutation (%)	Cluster
25	I-V	-	5 (3%)	IV
28	T-P	-	1 (1%)	V
30	T-P	-	1 (1%)	V
33	R-Q	-	2 (1%)	V
50	E-K	C	8 (5%)	I, III
53	D-G	C	133 (83%)	IV, V
53	D-N	C	21 (13%)	III
54	S-N	C	1 (1%)	V
56	H-Y	-	3 (2%)	II
60	D-E	-	1 (1%)	V
78	G-D	E	5 (3%)	IV, V
78	G-N	E	1 (1%)	IV
79	F-V	-	4 (2%)	I
96	N-S	-	20 (12%)	III
104	D-G	-	127 (79%)	V
104	D-N	-	1 (1%)	I
112	V-I	-	1 (1%)	V
124	S-N	A	2 (1%)	V
135	T-K	A	1 (1%)	III
140	I-M	A	10 (6%)	III, V
140	I-K	A	4 (2%)	I
150	R-K	A	1 (1%)	V
152	N-S	A	1 (1%)	III
156	**H-S**	**B**	**154 (96%)**	**III, IV, V**
157	L-I	B	8 (5%)	V
159	**Y-N**	**B**	**161 (100%)**	**I, II, III, IV, V**
160	K-I	B	161 (100%)	I, II, III, IV, V

Amino acid position	Amino acid substitution	Antigenic site	N. of sequences with mutation (%)	Cluster
164	L-Q	B	161 (100%)	I, II, III, IV, V
167	T-S	D	26 (16%)	V
171	N-K	D	161 (100%)	I, II, III, IV, V
175	D-E	D	1 (1%)	V
186	R-D	B	161 (100%)	I, II, III, IV, V
190	D-N	B	161 (100%)	I, II, III, IV, V
192	I-F	B	21 (13%)	III
198	P-S	B	161 (100%)	I, II, III, IV, V
201	R-K	D	5 (3%)	IV
205	S-F	-	3 (2%)	II
207	K-Q	D	1 (1%)	V
208	R-G	D	3 (2%)	V
212	A-T	D	3 (2%)	II
219	S-Y	D	5 (3%)	IV
220	R-K	-	1 (1%)	V
223	I-V	-	4 (2%)	III
227	P-S	D	1 (1%)	V
242	I-M	D	1 (1%)	II
259	K-R	-	1 (1%)	V
261	R-Q	E	1 (1%)	III
262	S-N	E	12 (7%)	I, V
270	S-T	-	3 (2%)	II
276	K-R	C	127 (79%)	V
299	R-K	C	1 (1%)	V
326	K-Q	-	2 (1%)	III
326	K-R	-	3 (2%)	V
328	T-I	-	1 (1%)	V
358	S-F	-	1 (1%)	V

Amino acid position	Amino acid substitution	Antigenic site	N. of sequences with mutation (%)	Cluster
358	S-Y	-	1 (1%)	IV
361	R-I	-	1 (1%)	V
378	N-S	-	21 (13%)	III
420	L-I	-	1 (1%)	V
426	E-D	-	1 (1%)	V
450	K-R	-	2 (1%)	V
476	A-T	-	1 (1%)	V
502	I-V	-	1 (1%)	III
503	K-N	-	1 (1%)	V
510	G-E	-	1 (1%)	V

Chapter 10

Epidemiological impact of human Adenoviruses as causative agent of respiratory infection: an Italian multicentre retrospective study, 2022-2023

Federica A.M. Giardina, Laura Pellegrinelli, Federica Novazzi, Elisa Vian, Valeria Biscaro, Cristina Russo, Stefania Ranno, Elisabetta Pagani, Elisa Masi, Claudia Tiberio, Martina Esposito, Sara Uceda Renteria, Anna Paola Callegaro, Giulia Piccirilli, Tiziana Lazzarotto, Francesca Rovida, Cristina Galli, Eleonora Lalle, Fabrizio Maggi, Nicasio Mancini, Carla Acciarri, Stefano Menzo, Anna Maria Colacicco, Maria Scarasciulli, Antonio Piralla, Fausto Baldanti, Elena Pariani and the AMCLI-GLIVIRE Working group

Role: First author

Author's contribution: Study design, acquisition, analysis, data interpretation, writing – original draft.

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Epidemiological impact of human adenoviruses as causative agent of respiratory infection: an Italian multicentre retrospective study, 2022-2023

Federica A.M. Giardina[a,1], Laura Pellegrinelli[b,1], Federica Novazzi[c,d,1], Elisa Vian[e], Valeria Biscaro[e], Cristina Russo[f], Cristina Stefania Ranno[f], Elisabetta Pagani[g], Elisa Masi[g], Claudia Tiberio[h], Martina Esposito [h], Sara Colonia Uceda Renteria[i], Anna Paola Callegaro[i], Giulia Piccirilli[j], Tiziana Lazzarotto[j,k], Francesca Rovida[a,l], Cristina Galli[b], Eleonora Lalle[m], Fabrizio Maggi[m], Nicasio Mancini[c,d], Carla Acciarri[n], Stefano Menzo[n,o], Anna Maria Colacicco[p], Maria Scarasciulli[p], Antonio Piralla[l], Fausto Baldanti[a,l], Elena Pariani[b], and the AMCLI GLiViRe Working group.

[a] *Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy;*
[b] *Department of Biomedical Sciences for Health, University of Milan, Milan, Italy;*
[c] *Department of Medicine and Innovation Technology, University of Insubria (DIMIT), Varese, Italy;*
[d] *Laboratory of Medical Microbiology and Virology University Hospital of Varese, Varese, Italy;*
[e] *UOC Microbiology, Department of specialist and laboratory medicine, AULSS 2 La Marca, Italy;*
[f] *Department of Diagnostic and Laboratory Medicine, Unit of Microbiology and Diagnostic Immunology, Bambino Gesù Children Hospital IRCCS, Roma, Italy;*
[g] *Laboratory of Microbiology and Virology, Provincial Hospital of Bolzano (SABES-ASDAA), Lehrkrankenhaus der Paracelsus Medizinischen Privatuniversität, Bolzano, Italy;*
[h] *Microbiology and Virology, Cotugno Hospital AORN dei Colli, Naples, Italy;*
[i] *Virology Unit, Clinical Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy;*
[j] *Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy;*
[k] *Section of Microbiology, Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy;*
[l] *Microbiology and Virology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy;*
[m] *Laboratory of Virology, National Institute for Infectious Diseases "Lazzaro Spallanzani" IRCCS, Rome, Italy;*
[n] *Virology Unit, Azienda Ospedaliero Universitaria delle Marche, Ancona, Italy;*
[o] *Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy;*
[p] *Virology Laboratory - Microbiology and Virology Unit - University of Bari - Policlinic of Bari, Bari, Italy.*

ABSTRACT

Human adenoviruses are the causative agents of 5-7% of viral respiratory infections, mainly caused by viruses from species B and C. They can infect all age groups, but children are usually at high risk of infections. Adenovirus epidemiology is well documented in East-Asian countries but little is known about adenovirus circulation in Europe in recent years. In our study, we investigated the epidemiology of adenovirus in respiratory infections in the Italian population. Our results showed that adenovirus was detected in 6.6% of all cases of acute respiratory infection included in the study and the median age of positive patients was 3 years, with male children in 1-2 years age group being the most affected. 43.5% of adenovirus cases were co-infected with at least one other respiratory virus, and rhinovirus was co-detected in 54% of cases. Genotyping of adenovirus allowed the identification of 6 different genotypes circulating in Italy, among which type B3 was the most frequently detected.

Keywords: Adenovirus, respiratory infection, molecular epidemiology, pediatric infections, multicenter surveillance.

1. BACKGROUND

Human adenoviruses (hAdVs) belong to the mammalian family *Adenoviridae* and are non-enveloped viruses. The hAdV genome is double-stranded DNA, approximately 34-36 kb in length, encoding for 40 different proteins, including the penton, hexon and fiber proteins that make up the viral capsid [1]. To date, the Adenoviridae family comprises 7 species, named from A to G, and more than 110 genotypes [2]. HAdV can be transmitted directly or indirectly by aerosol or by the faecal-oral route. HAdVs are highly contagious pathogens that cause a variety of clinical syndromes, including upper and lower respiratory tract infections, conjunctivitis and acute gastroenteritis [3,4]. In addition, some hAdV genotypes are associated with specific disease patterns. For example, types B and C are mainly associated with respiratory infections [5]. Upper respiratory tract infections (URTI) are characterised by signs and symptoms such as fever, sore throat, cervical adenopathy, headache, myalgia, cough and chills [6]. Sometimes, usually in children or immunocompromised patients, hAdV infection can progress to more severe disease such as bronchiolitis or pneumonia with severe and chronic lung damage [7,8]. No specific age limit for hAdV infection has been observed. However, hAdVs infections are particularly common in children under 5 years of age, accounting for at least 5-7% of respiratory diseases [9,10]. The epidemiology of hAdV-associated respiratory infections is well documented in East Asian countries such as China or Taiwan [11-13], but little is known about their circulation in Europe: in fact, very few data on hAdV epidemiology or molecular characterisation have been reported in the last decade [14-16]. Given the increasing interest in respiratory viruses and their role in causing severe respiratory syndromes, it is now necessary to improve our knowledge of the

epidemiology of these viruses by evaluating the presence of a molecular signature that may correlate with the clinical picture.

This multicentre retrospective study involved twelve microbiology laboratories in Italy with the aim of studying the circulation and molecular epidemiology of hAdVs over an 18-month period between 2022-2023.

2. METHODS

2.1 Study Design

Residual clinical specimens collected for diagnostic purposes from patients with respiratory tract infections between January 2022 and 30 June 2023 were used to assess hAdV epidemiology. Twelve clinical microbiology laboratories participated in this multicentre and retrospective study. The distribution of the participating laboratories on the Italian territory is shown in Figure 1. All these laboratories are members of the Working Group on Respiratory Virus Infections (GLIViRe) of the Italian Association of Clinical Microbiologists (AMCLI). All data included in this study were collected during routine clinical practice and analysed retrospectively and anonymously. All procedures were performed in accordance with ethical standards, the Declaration of Helsinki, and Italian law. Informed consent was not required because all data were de-identified.

Figure 1. Geographical distribution of clinical microbiology laboratories participating in the study. The following microbiology laboratories have participated in the study: Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia/ Microbiology and Virology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia; Department of Biomedical

Sciences for Health, University of Milan; Department of Medicine and Innovation Technology, University of Insubria (DIMIT)/Laboratory of Medical Microbiology and Virology University Hospital of Varese; Virology Unit, Clinical Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan; Laboratory of Microbiology and Virology, Provincial Hospital of Bolzano; UOC Microbiology- Dept. Specialist and laboratory medicine, Treviso; Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna/ Section of Microbiology, Department of Medical and Surgical Sciences, University of Bologna; Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona; Virology Unit, Azienda Ospedaliero Universitaria delle Marche, Ancona, Italy; Laboratory of Virology, National Institute for Infectious Diseases "Lazzaro Spallanzani" IRCCS, Rome; Department of Diagnostic and Laboratory Medicine, Unit of Microbiology and Diagnostic Immunology, Bambino Gesù Children Hospital IRCCS, Roma; Microbiology and Virology, Cotugno Hospital AORN dei Colli, Naples; Virology Laboratory - Microbiology And Virology Unit -University Of Bari - Policlinic Of Bari. For each center the total number of samples tested and HAdV-positive samples are indicated.

2.2 Molecular detection of respiratory viruses

The presence of respiratory viruses in clinical specimens was determined using commercial assays used in the participating laboratories: Allplex Respiratory Panel Assays (Seegene), BioFire® Respiratory 2.1 Panel (Biomerieux), QIAstat-Dx Respiratory SARS-CoV-2 Panel (Qiagen), Quanty HAdV (Clonit), HAdV ELITe MGB® kit (Elitech Group), HADV R-GENE® (Biomerieux), FTD Respiratory Panel 21 Assays (Siemens Healthineers). All assays were performed according to the manufacturer's instructions. Two laboratories used an in-house assay [17, 18]. In addition to hAdV, clinical samples were tested for the presence of influenza viruses (FluA/FluB), parainfluenza viruses (PIVs), enterovirus (EV), metapneumovirus (MPV), seasonal coronaviruses (hCoV), human rhinovirus (hRV), respiratory syncytial virus (hRSV), human bocavirus (hBoV) and SARS-CoV-2.

2.3 Adenovirus genotyping

HAdV DNA was amplified in a nested PCR targeting a portion of hexon gene (~750bp) according to Lu and Erdman 2006 [16]. Specifically, the first amplification was performed using AmpliTaqGold® with GeneAmp® (Life Technologies, NJ, USA) according to the manufacturer's instructions. The thermal profile was as follows 95°C for 10 min, then 50 cycles of 95°C for 30 s, 45°C for 30 s and 72°C for 1 min 30 s. The final step was 72°C for 5 min. Nested amplification was performed with the same thermal profile for a total of 40 cycles. The amplicons obtained have an expected size of approximately 800 nt. The amplicons were sequenced with the internal primers already used in the nested PCR using different sequencing platforms (3500 xL Dx Genetic Analyzer, SeqStudio Genetic Analyser system and ABI PRISM® 3100 GeneticAnalyser, Applied Biosystem, NJ, USA). The resulting sequences were analysed using Sequencher software version 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA).

BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to identify the hAdV genotype.

2.4 Statistical analysis

Comparisons between infection rates in different age groups were calculated with $\chi 2$ test. Statistical analyses were performed using GraphPad software version 8.3.0 (Prism).

3. RESULTS

3.1 Study samples

A total of 54463 respiratory specimens collected between 1 January 2022 and 20 June 2023 were tested for the presence of respiratory viruses. Of these, 3583 (6.6%) were hAdV positive. Of the 3583 r hAdV-positive samples, 2064 were nasal swabs (2064/3583, 57.7%), 1449 nasopharyngeal aspirates (1449/3583, 40.4%) and 70 bronchoalveolar lavages (70/3583, 1.9%). The median age of the hAdV-positive patients was 3 years (25th percentile: 1 year, 75th percentile: 5 years; range 6 days-98 years), including 1993 male and 1590 female subjects (55.6% and 44.4%, respectively). As shown in Figure 2, hAdV-positive cases were reported in all age groups in both males and females, with males in the 1-2 year age group being most affected. Overall, no statistically significant difference was observed between male and female subjects in all age groups (p=0.13). However, a statistically significant difference was observed between the number of hAdV-positive male subjects (1541/1993, 77.3%) compared to the number of hAdV-positive female subjects under 5 years of age (1184/1590, 74.4%, p=0.02).

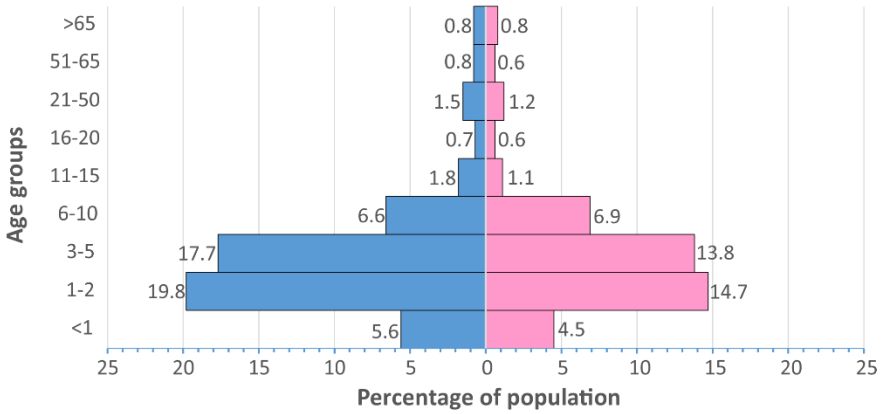

Figure 2. Distribution of HAdV-positive cases by age group and by gender. Blue bars represent male subjects, pink bars represent female subjects.

3.2 Temporal distribution of hAdV-positive cases

The majority of all hAdV cases (1648/3583, 46% of the total) were observed between weeks 10-2023 (early March) and 24-2023 (mid-June). It is noteworthy that the positivity rate was constantly above 10% since week 10-2023. (Fig. 3). The positivity rates varied throughout the study period, with the lowest value observed in week 6-2022 (1.7%, 8 positive samples out of 471 tested), while the highest value of the positivity rate was observed in week 22-2023 (15.8%, 97 positive samples out of 614 tested) (Fig. 3).

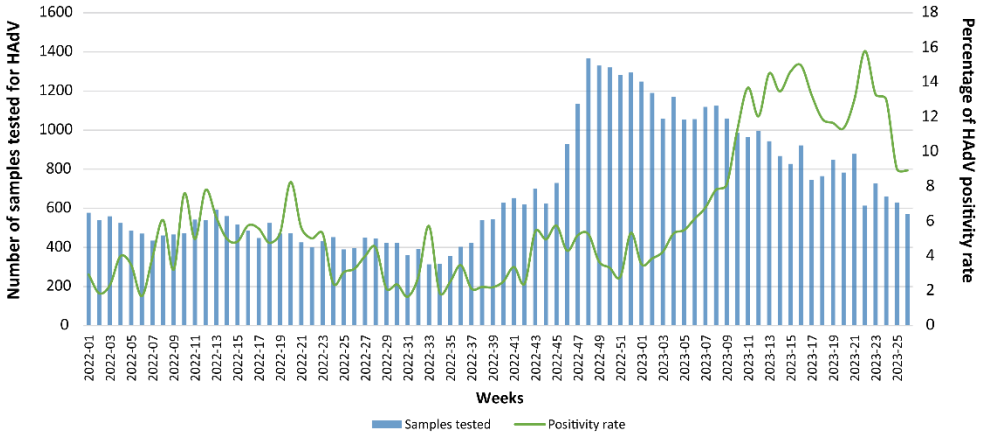

Figure 3. Weekly distribution of the number of samples tested for hAdV and hAdV positivity rates by week throughout the study period (January 2022-June 2023).

3.3 Co-detection of other respiratory viruses

At least one additional respiratory virus was detected in 1561/3583 (43.5%) hAdV-positive specimens. A single additional virus was detected in 1121/1561 cases (71.8%), two additional viruses in 348 (22.3%) and three additional viruses in 75 (4.8%). Co-detection of 4 or 6 other respiratory viruses was reported in less than 1% of cases. hRV was the most commonly co-detected virus (842/1561, 54%), followed by hRSV (206/1561, 13.2%), while PIV, MPV, EV and hBoV were detected in 12%, 11.3%, 9.7% and 9% of co-detection cases (187, 177, 152 and 141 of 1561 cases, respectively). Seasonal hCoV and influenza A viruses were co-detected in 8.6% and 6.3% of cases (134 and 99 out of 1561 cases, respectively). All other viruses detected, including influenza B virus, parechovirus and SARS-CoV-2, were detected in less than 6% of cases.

3.4 HAdV genotyping

Genotyping results were available for 158 samples collected between July 2022 and June 2023 from 3583 (4.4%) hAdV-positive cases. The genotypes detected were hAdV-B3 (98/158, 62%), -C2 (30/158, 19%), -C1 (19/158, 12%), -C5 (7/158, 4.4%), -B7 (3/158, 1.9%) and -C6 (1/158, 0.7%). From July 2022 to January 2023, hAdV-C strains were the most frequently detected: 34/41 (82.9%) strains sequenced belonged to hAdV-C1, -C2 or -C5, while 7/41 (17.1%) belonged to hAdV-B species. From February 2023 to summer 2023, hAdV-B3 strains were the predominant circulating strains in Italy, with 92/117 (78.6%) strains identified. The remaining hAdV strains, except for 2/117 (1.7%) strains identified as hAdV-B7, 23/117 (19.6%) strains belonged to hAdV-C species (Figure 4).

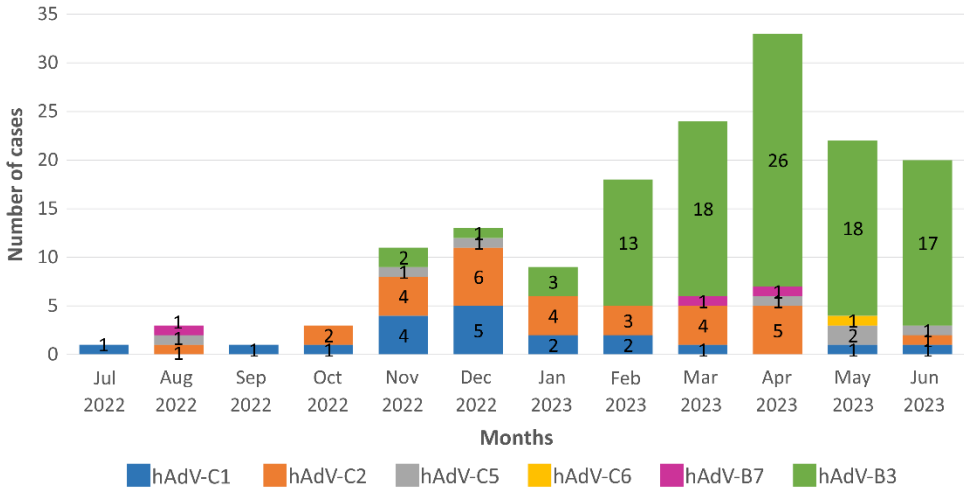

Figure 4. Number of hAdV genotypes identified by month from July 2022 to June 2023.

4. DISCUSSION

HAdV infection accounts for at least 5-7% of all viral respiratory infections, with children under 5 years of age being most affected [4]. The present study investigated the epidemiology of hAdV respiratory infections in adult and pediatric populations. In a large retrospective study involving 12 laboratories distributed throughout Italy and involving more than 50,000 specimens, hAdV was detected in 6.6% of the specimens included in the study. Our results are in agreement with those published by other groups [20, 21], who reported an overall positivity rate of 6.6% and 6.8%, respectively. In contrast, the percentage of hAdV-positive cases in our study was higher than that

reported by Radin et al [22] and Pscheidt et al [23], where hAdV accounted for approximately 2% of all respiratory infections. Positivity rates varied throughout the study period, ranging from 1.7% reported in February 2022 to 15.8% reported in June 2023. This can be explained by non-pharmaceutical interventions (NPIs): during the COVID-19 pandemic, from 2020 to the autumn season of 2022, the circulation of respiratory viruses decreased, mainly due to NPIs such as social distancing, smart working, school closure, hygiene measures, use of personal protective equipment such as face masks to control SARS-CoV-2 circulation. This also affected the circulation of other respiratory viruses [24, 25]. In the autumn of 2022, as health restrictive measures were gradually reduced, the circulation of respiratory viruses returned to pre-pandemic levels with an increasing number of samples analysed with a panel of respiratory viruses [26].

In our study population, the median age of hAdV-positive patients was 3 years, and especially male children in the age group 1-2 years were most affected. These data are in line with other reports [14, 23, 27]. These results also confirm that children under 5 years of age are more likely to be affected by hAdV than adults. There is a growing awareness of the importance of sex and gender in medicine and research. Females typically have stronger immune responses to self and foreign antigens than males, leading to sex differences in autoimmunity and infectious diseases [27]. In both animals and humans, males are generally more susceptible to bacterial infections than females. As hypothesised by Cheng et al [28], the difference in infection rates between males and females could be explained by the fact that a gene that regulates the natural killer response is located on the X chromosome and is therefore expressed twice as much in females as in males. At the same time, gender differences have been reported in health-seeking behaviour, quality of health care and adherence to treatment recommendations. As most infections in our studies were reported in children < 5 years of age, the difference in infection rate between males and females may depend on the recreational activities: males tend to play in larger groups than females and prefer rough-and-tumble games, which reduce personal distances and increase the likelihood of infection.

Viral co-infection may play a crucial role in the outcome of respiratory syndrome: it has been reported that patients (mostly immunocompromised individuals or adults >65 years) with more than one respiratory virus, including rhinovirus and SARS-CoV-2, are more likely to report cough or dyspnoea or to be admitted to intensive care units [30,31]. However, other studies have not observed a worsening of clinical syndromes due to viral co-detection, suggesting that disease severity is not correlated with the number of respiratory viruses detected [32,22]. In our study, the rate of co-infection accounted for at least 40% of hAdV episodes. This value is higher than that reported in a study conducted in Vietnam in 2022, where the co-detection rate was 20% [13]. This difference in co-infection rates is probably due to the different length of the study period. While our study looked at a period of 18 consecutive months, the study by Nguyen and colleagues in 2022 looked at only 1 month; moreover, as they reported, RSV was not

included in the testing panel. Therefore, some cases of co-detection may be missing. On the contrary, it is lower than the 81% cases of viral co-detection reported in Jordan between 2010 and 2013 [34]. This could be explained by the nature of this study. Indeed, it was conducted over three years in hospitalised children under 2 years of age with acute respiratory illness, including those with underlying diseases. In contrast, the population of our study included both paediatric and adult patients, irrespective of clinical syndromes. Similar to Probst and colleagues, rhinovirus was most commonly co-detected with hAdV in our study (54%), whereas co-detection of influenza A virus was observed in 6%. This may be explained by the seasonality of respiratory virus circulation: rhinovirus circulates throughout the year, with peaks in spring and autumn, whereas other viruses such as influenza circulate during the winter season. This study looked at an 18-month period from January 2022 to June 2023, including a period of reduced respiratory virus circulation due to NPIs.

Genotyping of the hAdV strains included in our study showed that hAdV-B, especially hAdV-B3, was the most commonly detected (64%), followed by hAdV-C strains (36%). This finding is similar to studies reporting hAdV-B types as the most commonly detected [11,13,35]. Some other studies have instead reported a different pattern of hAdV genotypes detected, with type C being the most commonly detected [34]. Nevertheless, our study confirms that hAdV types B and C are mainly associated with respiratory infections. Beyond the epidemiological purpose, genotyping of hAdV strains could also be useful as a predictor of treatment efficacy, as reported by Matthes-Martin et al. Some antiviral drugs showed higher in vitro efficacy for hAdV-C, with limited activity for species A, B and D [36].

Our study has several limitations: i) Due to the nature of the study, which focused on epidemiological patterns rather than clinical outcomes, clinical data from the patients included in the study were not available, even though they would provide valuable insights into the impact of adenovirus infections. Therefore, we were not able to establish a possible correlation between hAdV genotypes and severity of respiratory disease; ii) unfortunately, data on the total number of respiratory specimens were not available, so we could not calculate age-specific positivity rates; iii) some of the commercial assays used in the different laboratories for hAdV detection are qualitative and it was not possible to investigate the possible correlation between viral loads and severity of disease; and iv) genotyping was only performed from July 2022, so no data are available on hAdV types circulating before summer 2022. In addition, the number of genotyped strains is relatively small. Unfortunately, not all clinical laboratories are able to perform sequencing tests. In fact, the genotyping data reported in the study were obtained from three laboratories, all located in Lombardy (northern Italy). Unfortunately, due to the retrospective nature of the study, it was not even possible to collect positive samples from each laboratory. In addition, v) these results are based on local data from Italy and may not be applicable internationally, particularly with regard to genetic analysis.

5. CONCLUSIONS

The results of this multicentre retrospective study show that hAdV accounts for nearly 7% of all viral respiratory infections in the post-pandemic period and that children under 5 years of age are at high risk of infection. Furthermore, genotyping showed that hAdV-B3 was the most frequently detected genotype circulating in Italy in 2022-2023. The importance of hAdV as a causative agent of respiratory syndromes was highlighted, suggesting that further studies on circulating genotypes and their correlation with different clinical presentations are needed to complete the knowledge on hAdV circulation in our country and in Europe.

References

1. Arnold A, MacMahon E. Adenovirus infections. Medicine. 2021 Dec; 49(12):790–3.
2. Barnadas C, Schmidt DJ, Fischer TK, Fonager J. Molecular epidemiology of human adenovirus infections in Denmark, 2011-2016. J Clin Virol. 2018 Jul;104:16-22. doi: 10.1016/j.jcv.2018.04.012. Epub 2018 Apr 21. PMID: 29704734; PMCID: PMC7106356.
3. Brañas P, Muñoz-Gallego I, Espartosa E, Moral N, Abellán G, Folgueira L. Dynamics of respiratory viruses other than SARS-CoV-2 during the COVID-19 pandemic in Madrid, Spain. Influenza Other Respir Viruses. 2023 Sep;17(9):e13199. doi: 10.1111/irv.13199. PMID: 37752064; PMCID: PMC10520512.
4. Canela LNP, Magalhães-Barbosa MC, Raymundo CE, Carney S, Siqueira MM, Prata-Barbosa A, Cunha AJLAD. Viral detection profile in children with severe acute respiratory infection. Braz J Infect Dis. 2018 Sep-Oct;22(5):402-411. doi: 10.1016/j.bjid.2018.09.001. Epub 2018 Oct 23. PMID: 30365924; PMCID: PMC7138071.
5. Cederwall S, Påhlman LI. Respiratory adenovirus infections in immunocompetent and immunocompromised adult patients. Epidemiol Infect. 2020 Jan 3;147:e328. doi: 10.1017/S0950268819002176. PMID: 31896387; PMCID: PMC7006015.
6. Chen Y, Lin T, Wang CB, Liang WL, Lian GW, Zanin M, Wong SS, Tian XG, Zhong JY, Zhang YY, Xie JH, Zheng LL, Chen FY, Dang R, Zhao MQ, Yang YY, Zhou R, Zhu B. Human adenovirus (HAdV) infection in children with acute respiratory tract infections in Guangzhou, China, 2010-2021: a molecular epidemiology study. World J Pediatr. 2022 Aug;18(8):545-552. doi: 10.1007/s12519-022-00590-w. Epub 2022 Jul 21. PMID: 35861938; PMCID: PMC9302229.
7. Chow EJ, Uyeki TM, Chu HY. The effects of the COVID-19 pandemic on community respiratory virus activity. Nat Rev Microbiol. 2023 Mar;21(3):195-210. doi:

10.1038/s41579-022-00807-9. Epub 2022 Oct 17. PMID: 36253478; PMCID: PMC9574826.
8. Davison AJ, Benkő M, Harrach B. Genetic content and evolution of adenoviruses. J Gen Virol. 2003 Nov;84(Pt 11):2895-2908. doi: 10.1099/vir.0.19497-0. PMID: 14573794.
9. De Francesco MA, Pollara C, Gargiulo F, Giacomelli M, Caruso A. Circulation of Respiratory Viruses in Hospitalized Adults before and during the COVID-19 Pandemic in Brescia, Italy: A Retrospective Study. Int J Environ Res Public Health. 2021 Sep 9;18(18):9525. doi: 10.3390/ijerph18189525. PMID: 34574450; PMCID: PMC8469422.
10. Dominguez O, Rojo P, de Las Heras S, Folgueira D, Contreras JR. Clinical presentation and characteristics of pharyngeal adenovirus infections. Pediatr Infect Dis J. 2005 Aug;24(8):733-4. doi: 10.1097/01.inf.0000172942.96436.2d. PMID: 16094232.
11. Esposito S, Zampiero A, Bianchini S, Mori A, Scala A, Tagliabue C, Sciarrabba CS, Fossali E, Piralla A, Principi N. Epidemiology and Clinical Characteristics of Respiratory Infections Due to Adenovirus in Children Living in Milan, Italy, during 2013 and 2014. PLoS One. 2016 Apr 5;11(4):e0152375. doi: 10.1371/journal.pone.0152375. PMID: 27045588; PMCID: PMC4821614.
12. Guérin-El Khourouj V, Dalle JH, Pédron B, Yakouben K, Bensoussan D, Cordeiro DJ, Peltier L, Ouachée-Chardin M, Baruchel A, Sterkers G. Quantitative and qualitative CD4 T cell immune responses related to adenovirus DNAemia in hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2011 Apr;17(4):476-85. doi: 10.1016/j.bbmt.2010.09.010. Epub 2010 Nov 11. PMID: 20869455.
13. Jothikumar N, Cromeans TL, Hill VR, Lu X, Sobsey MD, Erdman DD. Quantitative real-time PCR assays for detection of human adenoviruses and identification of serotypes 40 and 41. Appl Environ Microbiol. 2005;71:3131-6.
14. Le Glass E, Hoang VT, Boschi C, Ninove L, Zandotti C, Boutin A, Bremond V, Dubourg G, Ranque S, Lagier JC, Million M, Fournier PE, Drancourt M, Gautret P, Colson P. Incidence and Outcome of Coinfections with SARS-CoV-2 and Rhinovirus. Viruses. 2021 Dec 16;13(12):2528. doi: 10.3390/v13122528. PMID: 34960797; PMCID: PMC8709236.
15. Lin TY, Tsai YS, Shen CF. Type 3 adenovirus respiratory infection complicated with catastrophic bronchiole damage and related barotrauma. Pediatr Neonatol. 2022 May;63(3):319-321. doi: 10.1016/j.pedneo.2021.11.011. Epub 2022 Feb 12. PMID: 35246370.
16. Lu X, Erdman DD. Molecular typing of human adenoviruses by PCR and sequencing of a partial region of the hexon gene. Arch Virol. 2006 Aug;151(8):1587-602. doi: 10.1007/s00705-005-0722-7. Epub 2006 Feb 26. PMID: 16502282.
17. Matthes-Martin S, Boztug H, Lion T. Diagnosis and treatment of adenovirus infection in immunocompromised patients. Expert Rev Anti Infect Ther. 2013

Oct;11(10):1017-28. doi: 10.1586/14787210.2013.836964. Epub 2013 Sep 27. PMID: 24073835.
18. Narra R, Bono P, Zoccoli A, Orlandi A, Piconi S, Grasselli G, Crotti S, Girello A, Piralla A, Baldanti F, Lunghi G. Acute respiratory distress syndrome in adenovirus type 4 pneumonia: A case report. J Clin Virol. 2016 Aug;81:78-81. doi: 10.1016/j.jcv.2016.06.005. Epub 2016 Jun 14. PMID: 27354307.
19. Nguyen DD, Phung LT, Thanh Tran HT, Ly HTT, Vo AHM, Dinh NP, Doan PM, Nguyen AT, Dang LD, Doan TT, Pham KT, Pham HL, Hoang DX, Pham TN, Tran BT, Tran TTT, Le HTM, Pham AN, Antoniou A, Ho NT. Molecular subtypes of Adenovirus-associated acute respiratory infection outbreak in children in Northern Vietnam and risk factors of more severe cases. PLoS Negl Trop Dis. 2023 Nov 7;17(11):e0011311. doi: 10.1371/journal.pntd.0011311. PMID: 37934746; PMCID: PMC10655982.
20. Parcell BJ, McIntyre PG, Yirrell DL, Fraser A, Quinn M, Templeton K, Christie S, Romanes F. Prison and community outbreak of severe respiratory infection due to adenovirus type 14p1 in Tayside, UK. J Public Health (Oxf). 2015 Mar;37(1):64-9. doi: 10.1093/pubmed/fdu009. Epub 2014 Feb 25. PMID: 24573364.
21. Perez A, Lively JY, Curns A, Weinberg GA, Halasa NB, Staat MA, Szilagyi PG, Stewart LS, McNeal MM, Clopper B, Zhou Y, Whitaker BL, LeMasters E, Harker E, Englund JA, Klein EJ, Selvarangan R, Harrison CJ, Boom JA, Sahni LC, Michaels MG, Williams JV, Langley GE, Gerber SI, Campbell A, Hall AJ, Rha B, McMorrow M; New Vaccine Surveillance Network Collaborators. Respiratory Virus Surveillance Among Children with Acute Respiratory Illnesses - New Vaccine Surveillance Network, United States, 2016-2021. MMWR Morb Mortal Wkly Rep. 2022 Oct 7;71(40):1253-1259. doi: 10.15585/mmwr.mm7140a1. PMID: 36201373; PMCID: PMC9541034.
22. Piralla A, Pariani E, Rovida F, Campanini G, Muzzi A, Emmi V, Iotti GA, Pesenti A, Conaldi PG, Zanetti A, Baldanti F; Severe Influenza A Task Force. Segregation of virulent influenza A(H1N1) variants in the lower respiratory tract of critically ill patients during the 2010-2011 seasonal epidemic. PLoS One. 2011;6(12):e28332. doi: 10.1371/journal.pone.0028332. Epub 2011 Dec 14. PMID: 22194826; PMCID: PMC3237448.
23. Probst V, Rankin DA, Haddadin Z, Hamdan L, Rahman HK, Yanis A, Talj R, Spieker AJ, Howard L, Stewart LS, Guevara C, Yepsen E, Faouri S, Shehabi A, Williams JV, Chappell J, Khuri-Bulos N, Halasa NB. Adenovirus Infection in Hospitalized Children with Acute Respiratory Infection in Jordan. Pediatr Infect Dis J. 2022 Apr 1;41(4):277-283. doi: 10.1097/INF.0000000000003423. PMID: 35315822; PMCID: PMC8943843.
24. Pscheidt VM, Gregianini TS, Martins LG, Veiga ABGD. Epidemiology of human adenovirus associated with respiratory infection in southern Brazil. Rev Med Virol. 2021 Jul;31(4):e2189. doi: 10.1002/rmv.2189. Epub 2020 Nov 6. PMID: 33156553.

25. Radin JM, Hawksworth AW, Kammerer PE, Balansay M, Raman R, Lindsay SP, Brice GT. Epidemiology of pathogen-specific respiratory infections among three US populations. PLoS One. 2014 Dec 30;9(12):e114871. doi: 10.1371/journal.pone.0114871. PMID: 25549089; PMCID: PMC4280218.
26. Saha R, Majumdar A, Chaudhuri RD, Chatterjee A, Lo M, Dutta S, Chawla-Sarkar M. Molecular epidemiology of circulating human adenoviruses among acute respiratory infection patients seeking healthcare facilities in West Bengal, India. Virology. 2023 Nov;588:109912. doi: 10.1016/j.virol.2023.109912. Epub 2023 Oct 20. PMID: 37913611.
27. Scotta MC, Chakr VC, de Moura A, Becker RG, de Souza AP, Jones MH, Pinto LA, Sarria EE, Pitrez PM, Stein RT, Mattiello R. Respiratory viral coinfection and disease severity in children: A systematic review and meta-analysis. J Clin Virol. 2016 Jul;80:45-56. doi: 10.1016/j.jcv.2016.04.019. Epub 2016 Apr 30. PMID: 27155055; PMCID: PMC7185664.
28. Shieh WJ. Human adenovirus infections in pediatric population - An update on clinico-pathologic correlation. Biomed J. 2022 Feb;45(1):38-49. doi: 10.1016/j.bj.2021.08.009. Epub 2021 Sep 10. PMID: 34506970; PMCID: PMC9133246.
29. Teoh Z, Conrey S, McNeal M, Burrell A, Burke RM, Mattison C, McMorrow M, Payne DC, Morrow AL, Staat MA. Burden of Respiratory Viruses in Children Less Than 2 Years Old in a Community-based Longitudinal US Birth Cohort. Clin Infect Dis. 2023 Sep 18;77(6):901-909. doi: 10.1093/cid/ciad289. PMID: 37157868.
30. Trifonova I, Christova I, Madzharova I, Angelova S, Voleva S, Yordanova R, Tcherveniakova T, Krumova S, Korsun N. Clinical significance and role of coinfections with respiratory pathogens among individuals with confirmed severe acute respiratory syndrome coronavirus-2 infection. Front Public Health. 2022 Sep 2;10:959319. doi: 10.3389/fpubh.2022.959319. PMID: 36117597; PMCID: PMC9479447.
31. Wang L, Hu X, Huang Z, Zhang Y, Zhao X, Liu X, Mao H, Hao H, Xue W. Analysis of the typing of adenovirus and its clinical characteristics in children with acute respiratory tract infection. BMC Pediatr. 2023 Jan 16;23(1):25. doi: 10.1186/s12887-023-03840-6. PMID: 36647010; PMCID: PMC9841135.
32. Wen S, Lin Z, Zhang Y, Lv F, Li H, Zhang X, Lin L, Zhu HH, Xu Z, Li C, Zhang H. The Epidemiology, Molecular, and Clinical of Human Adenoviruses in Children Hospitalized With Acute Respiratory Infections. Front Microbiol. 2021 Feb 16;12:629971. doi: 10.3389/fmicb.2021.629971. PMID: 33664719; PMCID: PMC7921318.
33. Xu D, Ji L, Chen L, Wu X. Molecular typing and epidemiology profiles of human adenovirus infection among children with severe acute respiratory infection in Huzhou, China. Lett Appl Microbiol. 2022 Nov;75(5):1225-1231. doi: 10.1111/lam.13790. Epub 2022 Aug 5. PMID: 35861697.
34. Zadheidar S, Yavarian J, Heydarifard Z, Nejati A, Sadeghi K, Ghavami N, Abbasi S, Shatizadeh Malekshahi S, Mokhtari-Azad T, Shafiei-Jandaghi NZ. Molecular

epidemiology of human adenoviruses in children with and without respiratory symptoms: Preliminary findings from a case-control study. BMC Pediatr. 2022 Oct 8;22(1):583. doi: 10.1186/s12887-022-03625-3. PMID: 36207696; PMCID: PMC9547415.

35. Zandvliet ML, Falkenburg JH, van Liempt E, Veltrop-Duits LA, Lankester AC, Kalpoe JS, Kester MG, van der Steen DM, van Tol MJ, Willemze R, Guchelaar HJ, Schilham MW, Meij P. Combined CD8+ and CD4+ adenovirus hexon-specific T cells associated with viral clearance after stem cell transplantation as treatment for adenovirus infection. Haematologica. 2010 Nov;95(11):1943-51. doi: 10.3324/haematol.2010.022947. Epub 2010 Jun 18. PMID: 20562315; PMCID: PMC2966918.

Chapter 11

General Discussion

In the first part of this PhD research, the main focus was to evaluate the epidemiology of SARS-CoV-2 variants in Lombardy region from the first pandemic wave to August 2022, eight months after the first Omicron lineage detection.
In February 2020, following the identification of the first SARS-CoV-2 case, Lombardy was the first Italian region to be heavily affected by the pandemic. The phylogenetic analysis performed on viral sequences collected during the initial phase of the pandemic indicated the simultaneous circulation of at least seven distinct SARS-CoV-2 lineages, with a different geographical distribution among Lombardy provinces (Alteri *et al.*, 2021). Similar results were also obtained from other studies evaluating the SARS-CoV-2 circulation in Italy and in Europe (Di Giallonardo et al., 2020; Alm et al., 2020). A reported, new lineages were detected in Lombardy between the first and the second pandemic waves, probably due to the partial restoration of social and commercial activities. However, this introduction did not result in an increased transmission between individuals. Several mutations were identified in the Spike protein, including D614G, but all of them were located outside the Receptor Binding Domain (RBD) region.
In September 2020, at least 25 different SARS-CoV-2 lineages were identified, confirming that national and international connections during the summer had contributed to the introduction of new lineages with a progressive evolution of the virus. All these lineages circulated in equal proportion until December 2020, when the first Alpha variant cases, characterized by the N501Y mutations, were detected before a rapid spread all over Europe (Lindstrøm *et al.*, 2022). In the same period, other variants were detected such as the Beta and Gamma lineages which caused large outbreaks in other countries but not in Italy (Umair *et al.*, 2022). A few months later, in May 2021, the first cases of Delta variants were reported. Delta variant spread very rapidly becoming predominant over Alpha strains; interestingly, by the time of first Delta identification, the vaccination campaign had already started and the majority of the Italian population, especially healthcare workers and older adults, had already received at least the first dose of vaccination. Thus, the rapid spread of the Delta variant was likely due to its ability to evade the host's vaccine-induced immune response, as suggested by Cassaniti and colleagues (Cassaniti *et al.*, 2022). From January 2022, all SARS-CoV-2 positive cases were instead sustained by the Omicron variant and its sublineages. As documented by the last epidemiological report of Italian Istituto Superiore di Sanità (ISS) published in May 2024, XBB, EG.5 and JN.1 were the latest SARS-Cov-2 variant detected (Istituto Superiore di Sanità, 2024).
It is well-known that SARS-CoV-2 symptoms may range from asymptomatic infection to severe respiratory disease, often requiring oxygen therapy or ICU admission (Ochani *et al.*, 2021). However, the majority of these studies reported clinical data collected from the adult population and little was known about the epidemiology of SARS-CoV-2 in children. As described in our study (Rovida *et al.*, 2022), between February 2020 and April 2021 5% of all SARS-CoV-2 cases detected were patients <15 years of age. Following the positivity rates in the general population, three different peaks were observed in the pediatric population (March 2020, November 2020, March 2021): the first one was correlated to the uncontrolled SARS-CoV-2 circulation while the second and third to the school re-opening after closure during lockdown. However, most of the

positive pediatric patients were asymptomatic or showed mild symptoms and thus could spread the infection to other family members (Castagnoli et al., 2020).
An NGS approach of the spike gene was used to gain a deeper insight into the genetic characteristics of SARS-CoV-2, such as genetic mutations in the spike protein that may be associated with the severity of the disease. In particular, sequences were obtained from paired samples collected from the upper (UTR) and lower respiratory tract (LRT) of patients with severe infection admitted to the Intensive Care Unit (ICU). The aims were to evaluate the genetic variability of SARS-CoV-2 in two different body compartments and identify minority variants within the viral quasispecies potentially associated with the progression from the upper to the lower respiratory tract. All the analyzed sequences harbored the mutation D614G associated to an increased fitness but not to increased disease severity (Korber *et al.*, 2020). Moreover, none of the polymorphisms identified was associated with the progression from upper to lower respiratory tract. This evidence was also supported by Rueca end colleagues who did not identify any compartment-specific pattern of mutations (Rueca *et al.*, 2020).
Spike gene sequences obtained showed greater variability, expressed as the number of haplotypes, in LRT than URT, probably due to independent replication in the two respiratory districts, as also suggested by Wölfel and colleagues (Wölfel *et al.*, 2020). Furthermore, since dN/dS ratio was higher than 1, a higher selective pressure was observed in the lung environment. A lot of nucleotidic changes were observed, most of which were probably associated with host editing mechanisms such as APOBEC and ADARs (Carpenter *et al.*, 2009; Di Giorgio *et al.*, 2020). T>G substitution was the most prevalent nucleotide change and was associated with URT. Lastly, a high concentration of mutation was observed around the cleavage site between the S1 and S2 subunits of S protein. The cleavage site is an important antigenic site that harbors mutations associated with viral spillover to humans (Andersen et al., 2020). Mutations in the cleavage site had been associated with virus attenuation in hamsters and milder symptoms in humans (Wang *et al.*, 2021; Andrés *et al.*, 2020).

The aim of the second part of this research was the investigation about the viruses that belong to *Picornaviridae* family, which comprises rhinoviruses and enteroviruses. HRVs are generally known to be the etiologic agents of the common cold but, in recent years, their role on causing severe respiratory syndrome has been re-evaluated (Drysdale *et al.*, 2017; Aydin *et al.*, 2019). In our analysis, almost 9% of all patients included were hRV-positive and most of them were less than 16 years old, suggesting that even if hRV can infect individuals in all age groups, it infects predominantly young patients. These data were also confirmed by other studies investigating the epidemiology of hRVs in the general population (Kenmoe *et al.*, 2021; Hung *et al.*, 2019). Most cases were caused by hRV-A species genotypes, which are the most numerous than other species. In 23.4% of hRV-positive samples, at least another respiratory virus was identified with hAdVs and hRSV being the most detected. This could be explained by the simultaneous circulation of hRVs and hAdVs all year round; moreover, as hRV infects mainly children, also hRSV circulates mainly among pediatric patients. Data about co-detection of hRVs and hAdVs have been reported in other studies (Bruning *et al.*, 2015; Hung *et al.*, 2019). 4.5% of patients with hRV had severe pneumonia and were admitted in the ICU. These

data confirm that also hRVs can cause severe infections, as reported by Jain and colleagues in 2015 who identified hRVs as the most detected in adult patients in ICU and the second one in the pediatric population after hRSV (Jain *et al.*, 2015; Jain *et al.*, 2015).
Furthermore, two intra-hospital transmission events were observed and both of them in the neonatal settings, probably for the contamination of inanimated surfaces or healthcare workers during viral shedding after the resolution of symptoms (Reese *et al.*, 2016).
An intra-hospital transmission event is probably the cause for the infection of newborn male twins with an echovirus 11 (E11) in April 2023, which resulted in severe hepatitis (Piralla *et al.*, 2023). E11 belongs to the species B of *Enterovirus* genus within the *Picornaviridae* family. It gained attention in early summer 2023 when an increased circulation among newborns and its association with severe and fatal infections were reported (Grapin *et al.*, 2023). In this study, Grapin and colleagues also supposed the recombinant origin of E11 strain, but couldn't identify the parental strain.
After the publication of a notification alert by WHO and ECDC, we promoted active surveillance, also in a retrospective way to the beginning of August 2021, on all enterovirus cases, especially focusing on pediatric cases (Pellegrinelli *et al.*, 2024). A total of 24 E11 cases were detected: all positive patients were children <10 years, whose symptoms ranged from Influenza-like Illness (ILI) to more severe clinical manifestations such as otitis, meningitis, respiratory distress and hepatitis. Phylogenetic analysis performed on complete genome sequences and partial sequences (P1+P2+3A-3B regions) of 16 E11 strains collected in 2023 confirmed that all these strains, together with 37 additional strains whose sequences were retrieved from GenBank, belonged to a new lineage within the genogroup D5 with a nucleotide identity of 98.7%. However, when compared to other E11 sequences, nucleotide identity decreased to 85.1%.
The phylogenetic analysis was then performed in $3C^{pro}$+$3D^{pol}$ regions confirming a higher similarity with an echovirus 6 strain circulating in France in 2022 and whose sequence was available online in December 2023. The subsequent analysis, including SimPlot analysis, confirmed the recombination event likely dating it to June 2018.
Recombination is one of the two major mechanisms for enteroviruses evolution and the circulation of several recombinant strains has been documented in the scientific literature (Chan *et al.*, 2004; Tapparel *et al.*, 2009; Li *et al.*, 2023). However, since enterovirus infections are not notifiable to the Health Authorities, data on the real number of cases and their molecular characterization may be missed.

The main focus of the third part is the circulation of influenza in the post-pandemic period and its molecular characterization.
As well described, the circulation of influenza virus was disrupted during the SARS-CoV-2 pandemic thanks to the implementation of NPIs such as facial masks, hand hygiene, social distancing and lockdown. The first cases of influenza detection were reported in early 2022, a few weeks after the usual beginning of circulation during winter season. The delayed circulation of influenza was also documented by the Italian ISS (Istituto Superiore di Sanità, 2022). Most of strains identified were A(H3N2) and belonged to clade 3C.2a, subclade 3C.2a1 and genetic group 3C.2a1b, with high

nucleotide and amino acid similarity. These findings were in agreement with European data (ECDC, 2022; ISS, 2022). A comparison between HA sequences of A(H3N2) strains and the vaccine strain for the Northern Hemisphere was performed resulting in 98.4% nucleotide identity and 97.5% in amino acid similarity. Furthermore, among all amino acid substitutions observed, mutations H156S and Y159N were located within the major antigenic sites of the receptor binding domain; thus, the circulating strains harboring these mutations could evade the vaccine-induced immune response and the vaccine efficacy could be sub-optimal. The sub-optimal vaccine effectiveness against H3N2 strains has been described also in other European studies, suggesting that this could be explained by the circulation of H3N2 drifted variants (Chung *et al.*, 2022; Emborg *et al.*, 2022; Kim *et al.*, 2022).

The last part is focused on preliminary results of a multicentric study of the epidemiological impact of adenoviruses on respiratory infections among adult and pediatric populations. The epidemiology of HAdV infections is well described in East-Asian countries (Chen *et al.*, 2022; Nguyen *et al.*, 2023) but little is known about its circulation in Europe. In our study, hAdVs accounted for 6.6% cases of respiratory infections, in agreement with other studies evaluating hAdVs epidemiology (Zadheidar *et al.*, 2022; Saha *et al.*, 2023). Moreover, even the circulation of hAdVs was affected by the use of NPIs, ad demonstrated by the variability of positivity rates ranging from 1.7% in February 2022 to 15.8% in June 2023. Since the median age of positive patients was 3 years old, with male patients in the age group 1-2 years as the most affected, HAdV confirmed to be infecting mainly children (Perez *et al.*, 2022). Results about coinfection rates are similar to those reported in the study evaluating the epidemiology of hRVs which are the most detected with hAdVs; however, since several studies did not report any worsening in clinical conditions in patients infected with two or more respiratory viruses, the role of coinfection on clinical syndromes needs to be cleared (Scotta *et al.*, 2016; Radin *et al.*, 2014). Genotyping of positive samples revealed that hAdV-B and hAdV-C species were the most prevalent, confirming their association with respiratory syndromes (Wang *et al.*, 2023; Chen *et al.*, 2022).
However, for the retrospective nature of this study, retrieving the clinical records of hAdV-positive patients was not possible so that we couldn't make any assumption on possible correlations between hAdV-related clinical syndromes, coinfection rates or circulating genotypes.

Chapter 12

Conclusions

Respiratory viral infections cause every year high rates of morbidity and mortality, in immunocompromised subjects as well as in otherwise healthy individuals. Thus, they represent a major concern for public health.
In all the studies included in this research project, we aimed to better understand the epidemiology and the genetic features of some of the main respiratory viruses.
For SARS-CoV-2, we described the epidemiology of different variants from June 2020 to August 2022 and the circulation in the pediatric population. Then we looked for molecular signatures possibly associated with the progression from upper to lower respiratory tract and the severity of the disease.
For picornaviruses, we described the epidemiology of rhinovirus and their ability to cause the common cold as well as severe infections. Moreover, we performed genetic analyses to confirm the recombinant origin of the echovirus 11 strain associated with severe and fatal neonatal infection.
For Influenza, a delayed circulation of influenza A/H3N2 in 2022 was observed; molecular characterization revealed the presence of mutations in hemagglutinin protein that could have an impact on the recognition of vaccine-induced immune response.
Lastly, the results of a preliminary study on adenovirus respiratory infections were reported, confirming higher infection rates among children.
In conclusion, all these studies highlight the importance of continuous surveillance of respiratory viruses and their molecular characterization, to promptly identify mutations or pathogenetic alterations correlated to severe clinical syndromes, tracing the outbreaks within and outside hospital setting or follow viral evolution in the context of vaccine effectiveness.

References

1. Alm E, Broberg EK, Connor T, Hodcroft EB, Komissarov AB, Maurer-Stroh S, Melidou A, Neher RA, O'Toole Á, Pereyaslov D; WHO European Region sequencing laboratories and GISAID EpiCoV group; WHO European Region sequencing laboratories and GISAID EpiCoV group*. Geographical and temporal distribution of SARS-CoV-2 clades in the WHO European Region, January to June 2020. Euro Surveill. 2020 Aug;25(32):2001410. doi: 10.2807/1560-7917.ES.2020.25.32.2001410. Erratum in: Euro Surveill. 2020 Aug;25(33). doi: 10.2807/1560-7917.ES.2020.25.33.200820c. PMID: 32794443; PMCID: PMC7427299.
2. Alteri C, Cento V, Piralla A, Costabile V, Tallarita M, Colagrossi L, Renica S, Giardina F, Novazzi F, Gaiarsa S, Matarazzo E, Antonello M, Vismara C, Fumagalli R, Epis OM, Puoti M, Perno CF, Baldanti F. Genomic epidemiology of SARS-CoV-2 reveals multiple lineages and early spread of SARS-CoV-2 infections in Lombardy, Italy. Nat Commun. 2021 Jan 19;12(1):434. doi: 10.1038/s41467-020-20688-x. PMID: 33469026; PMCID: PMC7815831.
3. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. The proximal origin of SARS-CoV-2. Nat Med. 2020 Apr;26(4):450-452. doi: 10.1038/s41591-020-0820-9. PMID: 32284615; PMCID: PMC7095063.
4. Andrés C, Garcia-Cehic D, Gregori J, Piñana M, Rodriguez-Frias F, Guerrero-Murillo M, Esperalba J, Rando A, Goterris L, Codina MG, Quer S, Martín MC, Campins M, Ferrer R, Almirante B, Esteban JI, Pumarola T, Antón A, Quer J. Naturally occurring SARS-CoV-2 gene deletions close to the spike S1/S2 cleavage site in the viral quasispecies of COVID19 patients. Emerg Microbes Infect. 2020 Dec;9(1):1900-1911. doi: 10.1080/22221751.2020.1806735. PMID: 32752979; PMCID: PMC8284971.
5. Antalis E, Oikonomopoulou Z, Kottaridi C, Kossyvakis A, Spathis A, Magkana M, Katsouli A, Tsagris V, Papaevangelou V, Mentis A, Tsiodras S. Mixed viral infections of the respiratory tract; an epidemiological study during consecutive winter seasons. J Med Virol. 2018 Apr;90(4):663-670. doi: 10.1002/jmv.25006. Epub 2018 Jan 17. PMID: 29244214; PMCID: PMC7167177.
6. Aydin Köker S, Demirağ B, Tahta N, Bayram N, Oymak Y, Karapinar TH, Gözmen S, Düzgöl M, Erçan Bozyer H, Vergin C, Devrim İ. A 3-Year Retrospective Study of the Epidemiology of Acute Respiratory Viral Infections in Pediatric Patients With Cancer Undergoing Chemotherapy. J Pediatr Hematol Oncol. 2019 May;41(4):e242-e246. doi: 10.1097/MPH.0000000000001418. PMID: 30688827.
7. Azoulay E, Russell L, Van de Louw A, Metaxa V, Bauer P, Povoa P, Montero JG, Loeches IM, Mehta S, Puxty K, Schellongowski P, Rello J, Mokart D, Lemiale V, Mirouse A; Nine-i Investigators. Diagnosis of severe respiratory infections in immunocompromised patients. Intensive Care Med. 2020

Feb;46(2):298-314. doi: 10.1007/s00134-019-05906-5. Epub 2020 Feb 7. PMID: 32034433; PMCID: PMC7080052.
8. Baden LR, El Sahly HM, Essink B, Kotloff K, Frey S, Novak R, Diemert D, Spector SA, Rouphael N, Creech CB, McGettigan J, Khetan S, Segall N, Solis J, Brosz A, Fierro C, Schwartz H, Neuzil K, Corey L, Gilbert P, Janes H, Follmann D, Marovich M, Mascola J, Polakowski L, Ledgerwood J, Graham BS, Bennett H, Pajon R, Knightly C, Leav B, Deng W, Zhou H, Han S, Ivarsson M, Miller J, Zaks T; COVE Study Group. Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. New England Journal of Medicine. 2021 Feb 4;384(5):403-416. doi: 10.1056/NEJMoa2035389. Epub 2020 Dec 30. PMID: 33378609; PMCID: PMC7787219
9. Bai L, Zhao Y, Dong J, Liang S, Guo M, Liu X, Wang X, Huang Z, Sun X, Zhang Z, Dong L, Liu Q, Zheng Y, Niu D, Xiang M, Song K, Ye J, Zheng W, Tang Z, Tang M, Zhou Y, Shen C, Dai M, Zhou L, Chen Y, Yan H, Lan K, Xu K. Coinfection with influenza A virus enhances SARS-CoV-2 infectivity. Cell Res. 2021 Apr;31(4):395-403. doi: 10.1038/s41422-021-00473-1. Epub 2021 Feb 18. PMID: 33603116; PMCID: PMC7890106.
10. Baker RE, Park SW, Yang W, Vecchi GA, Metcalf CJE, Grenfell BT. The impact of COVID-19 nonpharmaceutical interventions on the future dynamics of endemic infections. Proc Natl Acad Sci U S A. 2020 Dec 1;117(48):30547-30553. doi: 10.1073/pnas.2013182117. Epub 2020 Nov 9. PMID: 33168723; PMCID: PMC7720203.
11. Barker JH, Luby JP, Sean Dalley A, Bartek WM, Burns DK, Erdman DD. Fatal type 3 adenoviral pneumonia in immunocompetent adult identical twins. Clin Infect Dis. 2003 Nov 15;37(10):e142-6. doi: 10.1086/379127. Epub 2003 Oct 14. PMID: 14583886.
12. Bianchini S, Silvestri E, Argentiero A, Fainardi V, Pisi G, Esposito S. Role of Respiratory Syncytial Virus in Pediatric Pneumonia. Microorganisms. 2020 Dec 21;8(12):2048. doi: 10.3390/microorganisms8122048. PMID: 33371276; PMCID: PMC7766387.
13. Branche AR, Falsey AR. Parainfluenza Virus Infection. Semin Respir Crit Care Med. 2016 Aug;37(4):538-54. doi: 10.1055/s-0036-1584798. Epub 2016 Aug 3. PMID: 27486735; PMCID: PMC7171724.
14. Branche AR, Saiman L, Walsh EE, Falsey AR, Jia H, Barrett A, Alba L, Phillips M, Finelli L. Change in functional status associated with respiratory syncytial virus infection in hospitalized older adults. Influenza Other Respir Viruses. 2022 Nov;16(6):1151-1160. doi: 10.1111/irv.13043. Epub 2022 Sep 7. PMID: 36069297; PMCID: PMC9530534.
15. Brankston G, Gitterman L, Hirji Z, Lemieux C, Gardam M. Transmission of influenza A in human beings. Lancet Infect Dis. 2007 Apr;7(4):257-65. doi: 10.1016/S1473-3099(07)70029-4. PMID: 17376383.
16. Bruning AHL, Thomas XV, van der Linden L, Wildenbeest JG, Minnaar RP, Jansen RR, de Jong MD, Sterk PJ, van der Schee MP, Wolthers KC, Pajkrt D. Clinical, virological and epidemiological characteristics of rhinovirus infections in early childhood: A comparison between non-hospitalised and hospitalised

children. J Clin Virol. 2015 Dec;73:120-126. doi: 10.1016/j.jcv.2015.10.024. Epub 2015 Nov 10. PMID: 26599608; PMCID: PMC7185867.
17. Bulloch MN. Treatment and prevention of influenza in geriatric patients. Expert Rev Clin Pharmacol. 2023 Jul-Dec;16(9):825-841. doi: 10.1080/17512433.2023.2243221. Epub 2023 Aug 7. PMID: 37526068.
18. Burrough ER, Magstadt DR, Petersen B, Timmermans SJ, Gauger PC, Zhang J, Siepker C, Mainenti M, Li G, Thompson AC, Gorden PJ, Plummer PJ, Main R. Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4b Virus Infection in Domestic Dairy Cattle and Cats, United States, 2024. Emerg Infect Dis. 2024 Jul;30(7):1335-1343. doi: 10.3201/eid3007.240508. Epub 2024 Apr 29. PMID: 38683888; PMCID: PMC11210653.
19. Calvo C, Garcia ML, Pozo F, Reyes N, Pérez-Breña P, Casas I. Role of rhinovirus C in apparently life-threatening events in infants, Spain. Emerg Infect Dis. 2009 Sep;15(9):1506-8. doi: 10.3201/eid1509.090453. PMID: 19788827; PMCID: PMC2819893.
20. Carpenter JA, Keegan LP, Wilfert L, O'Connell MA, Jiggins FM. Evidence for ADAR-induced hypermutation of the Drosophila sigma virus (Rhabdoviridae). BMC Genet. 2009 Nov 26;10:75. doi: 10.1186/1471-2156-10-75. PMID: 19941656; PMCID: PMC2790459.
21. Casalegno JS, Ploin D, Cantais A, Masson E, Bard E, Valette M, Fanget R, Targe SC, Myar-Dury AF, Doret-Dion M, Massoud M, Queromes G, Vanhems P, Claris O, Butin M, Pillet S, Ader F, Bin S, Gaymard A, Lina B, Morfin F; VRS study group in Lyon; Javouhey E, Gillet Y. Characteristics of the delayed respiratory syncytial virus epidemic, 2020/2021, Rhône Loire, France. Euro Surveill. 2021 Jul;26(29):2100630. doi: 10.2807/1560-7917.ES.2021.26.29.2100630. PMID: 34296674; PMCID: PMC8299747.
22. Caserta MT, O'Leary ST, Munoz FM, Ralston SL; COMMITTEE ON INFECTIOUS DISEASES. Palivizumab Prophylaxis in Infants and Young Children at Increased Risk of Hospitalization for Respiratory Syncytial Virus Infection. Pediatrics. 2023 Jul 1;152(1):e2023061803. doi: 10.1542/peds.2023-061803. PMID: 37357729.
23. Cassaniti I, Bergami F, Percivalle E, Gabanti E, Sammartino JC, Ferrari A, Adzasehoun KMG, Zavaglio F, Zelini P, Comolli G, Sarasini A, Piralla A, Ricciardi A, Zuccaro V, Maggi F, Novazzi F, Simonelli L, Varani L, Lilleri D, Baldanti F. Humoral and cell-mediated response against SARS-CoV-2 variants elicited by mRNA vaccine BNT162b2 in healthcare workers: a longitudinal observational study. Clin Microbiol Infect. 2022 Feb;28(2):301.e1-301.e8. doi: 10.1016/j.cmi.2021.09.016. Epub 2021 Sep 25. PMID: 34582981; PMCID: PMC8464022.
24. Castagnoli R, Votto M, Licari A, Brambilla I, Bruno R, Perlini S, Rovida F, Baldanti F, Marseglia GL. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Infection in Children and Adolescents: A Systematic Review. JAMA Pediatr. 2020 Sep 1;174(9):882-889. doi: 10.1001/jamapediatrics.2020.1467. PMID: 32320004.

25. Cecchetto R, Tonon E, Medaina N, Turri G, Diani E, Piccaluga PP, Salomoni A, Conti M, Tacconelli E, Lagni A, Lotti V, Favarato M, Gibellini D. Detection of SARS-CoV-2 Δ426 ORF8 Deletion Mutant Cluster in NGS Screening. Microorganisms. 2023 Sep 23;11(10):2378. doi: 10.3390/microorganisms11102378. PMID: 37894036; PMCID: PMC10609088.
26. Chan KKP, Hui DSC. Antiviral therapies for influenza. Curr Opin Infect Dis. 2023 Apr 1;36(2):124-131. doi: 10.1097/QCO.0000000000000910. Epub 2023 Jan 30. PMID: 36752709.
27. Chan YF, AbuBaker S. Recombinant human enterovirus 71 in hand, foot and mouth disease patients. Emerg Infect Dis. 2004 Aug;10(8):1468-70. doi: 10.3201/eid1008.040059. PMID: 15496251; PMCID: PMC3320397.
28. Channappanavar R, Perlman S. Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology. Semin Immunopathol. 2017 Jul;39(5):529-539. doi: 10.1007/s00281-017-0629-x. Epub 2017 May 2. PMID: 28466096; PMCID: PMC7079893.
29. Charre C, Ginevra C, Sabatier M, Regue H, Destras G, Brun S, Burfin G, Scholtes C, Morfin F, Valette M, Lina B, Bal A, Josset L. Evaluation of NGS-based approaches for SARS-CoV-2 whole genome characterisation. Virus Evol. 2020 Oct 5;6(2):veaa075. doi: 10.1093/ve/veaa075. PMID: 33318859; PMCID: PMC7665770.
30. Chen Y, Lin T, Wang CB, Liang WL, Lian GW, Zanin M, Wong SS, Tian XG, Zhong JY, Zhang YY, Xie JH, Zheng LL, Chen FY, Dang R, Zhao MQ, Yang YY, Zhou R, Zhu B. Human adenovirus (HAdV) infection in children with acute respiratory tract infections in Guangzhou, China, 2010-2021: a molecular epidemiology study. World J Pediatr. 2022 Aug;18(8):545-552. doi: 10.1007/s12519-022-00590-w. Epub 2022 Jul 21. PMID: 35861938; PMCID: PMC9302229.
31. Cho HJ, Rhee JE, Kang D, Choi EH, Lee NJ, Woo S, Lee J, Lee SW, Kim EJ, Yun KW. Epidemiology of Respiratory Viruses in Korean Children Before and After the COVID-19 Pandemic: A Prospective Study From National Surveillance System. J Korean Med Sci. 2024 May 20;39(19):e171. doi: 10.3346/jkms.2024.39.e171. PMID: 38769924; PMCID: PMC11106558.
32. Chow EJ, Uyeki TM, Chu HY. The effects of the COVID-19 pandemic on community respiratory virus activity. Nat Rev Microbiol. 2023 Mar;21(3):195-210. doi: 10.1038/s41579-022-00807-9. Epub 2022 Oct 17. PMID: 36253478; PMCID: PMC9574826.
33. Chung JR, Kim SS, Kondor RJ, Smith C, Budd AP, Tartof SY, Florea A, Talbot HK, Grijalva CG, Wernli KJ, Phillips CH, Monto AS, Martin ET, Belongia EA, McLean HQ, Gaglani M, Reis M, Geffel KM, Nowalk MP, DaSilva J, Keong LM, Stark TJ, Barnes JR, Wentworth DE, Brammer L, Burns E, Fry AM, Patel MM, Flannery B. Interim Estimates of 2021-22 Seasonal Influenza Vaccine Effectiveness - United States, February 2022. MMWR Morb Mortal Wkly Rep.

2022 Mar 11;71(10):365-370. doi: 10.15585/mmwr.mm7110a1. PMID: 35271561; PMCID: PMC8911998.
34. Cieślak K, Kowalczyk D, Szymański K, Hallmann-Szelińska E, Brydak LB. Influenza and Influenza-Like Viruses: Frequent Infections in Children Under 14 Years of Age During the 2016/2017 Epidemic Season. Adv Exp Med Biol. 2018;1114:83-87. doi: 10.1007/5584_2018_229. PMID: 30117125; PMCID: PMC7124040.
35. Cillóniz C, Pericàs JM, Rojas JR, Torres A. Severe Infections Due to Respiratory Viruses. Semin Respir Crit Care Med. 2022 Feb;43(1):60-74. doi: 10.1055/s-0041-1740982. Epub 2022 Feb 16. PMID: 35172359.
36. Cimolai N. In pursuit of the right tail for the COVID-19 incubation period. Public Health. 2021 May;194:149-155. doi: 10.1016/j.puhe.2021.03.011. Epub 2021 Mar 26. PMID: 33915459; PMCID: PMC7997403.
37. Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet. 1998 Feb 14;351(9101):472-7. doi: 10.1016/S0140-6736(97)11212-0. Erratum in: Lancet 1998 Apr 25;351(9111):1292. PMID: 9482438.
38. Collins JP, Campbell AP, Openo K, Farley MM, Cummings CN, Hill M, Schaffner W, Lindegren ML, Thomas A, Billing L, Bennett N, Spina N, Bargsten M, Lynfield R, Eckel S, Ryan P, Yousey-Hindes K, Herlihy R, Kirley PD, Garg S, Anderson EJ. Outcomes of Immunocompromised Adults Hospitalized With Laboratory-confirmed Influenza in the United States, 2011-2015. Clin Infect Dis. 2020 May 6;70(10):2121-2130. doi: 10.1093/cid/ciz638. PMID: 31298691; PMCID: PMC7201407.
39. Cox DW, Bizzintino J, Ferrari G, Khoo SK, Zhang G, Whelan S, Lee WM, Bochkov YA, Geelhoed GC, Goldblatt J, Gern JE, Laing IA, Le Souëf PN. Human rhinovirus species C infection in young children with acute wheeze is associated with increased acute respiratory hospital admissions. Am J Respir Crit Care Med. 2013 Dec 1;188(11):1358-64. doi: 10.1164/rccm.201303-0498OC. PMID: 23992536; PMCID: PMC5447292.
40. De Conto F, Conversano F, Medici MC, Ferraglia F, Pinardi F, Arcangeletti MC, Chezzi C, Calderaro A. Epidemiology of human respiratory viruses in children with acute respiratory tract infection in a 3-year hospital-based survey in Northern Italy. Diagn Microbiol Infect Dis. 2019 Jul;94(3):260-267. doi: 10.1016/j.diagmicrobio.2019.01.008. Epub 2019 Jan 17. PMID: 30745224; PMCID: PMC7126416.
41. De Francesco MA, Pollara C, Gargiulo F, Giacomelli M, Caruso A. Circulation of Respiratory Viruses in Hospitalized Adults before and during the COVID-19 Pandemic in Brescia, Italy: A Retrospective Study. Int J Environ Res Public Health. 2021 Sep 9;18(18):9525. doi: 10.3390/ijerph18189525. PMID: 34574450; PMCID: PMC8469422.
42. de Crom SC, Obihara CC, de Moor RA, Veldkamp EJ, van Furth AM, Rossen JW. Prospective comparison of the detection rates of human enterovirus and

parechovirus RT-qPCR and viral culture in different pediatric specimens. J Clin Virol. 2013 Oct;58(2):449-54. doi: 10.1016/j.jcv.2013.07.017. Epub 2013 Aug 21. PMID: 23973350.
43. Di Giallonardo F, Duchene S, Puglia I, Curini V, Profeta F, Cammà C, Marcacci M, Calistri P, Holmes EC, Lorusso A. Genomic Epidemiology of the First Wave of SARS-CoV-2 in Italy. Viruses. 2020 Dec 14;12(12):1438. doi: 10.3390/v12121438. PMID: 33327566; PMCID: PMC7765063.
44. Di Giorgio S, Martignano F, Torcia MG, Mattiuz G, Conticello SG. Evidence for host-dependent RNA editing in the transcriptome of SARS-CoV-2. Sci Adv. 2020 Jun 17;6(25):eabb5813. doi: 10.1126/sciadv.abb5813. PMID: 32596474; PMCID: PMC7299625.
45. Dominguez F, Blodget E. Community-acquired respiratory viruses. Curr Opin Organ Transplant. 2019 Aug;24(4):511-514. doi: 10.1097/MOT.0000000000000667. PMID: 31192816.
46. Dreitlein WB, Maratos J, Brocavich J. Zanamivir and oseltamivir: two new options for the treatment and prevention of influenza. Clin Ther. 2001 Mar;23(3):327-55. doi: 10.1016/s0149-2918(01)80042-4. PMID: 11318072.
47. Drysdale SB, Mejias A, Ramilo O. Rhinovirus - not just the common cold. J Infect. 2017 Jun;74 Suppl 1:S41-S46. doi: 10.1016/S0163-4453(17)30190-1. PMID: 28646961.
48. Drysdale SB, Cathie K, Flamein F, Knuf M, Collins AM, Hill HC, Kaiser F, Cohen R, Pinquier D, Felter CT, Vassilouthis NC, Jin J, Bangert M, Mari K, Nteene R, Wague S, Roberts M, Tissières P, Royal S, Faust SN; HARMONIE Study Group. Nirsevimab for Prevention of Hospitalizations Due to RSV in Infants. N Engl J Med. 2023 Dec 28;389(26):2425-2435. doi: 10.1056/NEJMoa2309189. PMID: 38157500.
49. Emborg HD, Vestergaard LS, Botnen AB, Nielsen J, Krause TG, Trebbien R. A late sharp increase in influenza detections and low interim vaccine effectiveness against the circulating A(H3N2) strain, Denmark, 2021/22 influenza season up to 25 March 2022. Euro Surveill. 2022 Apr;27(15):2200278. doi: 10.2807/1560-7917.ES.2022.27.15.2200278. PMID: 35426361; PMCID: PMC9012088.
50. European Centre for Disease Prevention and Control (ECDC). Influenza Virus Characterisation Reports, summary Europe. Available at: https://www.ecdc.europa.eu/en/seasonal-influenza/surveillance-and-disease-data/influenza-virus-characterisation..
51. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. N Engl J Med. 2005 Apr 28;352(17):1749-59. doi: 10.1056/NEJMoa043951. PMID: 15858184.
52. Fillatre A, François C, Segard C, Duverlie G, Hecquet D, Pannier C, Roussel C, Zawadzki P, Brochot E, Castelain S. Epidemiology and seasonality of acute respiratory infections in hospitalized children over four consecutive years (2012-2016). J Clin Virol. 2018 May;102:27-31. doi:

10.1016/j.jcv.2018.02.010. Epub 2018 Feb 20. PMID: 29477833; PMCID: PMC7106524.
53. Fragkou PC, Moschopoulos CD, Karofylakis E, Kelesidis T, Tsiodras S. Update in Viral Infections in the Intensive Care Unit. Front Med (Lausanne). 2021 Feb 23;8:575580. doi: 10.3389/fmed.2021.575580. PMID: 33708775; PMCID: PMC7940368.
54. Food and Drug Administration, 2018. FDA approves new drug to treat influenza (https://www.fda.gov/news-events/press-announcements/fda-approves-new-drug-treat-influenza).
55. Gerna G, Percivalle E, Piralla A, Rognoni V, Marchi A, Baldanti F. Surveillance of influenza virus B circulation in Northern Italy: summer-fall 2008 isolation of three strains and phylogenetic analysis. New Microbiol. 2009 Oct;32(4):405-10. PMID: 20128448.
56. Gooskens J, Jonges M, Claas EC, Meijer A, Kroes AC. Prolonged influenza virus infection during lymphocytopenia and frequent detection of drug-resistant viruses. J Infect Dis. 2009 May 15;199(10):1435-41. doi: 10.1086/598684. PMID: 19392620.
57. Grapin M, Mirand A, Pinquier D, Basset A, Bendavid M, Bisseux M, Jeannoël M, Kireche B, Kossorotoff M, L'Honneur AS, Robin L, Ville Y, Renolleau S, Lemee V, Jarreau PH, Desguerre I, Lacaille F, Leruez-Ville M, Guillaume C, Henquell C, Lapillonne A, Schuffenecker I, Aubart M. Severe and fatal neonatal infections linked to a new variant of echovirus 11, France, July 2022 to April 2023. Euro Surveill. 2023 Jun;28(22):2300253. doi: 10.2807/1560-7917.ES.2023.28.22.2300253. PMID: 37261730; PMCID: PMC10236930.
58. Hammitt LL, Dagan R, Yuan Y, Baca Cots M, Bosheva M, Madhi SA, Muller WJ, Zar HJ, Brooks D, Grenham A, Wählby Hamrén U, Mankad VS, Ren P, Takas T, Abram ME, Leach A, Griffin MP, Villafana T; MELODY Study Group. Nirsevimab for Prevention of RSV in Healthy Late-Preterm and Term Infants. N Engl J Med. 2022 Mar 3;386(9):837-846. doi: 10.1056/NEJMoa2110275. PMID: 35235726.
59. Hayden FG, Sugaya N, Hirotsu N, Lee N, de Jong MD, Hurt AC, Ishida T, Sekino H, Yamada K, Portsmouth S, Kawaguchi K, Shishido T, Arai M, Tsuchiya K, Uehara T, Watanabe A; Baloxavir Marboxil Investigators Group. Baloxavir Marboxil for Uncomplicated Influenza in Adults and Adolescents. N Engl J Med. 2018 Sep 6;379(10):913-923. doi: 10.1056/NEJMoa1716197. PMID: 30184455.
60. Hei H, Bezpalko O, Smathers SA, Coffin SE, Sammons JS. Development of a novel prevention bundle for pediatric healthcare-associated viral infections. Infect Control Hosp Epidemiol. 2018 Sep;39(9):1086-1092. doi: 10.1017/ice.2018.149. Epub 2018 Jul 20. PMID: 30027857.
61. Hon KL, Leung AKC, Wong AHC, Dudi A, Leung KKY. Respiratory Syncytial Virus is the Most Common Causative Agent of Viral Bronchiolitis in Young Children: An Updated Review. Curr Pediatr Rev. 2023;19(2):139-149. doi: 10.2174/1573396318666220810161945. PMID: 35950255.

62. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020 Feb 15;395(10223):497-506. doi: 10.1016/S0140-6736(20)30183-5. Epub 2020 Jan 24. Erratum in: Lancet. 2020 Feb 15;395(10223):496. doi: 10.1016/S0140-6736(20)30252-X. PMID: 31986264; PMCID: PMC7159299.
63. Hung HM, Yang SL, Chen CJ, Chiu CH, Kuo CY, Huang KA, Lin TY, Hsieh YC, Gong YN, Tsao KC, Huang YC. Molecular epidemiology and clinical features of rhinovirus infections among hospitalized patients in a medical center in Taiwan. J Microbiol Immunol Infect. 2019 Apr;52(2):233-241. doi: 10.1016/j.jmii.2018.08.009. Epub 2018 Aug 30. PMID: 30201131.
64. Ikematsu H, Hayden FG, Kawaguchi K, Kinoshita M, de Jong MD, Lee N, Takashima S, Noshi T, Tsuchiya K, Uehara T. Baloxavir Marboxil for Prophylaxis against Influenza in Household Contacts. N Engl J Med. 2020 Jul 23;383(4):309-320. doi: 10.1056/NEJMoa1915341. Epub 2020 Jul 8. PMID: 32640124.
65. Ippolito G, La Vecchia A, Umbrello G, Di Pietro G, Bono P, Scalia Catenacci S, Pinzani R, Tagliabue C, Bosis S, Agostoni C, Marchisio PG. Disappearance of Seasonal Respiratory Viruses in Children Under Two Years Old During COVID-19 Pandemic: A Monocentric Retrospective Study in Milan, Italy. Front Pediatr. 2021 Aug 5;9:721005. doi: 10.3389/fped.2021.721005. Erratum in: Front Pediatr. 2022 Aug 23;10:1006948. doi: 10.3389/fped.2022.1006948. PMID: 34422733; PMCID: PMC8374241.
66. Istituto Superiore di Sanità. ISS: Sorveglianza virologica dell'influenza (rete InfluNet). Available at: https://www.iss.it/sorveglianza-virologica-dell-influenza-influnet.
67. Istituto Superiore di Sanità (ISS) Epicentro. Influnet. 2022 https://www.epicentro.iss.it/influenza/influnet.
68. Istituto Superiore di Sanità. Aggiornamento Nazionale relativo al periodo 29/04/2024 – 05/05/2024 dei dati della Sorveglianza integrata COVID-19. 8 Maggio 2024.
69. Jain S, Self WH, Wunderink RG, Fakhran S, Balk R, Bramley AM, Reed C, Grijalva CG, Anderson EJ, Courtney DM, Chappell JD, Qi C, Hart EM, Carroll F, Trabue C, Donnelly HK, Williams DJ, Zhu Y, Arnold SR, Ampofo K, Waterer GW, Levine M, Lindstrom S, Winchell JM, Katz JM, Erdman D, Schneider E, Hicks LA, McCullers JA, Pavia AT, Edwards KM, Finelli L; CDC EPIC Study Team. Community-Acquired Pneumonia Requiring Hospitalization among U.S. Adults. N Engl J Med. 2015 Jul 30;373(5):415-27. doi: 10.1056/NEJMoa1500245. Epub 2015 Jul 14. PMID: 26172429; PMCID: PMC4728150.
70. Jain S., Williams D.J., Arnold S.R., Ampofo K., Bramley A.M., Reed C., Stockmann C., Anderson E.J., Grijalva C.G., Self W.H., et al. Community-

acquired pneumonia requiring hospitalization among, U.S. children. N. Engl. J. Med. 2015;372:835–845. doi: 10.1056/NEJMoa1405870.
71. Jackson RJ, Cooper KL, Tappenden P, Rees A, Simpson EL, Read RC, Nicholson KG. Oseltamivir, zanamivir and amantadine in the prevention of influenza: a systematic review. J Infect. 2011 Jan;62(1):14-25. doi: 10.1016/j.jinf.2010.10.003. Epub 2010 Oct 13. PMID: 20950645.
72. Javanian M, Barary M, Ghebrehewet S, Koppolu V, Vasigala V, Ebrahimpour S. A brief review of influenza virus infection. J Med Virol. 2021 Aug;93(8):4638-4646. doi: 10.1002/jmv.26990. Epub 2021 Apr 14. PMID: 33792930.
73. John G, Sahajpal NS, Mondal AK, Ananth S, Williams C, Chaubey A, Rojiani AM, Kolhe R. Next-Generation Sequencing (NGS) in COVID-19: A Tool for SARS-CoV-2 Diagnosis, Monitoring New Strains and Phylodynamic Modeling in Molecular Epidemiology. Curr Issues Mol Biol. 2021 Jul 30;43(2):845-867. doi: 10.3390/cimb43020061. PMID: 34449545; PMCID: PMC8929009.
74. Kampmann B, Madhi SA, Munjal I, Simões EAF, Pahud BA, Llapur C, Baker J, Pérez Marc G, Radley D, Shittu E, Glanternik J, Snaggs H, Baber J, Zachariah P, Barnabas SL, Fausett M, Adam T, Perreras N, Van Houten MA, Kantele A, Huang LM, Bont LJ, Otsuki T, Vargas SL, Gullam J, Tapiero B, Stein RT, Polack FP, Zar HJ, Staerke NB, Duron Padilla M, Richmond PC, Koury K, Schneider K, Kalinina EV, Cooper D, Jansen KU, Anderson AS, Swanson KA, Gruber WC, Gurtman A; MATISSE Study Group. Bivalent Prefusion F Vaccine in Pregnancy to Prevent RSV Illness in Infants. N Engl J Med. 2023 Apr 20;388(16):1451-1464. doi: 10.1056/NEJMoa2216480. Epub 2023 Apr 5. PMID: 37018474.
75. Kalil AC, Thomas PG. Influenza virus-related critical illness: pathophysiology and epidemiology. Crit Care. 2019 Jul 19;23(1):258. doi: 10.1186/s13054-019-2539-x. PMID: 31324202; PMCID: PMC6642581.
76. Keam SJ. Nirsevimab: First Approval. Drugs. 2023 Feb;83(2):181-187. doi: 10.1007/s40265-022-01829-6. PMID: 36577878.
77. Kenmoe S, Sadeuh-Mba SA, Vernet MA, Penlap Beng V, Vabret A, Njouom R. Molecular epidemiology of Enteroviruses and Rhinoviruses in patients with acute respiratory infections in Yaounde, Cameroon. Influenza Other Respir Viruses. 2021 Sep;15(5):641-650. doi: 10.1111/irv.12851. Epub 2021 Mar 10. PMID: 33694322; PMCID: PMC8404047.
78. Kim SR, Ki CS, Lee NY. Rapid detection and identification of 12 respiratory viruses using a dual priming oligonucleotide system-based multiplex PCR assay. J Virol Methods. 2009 Mar;156(1-2):111-6. doi: 10.1016/j.jviromet.2008.11.007. Epub 2008 Dec 24. PMID: 19063921; PMCID: PMC7112863.
79. Kim S, Chuang ES, Sabaiduc S, Olsha R, Kaweski SE, Zelyas N, Gubbay JB, Jassem AN, Charest H, De Serres G, Dickinson JA, Skowronski DM. Influenza vaccine effectiveness against A(H3N2) during the delayed 2021/22 epidemic in

Canada. Euro Surveill. 2022 Sep;27(38):2200720. doi: 10.2807/1560-7917.ES.2022.27.38.2200720. PMID: 36148674; PMCID: PMC9511683.
80. Kıymet E, Böncüoğlu E, Şahinkaya Ş, Cem E, Çelebi MY, Düzgöl M, Kara AA, Arıkan KÖ, Aydın T, İşgüder R, Yılmazer MM, Ayhan Y, Gülfidan G, Bayram A, Bayram N, Çelik T, Alp YT, Devrim İ. Distribution of spreading viruses during COVID-19 pandemic: Effect of mitigation strategies. Am J Infect Control. 2021 Sep;49(9):1142-1145. doi: 10.1016/j.ajic.2021.06.002. Epub 2021 Jun 9. PMID: 34116082; PMCID: PMC8187742.
81. Ko JH, Lim JU, Choi JY, Oh HS, Yoo H, Jhun BW, Huh K, Peck KR. Early cidofovir administration might be associated with a lower probability of respiratory failure in treating human adenovirus pneumonia: a retrospective cohort study. Clin Microbiol Infect. 2020 May;26(5):646.e9-646.e14. doi: 10.1016/j.cmi.2019.10.012. Epub 2019 Oct 21. PMID: 31648000; PMCID: PMC7129699.
82. Korber B, Fischer WM, Gnanakaran S, Yoon H, Theiler J, Abfalterer W, Hengartner N, Giorgi EE, Bhattacharya T, Foley B, Hastie KM, Parker MD, Partridge DG, Evans CM, Freeman TM, de Silva TI; Sheffield COVID-19 Genomics Group; McDanal C, Perez LG, Tang H, Moon-Walker A, Whelan SP, LaBranche CC, Saphire EO, Montefiori DC. Tracking Changes in SARS-CoV-2 Spike: Evidence that D614G Increases Infectivity of the COVID-19 Virus. Cell. 2020 Aug 20;182(4):812-827.e19. doi: 10.1016/j.cell.2020.06.043. Epub 2020 Jul 3. PMID: 32697968; PMCID: PMC7332439.
83. Krumbein H, Kümmel LS, Fragkou PC, Thölken C, Hünerbein BL, Reiter R, Papathanasiou KA, Renz H, Skevaki C. Respiratory viral co-infections in patients with COVID-19 and associated outcomes: A systematic review and meta-analysis. Rev Med Virol. 2023 Jan;33(1):e2365. doi: 10.1002/rmv.2365. Epub 2022 Jun 10. PMID: 35686619; PMCID: PMC9347814.
84. Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N. The real-time polymerase chain reaction. Mol Aspects Med. 2006 Apr-Jun;27(2-3):95-125. doi: 10.1016/j.mam.2005.12.007. Epub 2006 Feb 3. PMID: 16460794.
85. Landry ML. Rapid viral diagnosis. In: Rose NR, Conway de Macario E, Folds JD, Lane HC, Nakamura RM, editors. Manual of clinical laboratory immunology. 5th ed. Washington DC: ASM Press; 1997. p. 608–17.
86. Le TK, Brown BK, Namtu KC, Berman DM, Kiskaddon AL. Use of cidofovir with extracorporeal membrane oxygenation to treat adenovirus-associated acute respiratory distress syndrome in paediatric patients- a case series. J Clin Pharm Ther. 2020 Dec;45(6):1505-1510. doi: 10.1111/jcpt.13244. Epub 2020 Aug 15. PMID: 33164241.
87. Leli C, Di Matteo L, Gotta F, Vay D, Piceghello A, Cornaglia E, Cavallo V, Busso S, Carrabba L, Mazzeo R, Rocchetti A. Prevalence of respiratory viruses by Multiplex PCR: a four-and-a-half year retrospective study in an Italian general hospital. Infez Med. 2021 Mar 1;29(1):94-101. PMID: 33664178.

88. Lerman AS, Navarro Albarracín LF, Figari AB, Macías Lainez V, Uez OC. Rhinovirus and metapneumovirus in patients with severe acute respiratory infection. Arch Argent Pediatr. 2023 Jun 1;121(3):e202202605. English, Spanish. doi: 10.5546/aap.2022-02605.eng. Epub 2022 Dec 1. PMID: 36445064.
89. Li J, Zhou CE, Wei SC, Wang LN, Shi MW, Sun CP, Lin LJ, Liu XM. Diagnostic Value of Metagenomic Next-Generation Sequencing for Pneumonia in Immunocompromised Patients. Can J Infect Dis Med Microbiol. 2022 Dec 1;2022:5884568. doi: 10.1155/2022/5884568. PMID: 36507192; PMCID: PMC9731749.
90. Li X, Lu H, Sun Q, Zheng P, Zhang B, Cui H, Tang H, Zhao H, Liu Y, Jiang J, Xiao J, Zhang Y. Transmission of recombinant enterovirus A76 (EV-A76) in Xinjiang Uighur autonomous region of China. Emerg Microbes Infect. 2023 Dec;12(1):2149350. doi: 10.1080/22221751.2022.2149350. PMID: 36394478; PMCID: PMC9788713.
91. Li Y, Wang X, Blau DM, Caballero MT, Feikin DR, Gill CJ, Madhi SA, Omer SB, Simões EAF, Campbell H, Pariente AB, Bardach D, Bassat Q, Casalegno JS, Chakhunashvili G, Crawford N, Danilenko D, Do LAH, Echavarria M, Gentile A, Gordon A, Heikkinen T, Huang QS, Jullien S, Krishnan A, Lopez EL, Markić J, Mira-Iglesias A, Moore HC, Moyes J, Mwananyanda L, Nokes DJ, Noordeen F, Obodai E, Palani N, Romero C, Salimi V, Satav A, Seo E, Shchomak Z, Singleton R, Stolyarov K, Stoszek SK, von Gottberg A, Wurzel D, Yoshida LM, Yung CF, Zar HJ; Respiratory Virus Global Epidemiology Network; Nair H; RESCEU investigators. Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in children younger than 5 years in 2019: a systematic analysis. Lancet. 2022 May 28;399(10340):2047-2064. doi: 10.1016/S0140-6736(22)00478-0. Epub 2022 May 19. PMID: 35598608; PMCID: PMC7613574.
92. Liang G, Bushman FD. The human virome: assembly, composition and host interactions. Nat Rev Microbiol. 2021 Aug;19(8):514-527. doi: 10.1038/s41579-021-00536-5. Epub 2021 Mar 30. PMID: 33785903; PMCID: PMC8008777.
93. Lindstrøm JC, Engebretsen S, Kristoffersen AB, Rø GØI, Palomares AD, Engø-Monsen K, Madslien EH, Forland F, Nygård KM, Hagen F, Gantzel G, Wiklund O, Frigessi A, de Blasio BF. Increased transmissibility of the alpha SARS-CoV-2 variant: evidence from contact tracing data in Oslo, January to February 2021. Infect Dis (Lond). 2022 Jan;54(1):72-77. doi: 10.1080/23744235.2021.1977382. Epub 2021 Oct 7. PMID: 34618665.
94. Loconsole D, Centrone F, Aprile V, Sallustio A, Casulli D, Accogli M, Sacco D, Zagaria R, Chironna M. What's Next for Flu? Out-of-Season Circulation of Influenza Viruses in Southern Italy, August 2022. Viruses. 2022 Nov

30;14(12):2689. doi: 10.3390/v14122689. PMID: 36560693; PMCID: PMC9783519.
95. Ly H. Highly pathogenic avian influenza H5N1 virus infection of companion animals. Virulence. 2024 Dec;15(1):2289780. doi: 10.1080/21505594.2023.2289780. Epub 2023 Dec 8. PMID: 38064414; PMCID: PMC10761027.
96. Lynch JP 3rd, Kajon AE. Adenovirus: Epidemiology, Global Spread of Novel Serotypes, and Advances in Treatment and Prevention. Semin Respir Crit Care Med. 2016 Aug;37(4):586-602. doi: 10.1055/s-0036-1584923. Epub 2016 Aug 3. PMID: 27486739; PMCID: PMC7171713.
97. Matias G, Taylor R, Haguinet F, Schuck-Paim C, Lustig R, Shinde V. Estimates of mortality attributable to influenza and RSV in the United States during 1997-2009 by influenza type or subtype, age, cause of death, and risk status. Influenza Other Respir Viruses. 2014 Sep;8(5):507-15. doi: 10.1111/irv.12258. Epub 2014 Jun 27. PMID: 24975705; PMCID: PMC4181813.
98. McClure DL, Kieke BA, Sundaram ME, Simpson MD, Meece JK, Sifakis F, Gasser RA Jr, Belongia EA. Seasonal incidence of medically attended respiratory syncytial virus infection in a community cohort of adults ≥50 years old. PLoS One. 2014 Jul 15;9(7):e102586. doi: 10.1371/journal.pone.0102586. PMID: 25025344; PMCID: PMC4099308.
99. Meijer A, Lackenby A, Hungnes O, Lina B, van-der-Werf S, Schweiger B, Opp M, Paget J, van-de-Kassteele J, Hay A, Zambon M; European Influenza Surveillance Scheme. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-08 season. Emerg Infect Dis. 2009 Apr;15(4):552-60. doi: 10.3201/eid1504.181280. PMID: 19331731; PMCID: PMC2671453.
100. Ministero della Salute, 2024. https://www.salute.gov.it/portale/nuovocoronavirus/dettaglioContenutiNuovoCoronavirus.jsp?lingua=italiano&id=5351&area=nuovoCoronavirus&menu=vuoto.
101. Moriyama M, Hugentobler WJ, Iwasaki A. Seasonality of Respiratory Viral Infections. Annu Rev Virol. 2020 Sep 29;7(1):83-101. doi: 10.1146/annurev-virology-012420-022445. Epub 2020 Mar 20. PMID: 32196426.
102. Neumann G, Kawaoka Y. Seasonality of influenza and other respiratory viruses. EMBO Mol Med. 2022 Apr 7;14(4):e15352. doi: 10.15252/emmm.202115352. Epub 2022 Feb 14. PMID: 35157360; PMCID: PMC8988196.
103. Nguyen DD, Phung LT, Thanh Tran HT, Ly HTT, Vo AHM, Dinh NP, Doan PM, Nguyen AT, Dang LD, Doan TT, Pham KT, Pham HL, Hoang DX, Pham TN, Tran BT, Tran TTT, Le HTM, Pham AN, Antoniou A, Ho NT. Molecular subtypes of Adenovirus-associated acute respiratory infection outbreak in children in Northern Vietnam and risk factors of more severe cases. PLoS Negl Trop Dis. 2023 Nov 7;17(11):e0011311. doi: 10.1371/journal.pntd.0011311. PMID: 37934746; PMCID: PMC10655982.

104. Nguyen-Van-Tam JS, O'Leary M, Martin ET, Heijnen E, Callendret B, Fleischhackl R, Comeaux C, Tran TMP, Weber K. Burden of respiratory syncytial virus infection in older and high-risk adults: a systematic review and meta-analysis of the evidence from developed countries. Eur Respir Rev. 2022 Nov 15;31(166):220105. doi: 10.1183/16000617.0105-2022. PMID: 36384703; PMCID: PMC9724807.
105. Ochani R, Asad A, Yasmin F, Shaikh S, Khalid H, Batra S, Sohail MR, Mahmood SF, Ochani R, Hussham Arshad M, Kumar A, Surani S. COVID-19 pandemic: from origins to outcomes. A comprehensive review of viral pathogenesis, clinical manifestations, diagnostic evaluation, and management. Infez Med. 2021 Mar 1;29(1):20-36. PMID: 33664170.
106. Olsen SJ, Winn AK, Budd AP, Prill MM, Steel J, Midgley CM, Kniss K, Burns E, Rowe T, Foust A, Jasso G, Merced-Morales A, Davis CT, Jang Y, Jones J, Daly P, Gubareva L, Barnes J, Kondor R, Sessions W, Smith C, Wentworth DE, Garg S, Havers FP, Fry AM, Hall AJ, Brammer L, Silk BJ. Changes in Influenza and Other Respiratory Virus Activity During the COVID-19 Pandemic - United States, 2020-2021. MMWR Morb Mortal Wkly Rep. 2021 Jul 23;70(29):1013-1019. doi: 10.15585/mmwr.mm7029a1. PMID: 34292924; PMCID: PMC8297694.
107. Palmas G, Trapani S, Agosti M, Alberti I, Aricò M, Azzari C, Bresesti I, Bressan S, Caselli D, Cazzato S, Da Dalt L, Felici E, Garazzino S, Giannattasio A, Iudica G, Lanari M, Marchisio P, Martelli G, Milani GP, Soro F, Piccotti E, Tirelli F, Resti M, Indolfi G; Associazione Ospedali Pediatrici Italiani (AOPI) Network. Disrupted Seasonality of Respiratory Viruses: Retrospective Analysis of Pediatric Hospitalizations in Italy from 2019 to 2023. J Pediatr. 2024 May;268:113932. doi: 10.1016/j.jpeds.2024.113932. Epub 2024 Feb 1. PMID: 38309520.
108. Park JE, Ryu Y. Transmissibility and severity of influenza virus by subtype. Infect Genet Evol. 2018 Nov;65:288-292. doi: 10.1016/j.meegid.2018.08.007. Epub 2018 Aug 10. PMID: 30103034.
109. Park KY, Seo S, Han J, Park JY. Respiratory virus surveillance in Canada during the COVID-19 pandemic: An epidemiological analysis of the effectiveness of pandemic-related public health measures in reducing seasonal respiratory viruses test positivity. PLoS One. 2021 Jun 18;16(6):e0253451. doi: 10.1371/journal.pone.0253451. PMID: 34143839; PMCID: PMC8213179.
110. Parker J, Fowler N, Walmsley ML, Schmidt T, Scharrer J, Kowaleski J, Grimes T, Hoyos S, Chen J. Analytical Sensitivity Comparison between Singleplex Real-Time PCR and a Multiplex PCR Platform for Detecting Respiratory Viruses. PLoS One. 2015 Nov 16;10(11):e0143164. doi: 10.1371/journal.pone.0143164.
111. Peiris JS, Yuen KY, Osterhaus AD, Stöhr K. The severe acute respiratory syndrome. N Engl J Med. 2003 Dec 18;349(25):2431-41. doi: 10.1056/NEJMra032498. PMID: 14681510.

112. Pellegrinelli L, Galli C, Giardina F, Ferrari G, Uceda Renteria SC, Ceriotti F, Seiti A, Binda S, Pitrolo AMG, Schiavo R, Malandrin SMI, Cavallero A, Arosio M, Farina C, Oggioni M, Congedo P, Cereda D, Rovida F, Piralla A, Pariani E, Baldanti F; Non-polio community-based surveillance group; Non-polio hospital-based surveillance group. Increased circulation of echovirus 11 in the general population and hospital patients as elicited by the non-polio enterovirus laboratory-based sentinel surveillance in northern Italy, 2023. Int J Infect Dis. 2024 May;142:106998. doi: 10.1016/j.ijid.2024.106998. Epub 2024 Mar 7. PMID: 38458420.
113. Perez A, Lively JY, Curns A, Weinberg GA, Halasa NB, Staat MA, Szilagyi PG, Stewart LS, McNeal MM, Clopper B, Zhou Y, Whitaker BL, LeMasters E, Harker E, Englund JA, Klein EJ, Selvarangan R, Harrison CJ, Boom JA, Sahni LC, Michaels MG, Williams JV, Langley GE, Gerber SI, Campbell A, Hall AJ, Rha B, McMorrow M; New Vaccine Surveillance Network Collaborators. Respiratory Virus Surveillance Among Children with Acute Respiratory Illnesses - New Vaccine Surveillance Network, United States, 2016-2021. MMWR Morb Mortal Wkly Rep. 2022 Oct 7;71(40):1253-1259. doi: 10.15585/mmwr.mm7140a1. PMID: 36201373; PMCID: PMC9541034.
114. Perotti C, Del Fante C, Baldanti F, Franchini M, Percivalle E, Vecchio Nepita E, Seminari E, De Silvestri A, Bruno R, Klersy C. Plasma from donors recovered from the new Coronavirus 2019 as therapy for critical patients with COVID-19 (COVID-19 plasma study): a multicentre study protocol. Intern Emerg Med. 2020 Aug;15(5):819-824. doi: 10.1007/s11739-020-02384-2. Epub 2020 May 28. PMID: 32468508; PMCID: PMC8849045.
115. Piralla A, Zecca M, Comoli P, Girello A, Maccario R, Baldanti F. Persistent rhinovirus infection in pediatric hematopoietic stem cell transplant recipients with impaired cellular immunity. J Clin Virol. 2015 Jun;67:38-42. doi: 10.1016/j.jcv.2015.03.022. Epub 2015 Mar 31. PMID: 25959156; PMCID: PMC7172262.
116. Piralla A, Borghesi A, Di Comite A, Giardina F, Ferrari G, Zanette S, Figar TA, Angelini M, Pisoni C, Pitrolo AMG, Paolucci S, Rovida F, Pellicioli I, Bonanomi E, Baldanti F, Ghirardello S. Fulminant echovirus 11 hepatitis in male non-identical twins in northern Italy, April 2023. Euro Surveill. 2023 Jun;28(24):2300289. doi: 10.2807/1560-7917.ES.2023.28.24.2300289. PMID: 37318763; PMCID: PMC10318938.
117. Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, Perez JL, Pérez Marc G, Moreira ED, Zerbini C, Bailey R, Swanson KA, Roychoudhury S, Koury K, Li P, Kalina WV, Cooper D, Frenck RW Jr, Hammitt LL, Türeci Ö, Nell H, Schaefer A, Ünal S, Tresnan DB, Mather S, Dormitzer PR, Şahin U, Jansen KU, Gruber WC; C4591001 Clinical Trial Group. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. New England Journal of Medicine. 2020 Dec 31;383(27):2603-2615. doi:

10.1056/NEJMoa2034577. Epub 2020 Dec 10. PMID: 33301246; PMCID: PMC7745181.

118. Prasad N, Walker TA, Waite B, Wood T, Trenholme AA, Baker MG, McArthur C, Wong CA, Grant CC, Huang QS, Newbern EC. Respiratory Syncytial Virus-Associated Hospitalizations Among Adults With Chronic Medical Conditions. Clin Infect Dis. 2021 Jul 1;73(1):e158-e163. doi: 10.1093/cid/ciaa730. PMID: 32531019.

119. Pscheidt VM, Gregianini TS, Martins LG, Veiga ABGD. Epidemiology of human adenovirus associated with respiratory infection in southern Brazil. Rev Med Virol. 2021 Jul;31(4):e2189. doi: 10.1002/rmv.2189. Epub 2020 Nov 6. PMID: 33156553.

120. Radin JM, Hawksworth AW, Kammerer PE, Balansay M, Raman R, Lindsay SP, Brice GT. Epidemiology of pathogen-specific respiratory infections among three US populations. PLoS One. 2014 Dec 30;9(12):e114871. doi: 10.1371/journal.pone.0114871. PMID: 25549089; PMCID: PMC4280218.

121. Reese SM, Thompson M, Price CS, Young HL. Evidence of nosocomial transmission of human rhinovirus in a neonatal intensive care unit. Am J Infect Control. 2016 Mar 1;44(3):355-7. doi: 10.1016/j.ajic.2015.10.018. Epub 2016 Jan 5. PMID: 26775935; PMCID: PMC7115317.

122. Richter J, Panayiotou C, Tryfonos C, Koptides D, Koliou M, Kalogirou N, Georgiou E, Christodoulou C. Aetiology of Acute Respiratory Tract Infections in Hospitalised Children in Cyprus. PLoS One. 2016 Jan 13;11(1):e0147041. doi: 10.1371/journal.pone.0147041. PMID: 26761647; PMCID: PMC4720120.Visseaux B, Burdet C, Voiriot G, Lescure FX, Chougar T, Brugière O, Crestani B, Casalino E, Charpentier C, Descamps D, Timsit JF, Yazdanpanah Y, Houhou-Fidouh N. Prevalence of respiratory viruses among adults, by season, age, respiratory tract region and type of medical unit in Paris, France, from 2011 to 2016. PLoS One. 2017 Jul 14;12(7):e0180888. doi: 10.1371/journal.pone.0180888. PMID: 28708843; PMCID: PMC5510824.

123. Roh D, Jeon W, Lee J. Enterovirus Meningitis without Pleocytosis: a Retrospective Observational Study in Adults. Jpn J Infect Dis. 2023 Nov 22;76(6):329-334. doi: 10.7883/yoken.JJID.2023.123. Epub 2023 Jun 30. PMID: 37394460.

124. Rovida F, Giardina F, Ferrari G, Paolucci S, Piralla A, Baldanti F. SARS-CoV-2 infections in pediatric patients: A comparison of three pandemic waves. Pediatr Allergy Immunol. 2022 Jan;33 Suppl 27(Suppl 27):93-95. doi: 10.1111/pai.13642. PMID: 35080306; PMCID: PMC9545555.

125. Rueca M, Bartolini B, Gruber CEM, Piralla A, Baldanti F, Giombini E, Messina F, Marchioni L, Ippolito G, Di Caro A, Capobianchi MR. Compartmentalized Replication of SARS-Cov-2 in Upper vs. Lower Respiratory Tract Assessed by Whole Genome Quasispecies Analysis. Microorganisms. 2020 Aug 26;8(9):1302. doi: 10.3390/microorganisms8091302. PMID: 32858978; PMCID: PMC7563410.

126. Sadoff J, Gray G, Vandebosch A, Cárdenas V, Shukarev G, Grinsztejn B, Goepfert PA, Truyers C, Fennema H, Spiessens B, Offergeld K, Scheper G, Taylor KL, Robb ML, Treanor J, Barouch DH, Stoddard J, Ryser MF, Marovich MA, Neuzil KM, Corey L, Cauwenberghs N, Tanner T, Hardt K, Ruiz-Guiñazú J, Le Gars M, Schuitemaker H, Van Hoof J, Struyf F, Douoguih M; ENSEMBLE Study Group. Safety and Efficacy of Single-Dose Ad26.COV2.S Vaccine against Covid-19. New England Journal of Medicine. 2021 Jun 10;384(23):2187-2201. doi: 10.1056/NEJMoa2101544. Epub 2021 Apr 21. PMID: 33882225; PMCID: PMC8220996.

127. Saha R, Majumdar A, Chaudhuri RD, Chatterjee A, Lo M, Dutta S, Chawla-Sarkar M. Molecular epidemiology of circulating human adenoviruses among acute respiratory infection patients seeking healthcare facilities in West Bengal, India. Virology. 2023 Nov;588:109912. doi: 10.1016/j.virol.2023.109912. Epub 2023 Oct 20. PMID: 37913611.

128. Scotta MC, Chakr VC, de Moura A, Becker RG, de Souza AP, Jones MH, Pinto LA, Sarria EE, Pitrez PM, Stein RT, Mattiello R. Respiratory viral coinfection and disease severity in children: A systematic review and meta-analysis. J Clin Virol. 2016 Jul;80:45-56. doi: 10.1016/j.jcv.2016.04.019. Epub 2016 Apr 30. PMID: 27155055; PMCID: PMC7185664.

129. Selleri M, Piralla A, Rozera G, Giombini E, Bartolini B, Abbate I, Campanini G, Rovida F, Dossena L, Capobianchi MR, Baldanti F. Detection of haemagglutinin D222 polymorphisms in influenza A(H1N1)pdm09-infected patients by ultra-deep pyrosequencing. Clin Microbiol Infect. 2013 Jul;19(7):668-73. doi: 10.1111/j.1469-0691.2012.03984.x. Epub 2012 Aug 3. PMID: 22862843.

130. Seppälä E, Sillanpää S, Nurminen N, Huhtala H, Toppari J, Ilonen J, Veijola R, Knip M, Sipilä M, Laranne J, Oikarinen S, Hyöty H. Human enterovirus and rhinovirus infections are associated with otitis media in a prospective birth cohort study. J Clin Virol. 2016 Dec;85:1-6. doi: 10.1016/j.jcv.2016.10.010. Epub 2016 Oct 20. PMID: 27780081.

131. Skog E, Nykvist M, Naguib MM, Wille M, Bröjer C, Agarwal V, Ellström P, Westman G, Lundkvist Å, Järhult JD. An oseltamivir-resistant avian H1N1 influenza A virus can transmit from mallards to chickens similarly to a wild-type strain: implications for the risk of resistance transmission to humans. J Gen Virol. 2023 Apr;104(4). doi: 10.1099/jgv.0.001835. PMID: 37018118.

132. Slavov SN. Viral Metagenomics for Identification of Emerging Viruses in Transfusion Medicine. Viruses. 2022 Nov 4;14(11):2448. doi: 10.3390/v14112448. PMID: 36366546; PMCID: PMC9699440.

133. Tapparel C, Junier T, Gerlach D, Van-Belle S, Turin L, Cordey S, Mühlemann K, Regamey N, Aubert JD, Soccal PM, Eigenmann P, Zdobnov E, Kaiser L. New respiratory enterovirus and recombinant rhinoviruses among circulating picornaviruses. Emerg Infect Dis. 2009 May;15(5):719-26. doi: 10.3201/eid1505.081286. PMID: 19402957; PMCID: PMC2687021.

134. Tejada S, Martinez-Reviejo R, Karakoc HN, Peña-López Y, Manuel O, Rello J. Ribavirin for Treatment of Subjects with Respiratory Syncytial Virus-Related Infection: A Systematic Review and Meta-Analysis. Adv Ther. 2022 Sep;39(9):4037-4051. doi: 10.1007/s12325-022-02256-5. Epub 2022 Jul 25. PMID: 35876973.
135. Tempia S, Walaza S, Bhiman JN, McMorrow ML, Moyes J, Mkhencele T, Meiring S, Quan V, Bishop K, McAnerney JM, von Gottberg A, Wolter N, Du Plessis M, Treurnicht FK, Hellferscee O, Dawood H, Naby F, Variava E, Siwele C, Baute N, Nel J, Reubenson G, Zar HJ, Cohen C. Decline of influenza and respiratory syncytial virus detection in facility-based surveillance during the COVID-19 pandemic, South Africa, January to October 2020. Euro Surveill. 2021 Jul;26(29):2001600. doi: 10.2807/1560-7917.ES.2021.26.29.2001600. PMID: 34296675; PMCID: PMC8299743.
136. Teoh Z, Conrey S, McNeal M, Burrell A, Burke RM, Mattison CP, McMorrow M, Thornburg N, Payne DC, Morrow AL, Staat MA. Factors Associated With Prolonged Respiratory Virus Detection From Polymerase Chain Reaction of Nasal Specimens Collected Longitudinally in Healthy Children in a US Birth Cohort. J Pediatric Infect Dis Soc. 2024 Mar 19;13(3):189-195. doi: 10.1093/jpids/piae009. PMID: 38366142.
137. Thorburn K, Eisenhut M, Riordan A. Mortality and morbidity of nosocomial respiratory syncytial virus (RSV) infection in ventilated children--a ten year perspective. Minerva Anestesiol. 2012 Jul;78(7):782. Epub 2012 Mar 13. PMID: 22415435.
138. Trombetta H, Faggion HZ, Leotte J, Nogueira MB, Vidal LR, Raboni SM. Human coronavirus and severe acute respiratory infection in Southern Brazil. Pathog Glob Health. 2016 May;110(3):113-8. doi: 10.1080/20477724.2016.1181294. Epub 2016 May 19. PMID: 27195607; PMCID: PMC4984956.
139. Ujiie M, Tsuzuki S, Nakamoto T, Iwamoto N. Resurgence of Respiratory Syncytial Virus Infections during COVID-19 Pandemic, Tokyo, Japan. Emerg Infect Dis. 2021 Nov;27(11):2969-2970. doi: 10.3201/eid2711.211565. Epub 2021 Aug 13. PMID: 34388086; PMCID: PMC8544984.
140. Umair M, Ikram A, Salman M, Haider SA, Badar N, Rehman Z, Ammar M, Rana MS, Ali Q. Genomic surveillance reveals the detection of SARS-CoV-2 delta, beta, and gamma VOCs during the third wave in Pakistan. J Med Virol. 2022 Mar;94(3):1115-1129. doi: 10.1002/jmv.27429. Epub 2021 Nov 9. PMID: 34726786; PMCID: PMC8661651.
141. Uyeki TM, Milton S, Abdul Hamid C, Reinoso Webb C, Presley SM, Shetty V, Rollo SN, Martinez DL, Rai S, Gonzales ER, Kniss KL, Jang Y, Frederick JC, De La Cruz JA, Liddell J, Di H, Kirby MK, Barnes JR, Davis CT. Highly Pathogenic Avian Influenza A(H5N1) Virus Infection in a Dairy Farm Worker. N Engl J Med. 2024 Jun 6;390(21):2028-2029. doi: 10.1056/NEJMc2405371. Epub 2024 May 3. PMID: 38700506.

142. Vaira LA, Salzano G, Fois AG, Piombino P, De Riu G. Potential pathogenesis of ageusia and anosmia in COVID-19 patients. Int Forum Allergy Rhinol. 2020 Sep;10(9):1103-1104. doi: 10.1002/alr.22593. Epub 2020 Jun 15. PMID: 32342636; PMCID: PMC7267531.
143. Vanhems P, Bénet T, Munier-Marion E. Nosocomial influenza: encouraging insights and future challenges. Curr Opin Infect Dis. 2016 Aug;29(4):366-72. doi: 10.1097/QCO.0000000000000287. PMID: 27362641.
144. Volpe S, Irish J, Palumbo S, Lee E, Herbert J, Ramadan I, Chang EH. Viral infections and chronic rhinosinusitis. J Allergy Clin Immunol. 2023 Oct;152(4):819-826. doi: 10.1016/j.jaci.2023.07.018. Epub 2023 Aug 22. PMID: 37574080; PMCID: PMC10592176.
145. Voysey M, Clemens SAC, Madhi SA, Weckx LY, Folegatti PM, Aley PK, Angus B, Baillie VL, Barnabas SL, Bhorat QE, Bibi S, Briner C, Cicconi P, Collins AM, Colin-Jones R, Cutland CL, Darton TC, Dheda K, Duncan CJA, Emary KRW, Ewer KJ, Fairlie L, Faust SN, Feng S, Ferreira DM, Finn A, Goodman AL, Green CM, Green CA, Heath PT, Hill C, Hill H, Hirsch I, Hodgson SHC, Izu A, Jackson S, Jenkin D, Joe CCD, Kerridge S, Koen A, Kwatra G, Lazarus R, Lawrie AM, Lelliott A, Libri V, Lillie PJ, Mallory R, Mendes AVA, Milan EP, Minassian AM, McGregor A, Morrison H, Mujadidi YF, Nana A, O'Reilly PJ, Padayachee SD, Pittella A, Plested E, Pollock KM, Ramasamy MN, Rhead S, Schwarzbold AV, Singh N, Smith A, Song R, Snape MD, Sprinz E, Sutherland RK, Tarrant R, Thomson EC, Török ME, Toshner M, Turner DPJ, Vekemans J, Villafana TL, Watson MEE, Williams CJ, Douglas AD, Hill AVS, Lambe T, Gilbert SC, Pollard AJ; Oxford COVID Vaccine Trial Group. Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. Lancet. 2021 Jan 9;397(10269):99-111. doi: 10.1016/S0140-6736(20)32661-1. Epub 2020 Dec 8. Erratum in: Lancet. 2021 Jan 9;397(10269):98. PMID: 33306989; PMCID: PMC7723445.
146. Walsh EE, Pérez Marc G, Zareba AM, Falsey AR, Jiang Q, Patton M, Polack FP, Llapur C, Doreski PA, Ilangovan K, Rämet M, Fukushima Y, Hussen N, Bont LJ, Cardona J, DeHaan E, Castillo Villa G, Ingilizova M, Eiras D, Mikati T, Shah RN, Schneider K, Cooper D, Koury K, Lino MM, Anderson AS, Jansen KU, Swanson KA, Gurtman A, Gruber WC, Schmoele-Thoma B; RENOIR Clinical Trial Group. Efficacy and Safety of a Bivalent RSV Prefusion F Vaccine in Older Adults. N Engl J Med. 2023 Apr 20;388(16):1465-1477. doi: 10.1056/NEJMoa2213836. Epub 2023 Apr 5. PMID: 37018468.
147. Walter JM, Wunderink RG. Severe Respiratory Viral Infections: New Evidence and Changing Paradigms. Infect Dis Clin North Am. 2017 Sep;31(3):455-474. doi: 10.1016/j.idc.2017.05.004. Epub 2017 Jul 5. PMID: 28687214; PMCID: PMC7347414.
148. Wang D, Zhu W, Yang L, Shu Y. The Epidemiology, Virology, and Pathogenicity of Human Infections with Avian Influenza Viruses. Cold Spring

Harb Perspect Med. 2021 Apr 1;11(4):a038620. doi: 10.1101/cshperspect.a038620. PMID: 31964651; PMCID: PMC8015695.
149. Wang L, Hu X, Huang Z, Zhang Y, Zhao X, Liu X, Mao H, Hao H, Xue W. Analysis of the typing of adenovirus and its clinical characteristics in children with acute respiratory tract infection. BMC Pediatr. 2023 Jan 16;23(1):25. doi: 10.1186/s12887-023-03840-6. PMID: 36647010; PMCID: PMC9841135.
150. Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, Shi Z, Hu Z, Zhong W, Xiao G. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell Res. 2020 Mar;30(3):269-271. doi: 10.1038/s41422-020-0282-0. Epub 2020 Feb 4. PMID: 32020029; PMCID: PMC7054408.
151. Wang P, Lau SY, Deng S, Chen P, Mok BW, Zhang AJ, Lee AC, Chan KH, Tam RC, Xu H, Zhou R, Song W, Liu L, To KK, Chan JF, Chen Z, Yuen KY, Chen H. Characterization of an attenuated SARS-CoV-2 variant with a deletion at the S1/S2 junction of the spike protein. Nat Commun. 2021 May 13;12(1):2790. doi: 10.1038/s41467-021-23166-0. PMID: 33986286; PMCID: PMC8119425.
152. Wang W, He J, Liu Y, Xu L, Guan W, Hu Y. Molecular genotyping of human rhinovirus by using PCR and Sanger sequencing. Methods Mol Biol. 2015;1221:39-47. doi: 10.1007/978-1-4939-1571-2_4. PMID: 25261305.
153. Wang Y, Eccles R, Bell J, Chua AH, Salvi S, Schellack N, Marks P, Wong YC. Management of acute upper respiratory tract infection: the role of early intervention. Expert Rev Respir Med. 2021 Dec;15(12):1517-1523. doi: 10.1080/17476348.2021.1988569. Epub 2021 Oct 19. PMID: 34613861.
154. Wölfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Müller MA, Niemeyer D, Jones TC, Vollmar P, Rothe C, Hoelscher M, Bleicker T, Brünink S, Schneider J, Ehmann R, Zwirglmaier K, Drosten C, Wendtner C. Virological assessment of hospitalized patients with COVID-2019. Nature. 2020 May;581(7809):465-469. doi: 10.1038/s41586-020-2196-x. Epub 2020 Apr 1. Erratum in: Nature. 2020 Dec;588(7839):E35. doi: 10.1038/s41586-020-2984-3. PMID: 32235945.
155. World Health Organization. WHO MORTALITY DATABASE. Available at: https://platform.who.int/mortality/themes/theme-details/topics/topic-details/MDB/respiratory-infections.
156. World Health Organization. Research needs for the Battle against Respiratory Viruses (BRaVe). Published online 2013. Accessed September 1, 2021. Available at https://www.who.int/influenza/patient_care/clinical/BRaVe_Research_Agenda_2013.pdf?ua¼1.
157. World Health Organization. Weekly Epidemiological Record (WER), 25 March 2022, Vol. 97, No. 12, https://reliefweb.int/report/world/weekly-

epidemiologicalrecord-wer-25-march-2022-vol-97-no-12-pp109-132-enfr (2022).
158. World Health Organization, 2024. https://data.who.int/dashboards/covid19/cases.
159. Yu J, Xie Z, Zhang T, Lu Y, Fan H, Yang D, Bénet T, Vanhems P, Shen K, Huang F, Han J, Li T, Gao Z, Ren L, Wang J. Comparison of the prevalence of respiratory viruses in patients with acute respiratory infections at different hospital settings in North China, 2012-2015. BMC Infect Dis. 2018 Feb 8;18(1):72. doi: 10.1186/s12879-018-2982-3. PMID: 29422011; PMCID: PMC5806372.
160. Zadheidar S, Yavarian J, Heydarifard Z, Nejati A, Sadeghi K, Ghavami N, Abbasi S, Shatizadeh Malekshahi S, Mokhtari-Azad T, Shafiei-Jandaghi NZ. Molecular epidemiology of human adenoviruses in children with and without respiratory symptoms: Preliminary findings from a case-control study. BMC Pediatr. 2022 Oct 8;22(1):583. doi: 10.1186/s12887-022-03625-3. PMID: 36207696; PMCID: PMC9547415.
161. Zhao J, Yap A, Wu E, Low CY, Yap J. Severe community acquired adenovirus pneumonia in an immunocompetent host successfully treated with IV Cidofovir. Respir Med Case Rep. 2020 Mar 10;30:101037. doi: 10.1016/j.rmcr.2020.101037. PMID: 32195107; PMCID: PMC7078434.
162. Zumla A, Hui DS, Perlman S. Middle East respiratory syndrome. Lancet. 2015 Sep 5;386(9997):995-1007. doi: 10.1016/S0140-6736(15)60454-8. Epub 2015 Jun 3. PMID: 26049252; PMCID: PMC4721578.

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SUPPLEMENT ARTICLE

WILEY

Spread of multiple SARS-CoV-2 lineages April-August 2020 anticipated the second pandemic wave in Lombardy (Italy)

Monica Tallarita[1] | Federica Giardina[1] | Federica Novazzi[1] | Stefano Gaiarsa[1] | Gherard Batisti Biffignandi[2] | Stefania Paolucci[1] | Francesca Rovida[1] | Antonio Piralla[1] | Fausto Baldanti[1,2]

[1]Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

[2]Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy

Correspondence
Antonio Piralla, Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Via Tamelli 5, 27100 Pavia, Italy.
Email: a.piralla@smatteo.pv.it

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Abstract

During the early phase of the pandemic (20 February–4 April 2020), we have investigated the temporal and geographical evolution of the virus in Lombardy showing the circulation of at least seven lineages distributed differently in the Region. In the present study, the molecular epidemiology of SARS-CoV-2 was monitored in a period between two pandemic waves in order to track the circulation of new variants (April-August 2020). A great majority of SARS-CoV-2 strains (70.8%) belonged to lineages B, B.1, B.1.1 and B.1.1.1, and five strains belonging to four lineages were already reported in Italy (B.1.1.148, B.1.1.162, B.1.1.71, and B.1.425). In addition, 21 SARS-CoV-2 strains belonged to six lineages not previously observed in Italy were detected. No variants of concern were observed. A total of 152/1274 (11.3%) amino acid changes were observed among spike gene sequences and only 26/152 (17.1%) occurred in the receptor-binding domain region of the spike protein. Results of this study are indicative of ongoing transmission throughout the lockdown period, rather than re-introduction of novel lineages past lockdown. The use of molecular epidemiology in Italy should be promoted in order to provide additional understanding of the transmission of the disease and to have major effect on controlling the spread of disease.

KEYWORDS
COVID-19, molecular epidemiology, NGS, SARS-CoV-2, whole genome sequencing

1 | INTRODUCTION

Since coronavirus disease 2019 (COVID-19) was initially reported in China on 30th December 2019,[1,2] SARS-CoV-2 has been spreading worldwide. As of 5 February 2021, there have been 104 million confirmed infections and more than 2 million deaths have been reported worldwide.[1] Lombardy with a population of 10 million is the most densely populated and affected Region in Italy during the first wave with more than 90,000 cases at the end of May 2020.[3,4] The population density coupled with the high level of transportation links to Europe creates the conditions to host and favor the spread of a highly transmissible viruses such as SARS-CoV-2. During the early phase of the pandemic (20 February–4 April 2020), we have investigated the temporal and geographical evolution of the virus in Lombardy.[5] This

Monica Tallarita, Federica Giardina and Federica Novazzi contributed equally to the work and share first authorship.

study has documented the circulation of at least seven lineages distributed differently in the Region. The first pandemic wave showed as sharp down at the end of May 2020. However, a steading number of cases was reported (more than 10,000 June-August 2020) and international road transport to and from several EU states was re-established after 1 June 2020.[4,6] In the present study, the molecular epidemiology of SARS-CoV-2 was monitored in a period between two pandemic waves in order to track the circulation of new variants.

Key Message

Surveillance of SARS-CoV-2 circulating strains is crucial to provide important information on viral evolution and epidemiology. Tracking SARS-CoV-2 evolution is fundamental for the assessment of vaccine-induced immunity and antiviral therapy efficacy against emerging variants.

2 | MATERIAL AND METHODS

A total of 89 respiratory samples with SARS-CoV-2 cycle threshold values <24 were selected for sequencing. Clinical samples were collected between 15 April and 20 August 2020 and tested positive for SARS-CoV-2 as previously described.[5,7] Total RNAs were extracted from nasopharyngeal swabs by using QIAamp Viral RNA Mini Kit, followed by purification with Agencourt RNA Clean XP beads. Virus genomes (GISAID EPI_ISL_1133145-1133202 and 1166095–1166108) were generated by using a multiplex approach, using version 1 of the CleanPlex SARS-CoV-2 Research and Surveillance Panel (Paragon Genomics, Inc.), according to the manufacturer's protocol starting with 50 ng of total RNA and followed by Illumina sequencing on a MiSeq platform. NGS data were also analyzed with an in-house pipeline. Lineages were assigned from alignment file using the Phylogenetic Assignment of Named Global Outbreak LINeages tool PANGOLIN v1.07 (https://github.com/hCoV-2019/pangolin).[8] The study protocol was approved by the local Research Ethics Committee of Fondazione IRCCS Policlinico San Matteo (P_20200085574). This study was conducted in accordance with the principles of the 1964 Declaration of Helsinki. Informed consent was waived in accordance with Italian governmental regulations on observational retrospective studies.

3 | RESULTS

Based on the GISAID EpiCoV™ database (https://www.epicov.org/epi3/) as of 5 February 2021, a total of 3212 SARS-CoV Italian sequences were available and a comparison between them was performed and presented in Table 1 SARS-CoV-strains. A SARS-CoV-2 lineages analysis performed using the Pangolin web application suggested that the B lineages were the most common in Lombardy (Figure 1). In detail, a great majority of SARS-CoV-2 strains (63/89; 70.8%) belonged to lineages B, B.1, B.1.1 and B.1.1.1. Five strains grouped into clade A including sequences from China and many from South East Asia, Japan, South Korea, Australia, and the USA. The distribution of lineages in the early phase of pandemic, a similar distribution of lineages also continued until August.[5] This may suggest that the initial multiple lineage introduction in Lombardy was followed by local transmission events during the lockdown period. In addition, five strains belonging to four lineages, already reported in Italy (B.1.1.148, B.1.1.162, B.1.1.71, and B.1.425) were detected. Finally, 21 SARS-CoV-2 strains belonged to six lineages not previously observed in Italy were detected (Table 1). This finding showed the introduction of additional lineages in the context of a predominant circulation of previously introduced lineages.

Since summer 2020, a series of SARS-CoV-2 variants of concern (VOC) (eg, VOC 202012/01, and 501Y.V2) harboring several amino acidic changes were highlighted by ECDC as of potential increased pathogenicity.[9] Some of them were associated with increased infection cases in the UK (VOC 202012/01) and later in other countries and other few (VOC501Y.V2 and Brasil) were associated with a reduced neutralization by plasma. In the period from 15 April to 20 August 2020 of this analysis, none of these variants were observed.

A total of 152/1274 (11.3%) amino acid (aa) changes were observed among spike gene sequences; however, only 10/152 (6.6%, 0.8% of total aa) changes (T29I, E281G, Q564R, F565S, D614G, S640A, N641H, K964R) were observed in at least two SARS-CoV-2 strains. Of note, only 26/152 (17.1%, 2.0% of total) changes occurred in the receptor-binding domain (RBD) region of the spike protein.

4 | DISCUSSION

The phylogenetic analysis performed on the viral sequences collected during the first period of the pandemic in Italy, mainly originated in Lombardy, suggested a clear circulation of at least seven SARS-CoV-2 lineages.[5] Similar results were also observed in a subsequent study including 460 Italian strains previously reported[10] and in a more extended study aiming to describe SARS-CoV-2 circulation in European region.[11] The generated genomes in the present study provide additional insight into the SARS-CoV-2 lineages and variants circulating in Lombardy in the period between the first and the before second pandemic wave. Results of the analyses seem to indicate that additional lineages were introduced in Lombardy during the summer but these introductions did not lead to further transmission of the virus in the community, or a limited transmission has occurred. The segregation of specific lineages was observed probably as a result of the strict lockdown measures applied during the three-month state of emergency (March-May 2020). Several lineages mainly circulating in other countries have been identified, and few of them were not previously reported in Italy. None of "high risk" variants have been observed to circulate in Lombardy in the study

TABLE 1 Lineages identified in this study

Lineages	This study (no.)	Italian sequences (no.)	Most common countries	Earliest date	Count	Description[a]
A	5	16	United_Arab_Emirates 19.0%, Germany 13.0%, China 13.0%, United States of America 6.0%, Japan 5.0%	30–12–2019	1312	Root of the pandemic lies within lineage A
B	9	21	United Kingdom 36.0%, United States of America 16.0%, China 13.0%, Spain 4.0%, Singapore 3.0%	24–12–2019	4976	Base of this lineage also lies in China, with many global exports, two distinct SNPs `8782TC` and `28144CT` define this lineage
B.1	45	743	United States of America 46.0%, United Kingdom 13.0%, Canada 5.0%, Spain 4.0%, France 3.0%	24–01–2020	50516	A large European lineage that corresponds to the Italian outbreak.
B.1.1	1	29	United States of America 27.0%, United Kingdom 23.0%, Canada 6.0%, Germany 6.0%, Netherlands 5.0%	24–02–2020	2766	European lineage
B.1.1.1	3	150	United Kingdom 76.0%, Denmark 2.0%, Italy 2.0%, United States of America 2.0%, Switzerland 2.0%	02–03–2020	7604	UK/ Europe lineage
B.1.1.148	1	1	USA 89.0%, Canada 5.0%, Germany 2.0%, UK 2.0%, Mexico 1.0%	16–05–2020	87	USA lineage
B.1.1.161	10	0	Saudi_Arabia 24.0%, UK 21.0%, Switzerland 10.0%, Czech_republic 10.0%, Denmark 4.0%	09–03–2020	202	Saudi Arabian lineage
B.1.1.162	1	57	United Kingdom 23.0%, United States of America 15.0%, Japan 10.0%, Canada 9.0%, Spain 6.0%	26–02–2020	1927	Australian/UK lineage
B.1.1.38	1	0	United Kingdom 23.0%, United States of America 15.0%, Japan 10.0%, Canada 9.0%, Spain 6.0%	24–03–2020	260	UK lineage
B.1.1.44	1	0	United Kingdom 56.0%, Spain 20.0%, United States of America 11.0%, Denmark 2.0%, Switzerland 1.0%	23–03–2020	630	UK/Spain lineage
B.1.1.71	2	4	Netherlands 27.0%, United_Arab_Emirates 21.0%, UK 17.0%, Australia 8.0%, Belgium 7.0%	06–03–2020	146	The Netherlands lineage
B.1.1.74	7	0	United Kingdom 87.0%, United States of America 5.0%, Peru 3.0%, Ireland 1.0%, Russia 1.0%	23–05–2020	268	Northern Irish lineage
B.1.243	1	0	United States of America 98.0%, Mexico 1.0%, Canada 0.0%, Switzerland 0.0%, South_Korea 0.0%	23–03–2020	5256	USA lineage
B.1.379	1	0	United Kingdom 91.0%, France 3.0%, United States of America 1.0%, Sierra_Leone 1.0%, Germany 1.0%	16–03–2020	67	UK lineage, previously B.1.5.30
B.1.425	1	1	United States of America 90.0%, Turkey 10.0%, Italy 0.0%		296	USA lineage (UT), reassigned from part of B.1.370

[a]https://cov-lineages.org/lineages.html.

period. However, the recently circulation of UK variants (B.1.1.7) associated with a more transmissible virus raise the attention on global changing of SARS-CoV-2 circulation. The ongoing vaccine campaign should be supported with real-time surveillance of circulating variants in order to monitor the emergence of mutations associated with poorly antibody recognition.

Finally, the great majority of mutations within the spike gene region were observed in single strain and only D614G changes seem to

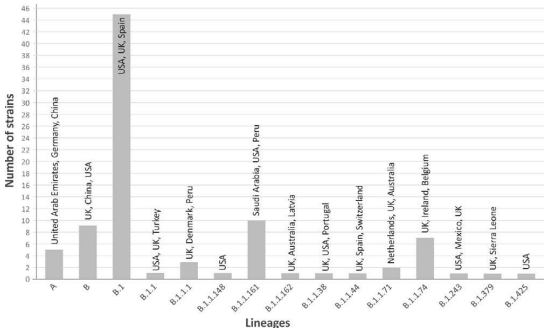

FIGURE 1 Distribution of SARS-CoV-2 strains according to lineages assigned by Pangolin COVID-19 Lineage Assigner online tool (https://github.com/hCoV-2019/pangolin). The most common countries in which each lineage was identified are reported above the bars

be fixed in the SARS-CoV-2 population analyzed. In addition, most of them located outside the RBD region with therefore a limited impact on antibodies recognition.

5 | CONCLUSION

We reported on the limited but ongoing within-region transmission during the first wave of SARS-CoV-2 in Lombardy. These features are indicative of ongoing transmission throughout the lockdown period, rather than re-introduction of novel lineages past lockdown. However, it is critical to acknowledge the extremely limited amount of genomic data from Italy compared with many other localities that clearly impacted the strength of the conclusions that can be drawn here. It is therefore vital that Italy build better structures for effective genomic epidemiology prior to any future major outbreaks of emerging infectious disease.

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CONFLICT OF INTERESTS

All authors have no conflicts of interest to disclose.

AUTHORS' CONTRIBUTION

Monica Tallarita: Methodology (equal), Writing-review & editing (equal). **Federica Giardina**: Methodology (equal), Writing-review & editing (equal). **Federica Novazzi**: Methodology (equal), Writing-review & editing (equal). **Stefano Gaiarsa**: Methodology (equal), Writing-review & editing (equal). **Gherard Batisti Biffignandi**: Methodology (equal), Writing-review & editing (equal). **Stefania Paolucci**: Supervision (equal); Methodology (equal), Writing-review & editing (equal). **Francesca Rovida**: Supervision (equal); Writing-review & editing (equal). **Antonio Piralla**: Conceptualization (equal); Supervision (lead); Writing-review & editing (equal). **Fausto Baldanti**: Conceptualization (equal); Supervision (equal); Writing-review & editing (equal).

ORCID

Monica Tallarita https://orcid.org/0000-0002-9191-7935
Federica Giardina https://orcid.org/0000-0001-6559-8099
Federica Novazzi https://orcid.org/0000-0002-2741-8840
Stefano Gaiarsa https://orcid.org/0000-0002-5847-6568
Gherard Batisti Biffignandi https://orcid.org/0000-0003-3108-6313
Stefania Paolucci https://orcid.org/0000-0001-8447-8586
Francesca Rovida https://orcid.org/0000-0003-2345-2790
Antonio Piralla https://orcid.org/0000-0002-6062-2579
Fausto Baldanti https://orcid.org/0000-0002-3358-8969

REFERENCES

1. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infect Dis*. 2020;20:533-534.
2. Wu F, Zhao S, Yu B, et al. A new coronavirus associated with human respiratory disease in China. *Nature*. 2020;579:265-269.
3. Eurostat. The statistical office of the European Union statistics. https://ec.europa.eu/Eurostat.web/cities/data/database (Accessed 4 April 2021)
4. Regione Lombardia - Dashboard COVID 19 update of cases. https://www.regione.lombardia.it/wps/portal/istituzionale/HP/servizi-e-informazioni/cittadini/salute-e-prevenzione/coronavirus/dashboard-covid19 (Accessed 4 April 2021)
5. Alteri C, Cento V, Piralla A, et al. Genomic epidemiology of SARS-CoV-2 reveals multiple lineages and early spread of SARS-CoV-2 infections in Lombardy, Italy. *Nat Commun*. 2021;12:434.
6. European Union. https://ec.europa.eu/info/live-work-travel-eu/coronavirus-response/travel-during-coronavirus-pandemic_en. (accessed 4 Apr 2021).
7. Piralla A, Ricchi M, Cusi MG, et al. Residual SARS-CoV-2 RNA in nasal swabs of convalescent COVID-19 patients: Is prolonged quarantine always justified? *Int J Infect Dis*. 2021;102:299-302.
8. Rambaut A, Holmes EC, O'Toole Á, et al. A dynamic nomenclature proposal for SARS-CoV-2 lineages to assist genomic epidemiology. *Nat Microbiol*. 2020;5:1403-1407.
9. European Centre for Disease Prevention and Control. *Risk Related to Spread of New SARS-CoV-2 Variants of Concern in the EU/EEA, First Update – 21 January 2021*. ECDC; 2021. https://www.ecdc.europa.eu/en/publications-data/covid-19-risk-assessment-spread-new-variants-concern-eueea-first-update (Accessed 4 April 2021).
10. Di Giallonardo F, Duchene S, Puglia I, et al. Genomic epidemiology of the first wave of SARS-CoV-2 in Italy. *Viruses*. 2020;12:1438.
11. Alm E, Broberg EK, Connor T, et al. Geographical and temporal distribution of SARS-CoV-2 clades in the WHO European Region, January to June 2020. *Euro Surveill*. 2020;25:2001410.

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Original Articles

An overview of SARS-CoV-2 variants circulating in the 2020–2022 period in Lombardy

Federica Giardina[a], Guglielmo Ferrari[b], Federica Zavaglio[b], Stefania Paolucci[b], Francesca Rovida[a,b], Giulia Campanini[b], Laura Pellegrinelli[c], Cristina Galli[c], Elena Pariani[c], Federica Bergami[b], Alice Nava[d], Elisa Matarazzo[d], Silvia Renica[e], Diana Fanti[d], Valeria Cento[f,g], Claudia Alteri[e], Francesco Scaglione[e], Chiara Vismara[d], Carlo Federico Perno[h], Antonio Piralla[b,*], Fausto Baldanti[a,b]

[a] Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy
[b] Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
[c] Department of Biomedical Sciences for Health, University of Milan, Milan, Italy
[d] Microbiological Analysis Unit, ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy
[e] Department of Oncology and Hemato-oncology, University of Milan, Milan, Italy
[f] Department of Biomedical Sciences, Humanitas University, Milan, Italy
[g] IRCCS Humanitas Research Hospital, Milan, Italy
[h] Multimodal Research Area, Unit of Microbiology and Diagnostic Immunology, Bambino Gesù Children Hospital IRCCS, Rome, Italy

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Next-generation sequencing

ABSTRACT

Since the beginning of the pandemic, SARS-CoV-2 has shown genetic variability. All the variants that have sustained pandemic waves have shown several mutations, especially in the Spike protein that could affect viral pathogenesis. A total of 15,729 respiratory samples, collected between December 2020 and August 2022, have been included in this study. We report the circulation of SARS-CoV-2 variants in the Lombardy region, Italy, in a 2-year study period. Alpha, Delta, and Omicron variants became predominant causing the majority of cases whereas Beta or Gamma variants mostly caused local outbreaks. Next-generation sequencing revealed several mutations and few deletions in all of the main variants. For example, 147 mutations were observed in the Spike protein of Omicron sublineages; 20% of these mutations occurred in the receptor-binding domain region.

1. Introduction

Since its first discovery at the end of 2019, SARS-CoV-2 spread rapidly all over the world. Italy, and in particular the Lombardy region (10 million inhabitants), was one of the first European countries heavily hit by the pandemic. Full genome sequencing of hundreds of SARS-CoV-2-positive respiratory samples revealed that the early phase of the pandemic was sustained by seven different lineages, with different geographical distributions within the Lombardy region [1]. The number of circulating lineages increased during the summer of 2020 [2]. At the same time, the first noteworthy amino acid change in the Spike protein (D614G) was associated with a high level of viral loads in clinical samples [3]. However, the concern about the emergence of lineages carrying new amino acid substitutions arose at the end of 2020, after the first identification of B.1.1.7 (Alpha variant) in the United Kingdom [4]. A set of new lineages continuously emerged including B.1.351, P.1, and B.1.617.2 (Beta, Gamma, and Delta variants, respectively) which were detected in South Africa, Brazil, and India, respectively [5–7]. All these lineages carried new mutations and some of them, in particular those that had occurred in the receptor-binding domain, affected the viral pathogenesis by causing high transmissibility rates, severe clinical syndromes, or immune escape [8–9]. The aim of this study was the evaluation of the presence and circulation of these lineages in Lombardy between September 2020 and August 2022 and the molecular characterization of new variants through next-generation sequencing.

2. Methods

2.1. Study design

To perform regional surveillance of circulating variants, from December 2020 to August 2022, specimens were collected in several hospitals in all Lombardy provinces. All the samples were tested for the presence of the SARS-CoV-2 genome; those which tested positive with a cycle threshold lower than 30 were then referred to

* Corresponding author. Tel: +39038250240; Fax: +390382502599.
E-mail address: a.piralla@smatteo.pv.it (A. Piralla).

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Fondazione I.R.C.C.S. Policlinico San Matteo, Pavia, Italy, as one of the reference centers for SARS-CoV-2 genotyping. In particular, as requested by the Italian Health Ministry, genotyping was recommended for subjects who tested positive after anti-SARS-CoV-2 vaccination, those with reinfection, and those returning to Italy from any country with a high prevalence of an emerging variant. Genotyping was also recommended for the following reasons: (1) increasing number of cases, (2) increasing viral transmissibility or virulence (Italian Ministry of Health, 0003787-31/01/2021-DGPRE-DGPRE-P).

Besides, to investigate the circulation of the SARS-CoV-2 variants from September to December 2020, a retrospective analysis was performed. Respiratory samples were mainly collected at Fondazione I.R.C.C.S. Policlinico San Matteo in Pavia, Grande Ospedale Metropolitano Niguarda in Milan, and the University of Milan. The sequence investigation of patient samples was approved by the Ethics Committee of our institution (P_20200085574) [10].

2.2. SARS-CoV-2 RNA detection

During September 2020–August 2022, 315,697 respiratory samples were analyzed at Fondazione Policlinico San Matteo in Pavia. RNA was extracted by using the MGISP-960 automated workstation and the MGI Easy Magnetic Beads Virus DNA/RNA Extraction Kit (MGI Technologies, Shenzhen, China). SARS-CoV-2 RNA was detected using the SARS-CoV-2 variants ELITe MGB® kit (ELITechGroup, Puteaux, France; cat. no. RTS170ING) targeting *ORF8* and *RdRp* gene. Reactions were carried out on the CFX96 Touch Real-time PCR detection system (BioRad, Mississauga, ON, Canada).

2.3. Molecular screening for detecting the SARS-CoV-2 variants

Genotyping was performed with a set of multiplex real-time PCR assays targeting specific mutations. To detect Alpha cases, including those carrying E484K mutation, a multiplex real-time PCR targeting mutations N501Y and E484K was performed. To detect the Delta variant, mutations T478K and L452R were used as targets. Finally, Omicron cases were detected by multiplex real-time PCR targeting mutations in positions 417, 484, and 501 and the deletion 368 to 370. All primers and probes used for genotyping are reported in Table 1.

Real-time PCR assays were performed using Quantifast® Pathogen RT-PCR+IC Kit (Qiagen, Heidelberg, Germany) using the following parameters: 52°C for 15 minutes, 95 °C for 10 seconds, then 45 cycles at 95 °C for 5 minutes and 60 °C for 30 seconds. If the real-time PCR results were not sufficient to discriminate between variants circulating in the same period, the *Spike* gene was sequenced as described below.

Table 1
Primers and probes used to identify SARS-CoV-2 Alpha, Delta, and Omicron variants.

Target	Primers/Probes	Sequence
B.1.1.7 + B.1.1.7/ E484K	E484K-For	TGAAATCTATCAGGCCGGTAGC
	N501Y-Rev	GTACTACTACTCTGTATGGTTGG
	N501-probe	FAM-AACCCACTAATGGTGTTGG-MGB
	501Y-probe	VIC-AACCCACTTATGGTGTTGG-MGB
	484K-probe	Cy5-GTAATGGTGTTAAAGGTTT-MGB
B.1.617.2	VAR-IND-For	AGGCTGCGTTATAGCTTGGAATT
	VAR-IND-Rev	AGTAACAATTAAAACCTTCAACACCATTAC
	452R-probe	FAM- CCGGTATAGATTGTTTAGGAAG-MGB
	478K-probe	HEX-CCGGTAGCAAACCT-MGB
B.1.1.529	S-E484KFor	TGAAATCTATCAGGCCGGTAGC
	S-N501YRev	GTACTACTACTCTGTATGGTTGG
	S417For2	CTCTGCTTTACTAATGTCTATGCA
	S-417Rev2	CGCAGCCTGTAAAATCATCTGG
	S368L-S370PFor	GGAAGAGAATCAGCAACTGTGTTG
	S368-S370Rev	TCTGCATAGACATTAGTAAAGCAGAGATC
	S-501Y-probe	VIC-AACCCACTTATGGTGTTGG-MGB
	E484A-probe	CY5-CCTTGTAATGGTGTTGCAGG-MGB
	S-417N-probe	TexasRed-CAAACTGGAAATATTG-MGB
	S368-S370-probe	FAM-CCTATATAATCTCGCACCATT-MGB

2.4. Sanger sequencing

To confirm the real-time PCR results, in 10% of all samples, randomly chosen, the Spike gene was amplified and then sequenced. Spike was amplified as described by Gaiarsa et al. [11]. Briefly, RNA was subjected to a one-step RT-PCR using the SuperScript IV One-Step RT-PCR System (Thermo Fisher Scientific, Waltham, MA, USA), using primer SARS-2-S-F3 and SARS-2-S-R3 (Supplementary Table S1) for full gene amplification (~4000 bp). The PCR parameters were as follows: 55 °C for 10 minutes, 98 °C for 2 minutes, then 42 cycles at 98 °C for 10 seconds, 60 °C for 10 seconds and 72 °C for 3 minutes. The final extension was at 72 °C for 5 minutes. Sequencing reaction of proper PCR products was performed with BigDye™ Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Thermo Fisher Scientific, CA, USA) on 3130xl Genetic Analyzer. The reaction was carried out with the same primers used for one-step RT-PCR and seven additional internal primers (Supplementary Table S1). Sequences obtained were analyzed with Sequencer 5.0 software.

2.5. Whole-genome sequencing

Genomic libraries were prepared using NEBNext® ARTIC SARS-CoV-2 library Prep kit (New England Biolabs) starting from 8 μL of viral RNA, according to the manufacturer's instructions. Libraries were quantified using Qubit™ 1X dsDNA HS kit (Invitrogen, Carlsbad, CA, USA) on Qubit 4 Fluorometer, normalized at the same concentration, and then pooled together. The pool was denatured with 0.2M NaOH and then diluted to 10 pM. Sequencing was performed using Miseq Reagent kit V2 300 cycles (Illumina, San Diego, CA) on the MiSeqDx platform. Fastq files were analyzed with Illumina® DRAGEN COVID Lineage App for sequence alignment and variant identification.

3. Results

3.1. Patients

A total of 15,729 SARS-CoV-2-positive respiratory samples were included in this study. The median age of patients was 46 years (range 1 day–106 years), including 7900 males (50.3%) and 7829 females (49.7%). Samples were collected in several hospitals in Lombardy provinces, from both inpatients and outpatients. In detail, 5919 samples (37.6%) were collected in Pavia province, 3,956 (25.2%) in Milano, 2,476 (15.7%) in Mantua, 1,534 (9.8%) in Lecco, 698 (4.4%) in Lodi and 609 (3.9%) in Cremona area. A total of 537 samples (3.4%) were collected in Bergamo, Brescia, Como, Monza Brianza, Sondrio, and Varese provinces.

3.2. Variant identification

SARS-CoV-2 was successfully genotyped in 14,075 samples (89.5% of the total). Delta was the most detected variant, identified in 8091 samples (57.5%), followed by Omicron variants (2719 cases, 19.3%) and Alpha variant (2333 cases, 16.6%). *Early variants*, as all those lineages circulating in the Lombardy region before the first Alpha variant circulation, were detected in 798 cases (5.7%) and, in particular, B.1.177 was detected in 67% of cases, B.1.160 in 7.5%, B.1.1.161 in 4.1%, B.1.177.52 and B.1.177.75 in 3.4% and 3.2%, respectively. All other lineages, including Beta, Eta, Gamma, Mu, and variants, were present in a frequency of less than 3%. A series of local clusters of these variants were observed, with less than 20 cases in each province. In 1,654 samples (10.5%) genotyping was unsuccessful due to low viral loads.

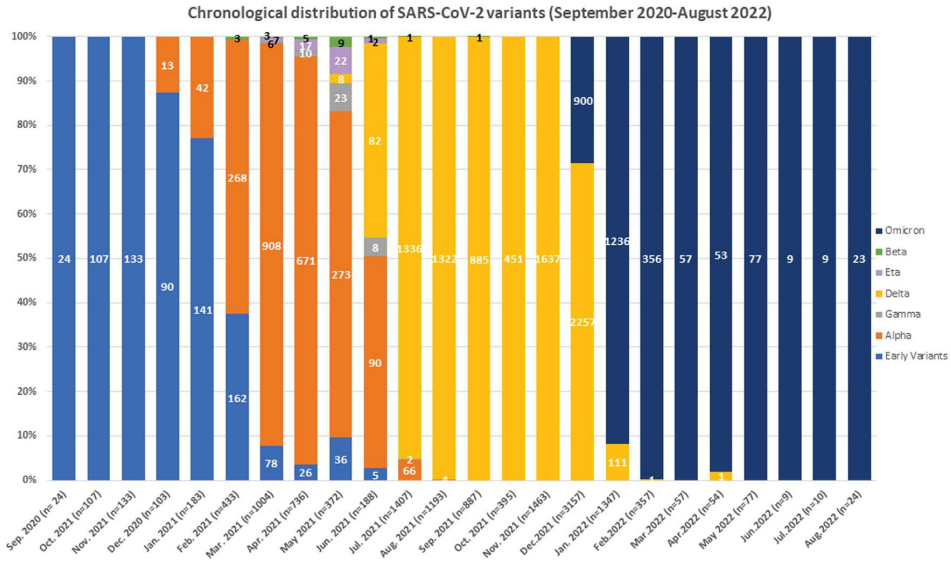

Fig. 1. Chronological distribution of SARS-CoV-2 variants during December 2020 to August 2022. "*Early variants*," as those circulating before the first Alpha identification, are represented by light blue; Alpha cases are represented by orange, Delta in yellow, and Omicron in dark blue. While Alpha, Delta, and Omicron predominated during the study period, Beta, Eta, and Gamma variants (here depicted in green, pink, and grey, respectively) only caused local clusters with few cases.

3.3. Chronological circulation of SARS-CoV-2 variants

As shown in Fig. 1, at the beginning of the study period, the only lineages detected were those we called *early variants*. In December 2020, the Alpha variant was detected for the first time causing 12.4% of all cases included in the study (December 2020–August 2022); in February 2021, the Alpha variant became predominant causing 49.2% of all cases. In the same period, circulation of Beta, Eta, and Gamma variants was observed. They caused 23, 48, and 49 cases, respectively (0.14%, 0.3%, and 0.3%) and were detected only from February to June 2021. In May, the Delta variant circulated, with 8 positive samples (2%) out of 372 samples sequenced in May 2021. Starting from June, the number of strains belonging to the Delta variant was more than 10-fold higher than in May, causing 27.5% of all cases tested in June. After a few weeks of co-circulation (from June to July 2021) of Alpha and Delta variants, in August Alpha was detected for the last time and Delta became the only variant detected until December 2021. Along with Delta, in December 2021, the first strain belonging to the Omicron clade (B.1.1.529) was detected in 900 cases (26%) out of 3461 patients tested. While the number of cases caused by Delta decreased in February 2022, the number of cases due to the Omicron variant increased rapidly becoming the predominant variant until the end of August 2022.

For the 3 main variants circulating in Lombardy, Alpha, Delta, and Omicron, the kinetics of frequency circulation were calculated and analyzed with respect to the vaccination campaign. As shown in Fig. 2, the number of Alpha, Delta, and Omicron cases increased when the number of distributed vaccine doses was at its lowest level.

3.4. Whole-genome sequencing

A total of 2613 (16.6% of total samples included in the study) strains were sequenced by next-generation sequencing. Among these, 670 were "*early variants*," 177 Alpha variant strains, 653 Delta, and 747 Omicron strains. Success rates of next-generation

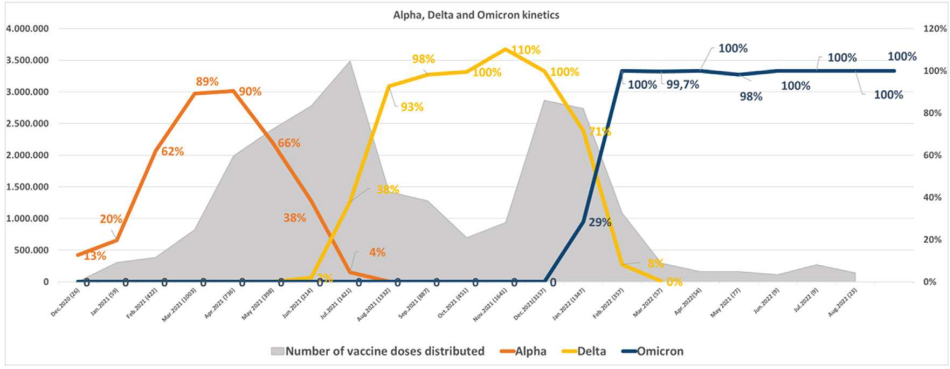

Fig. 2. Growth rates and kinetics of Alpha (orange), Delta (yellow), and Omicron (dark blue) cases. The grey area represents the number of distributed vaccine doses. Together with new amino acid changes and competition between variants, the decrease in the number of doses distributed contributed to the circulation of new variants.

sequencing for each of the main variants analyzed are reported in Supplementary Table S2.

According to the pattern of mutations for each *Variant of Concern*, in Alpha strains, N501Y was present in all the sequences. The most frequent mutations were A570D and T76I, P681H, D614G, and S982A, which were harbored by 90% of all Alpha cases. Interestingly, 18.6% of all Alpha sequences harbored E484K mutation.

Delta strains presented D614G, P681R, D950N, T478K, T19R, L452R, and G142D in more than 75% of sequences. Notably, 42.6% of all Delta sequences harbored G142D that will be later identified in Omicron strains, and none of them harbored E484K mutation. Moreover, about 40% of all Delta sequences harbored EFR156G deletions.

A total of 747 strains belonging to the Omicron clade were sequenced. The first Omicron lineage identified was the B.1.1.529 followed by BA.1, BA.2, BA.4, BA.5, BE.x, and BF.x. Overall, we observed 147 mutations in Spike protein; among these, 30/147 (20.4%) occurred in the receptor-binding domain. N501Y was identified from 21.4% for BA.4 sequences to 50.2% for BA.1 sequences. None of the Omicron strains harbored the substitution E484K, but in 37.2% of BA.2 sequences generated the glutamic acid residue in position 484 was substituted with alanine. Interestingly, H655Y mutation, that to date had been reported only in Gamma strains, was identified in more than 80% of the sequences.

Supplementary Table S3 summarizes all nonsynonymous nucleotide changes detected for each Omicron lineage. Moreover, the amino acid deletions identified are reported in Supplementary Table S4. In both tables, the number of cases is expressed as a percentage value.

4. Discussion

At the beginning of the surveillance period in September 2020, the pandemic in Lombardy was sustained by multiple lineages. This finding was already observed in the first weeks after the first SARS-CoV-2 detection in Italy, as described by Alteri et al. [1]. In particular, we observed the circulation of at least 25 different lineages, also suggesting that the restoration of national and international connections after the lockdown period promoted viral evolution and circulation. Overall, the epidemiological scenario in Lombardy reflected the national one. After its first detection, the Alpha variant spread rapidly all over Europe [12–13]. Indeed, it caused less than 15% of the cases in December 2020 but was identified in almost all the positive cases between January and March 2021, becoming the predominant variant. This means that all new mutations harbored in the Alpha variant, including N501Y, allowed a greater diffusion rate for this variant. Our data are consistent with those reported by Lai et al. [14] about other Italian regions. On the contrary, other variants like Beta or Gamma did not spread as easily as Alpha and caused only small clusters in Milan and Cremona provinces while in other countries they caused very large clusters [15]. In May 2021, the first cases of Delta variants were detected. By that time, most of the Italian population had received at least one dose of anti-SARS-CoV-2 vaccination. However, Delta circulation was rapid and widespread. Thus, the hypothesis was that the Delta variant could escape the host vaccine-induced immune response, as reported by Cassaniti et al [16]. Since the beginning of 2022, the Omicron variant and its sublineages were the last ones detected. The first isolation was in December 2021. As shown in Fig. 2, the kinetics of Alpha, Delta, and Omicron cases is inversely proportional to the number of distributed vaccine doses. In Italy, the vaccination campaign started on December 27, 2020 for healthcare workers; at the beginning of February 2021, vaccination was available for the general population starting from elderly people with a peak of doses distributed between June and July 2021. A new peak of vaccine distribution was recorded at the end of 2021, between November 2021 and January 2022 (regione.lombardia.it/wps/portal/istituzionale/HP/vaccinazionicovid/dashboard-vaccini). In this context, the reduction of vaccine coverage (grey area) together with other important factors such as the competition between variants promoted the circulation of the three main variants (Alpha in December 2020, Delta in May 2021, and Omicron in December 2021).

To date, all of the SARS-CoV-2 infections are due to Omicron and, in particular, to BA.4 and BA.5, as also demonstrated by other groups [17–19]. Moreover, next-generation sequencing of Omicron variants revealed 147 mutations occurring in different sub-lineages with different combinations and percentages.

This study has several limitations. It was conducted both retrospectively and prospectively. Patients included in this study were referred to several hospitals of every Lombardy province; thus, retrieving any clinical data was impossible in particular for outpatients. Real-time PCR assays performed in our laboratory for variant identification did not discriminate among variants with similar mutation pattern; moreover, *Spike* sequencing was possible only for samples with Ct lower than 30. For this reason, 10% of genotyping results are not available. Due to the epidemiological nature of the study, genetic results obtained by next-generation sequencing were discussed briefly.

5. Conclusions

In this study, insight into the circulation of SARS-CoV-2 variants in the Lombardy region during 2 years was reported. We observed the circulation of new variants every few months during the study period: each of them harbored new mutations that allowed them to become predominant thanks to higher transmissibility and the capacity for immune escape. Fortunately, not all of them have been associated with higher severity of the clinical manifestations [20–21]. From this perspective, the introduction of vaccination caused a reduction in severe cases.

From a future perspective, it would be very important to retrieve as many as possible clinical records from the patients included in this study to try to establish a correlation between the most frequent mutations and the clinical course of the infections.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

FG: Conceptualization, validation, data analysis, investigation, resources, data curation, writing - original draft. GF, FZ: samples collection, testing, data curation, sequencing, data analysis. SP, FR, GC: data curation. EP, LP: samples collection, testing, data curation, sequencing, data analysis. CG, FB, AN, EM, SR: samples collection, testing. DF, VC, CA, FS, CV, CFP: supervision, data curation. AP: Conceptualization, supervision, revision, and editing of the final draft. FB: Conceptualization, project administration, funding acquisition.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.diagmicrobio.2023.116070.

References

[1] Alteri C, Cento V, Piralla A, Costabile V, Tallarita M, Colagrossi L, et al. Genomic epidemiology of SARS-CoV-2 reveals multiple lineages and early spread of SARS-CoV-2 infections in Lombardy, Italy. Nat Commun 2021;12(1):434. doi: 10.1038/s41467-020-20688-x.

[2] Tallarita M, Giardina F, Novazzi F, Gaiarsa S, Batisti Biffignandi G, Paolucci S, et al. Spread of multiple SARS-CoV-2 lineages April-August 2020 anticipated the second pandemic wave in Lombardy (Italy). Pediatr Allergy Immunol 2022;33(Suppl 27):89–92. doi: 10.1111/pai.13641.

[3] Korber B, Fischer WM, Gnanakaran S, Yoon H, Theiler J, Abfalterer W, et al. Tracking changes in SARS-CoV-2 spike: evidence that D614G increases infectivity of the COVID-19 virus. Cell 2020;182(4):812–827.e19. doi: 10.1016/j.cell.2020.06.043.

[4] Rambaut A, Loman N, Pybus O, Barclay W, Barrett J, Carabelli A, et al. Preliminary genomic characterisation of an emergent SARS-CoV-2 lineage in the UK defined by a novel set of spike mutations. https://virological.org/t/preliminary-genomic-characterisation-ofan-emergent-sars-cov-2-lineage-in-the-uk-defined-by-a-novelset-of-spike-mutations/563 2020 (Accessed May 1, 2022).

[5] Tegally H, Wilkinson E, Giovanetti M, Iranzadeh A, Fonseca V, Giandhari J, et al. Detection of a SARS-CoV-2 variant of concern in South Africa. Nature 2021;592(7854):438–43. doi: 10.1038/s41586-021-03402-9.

[6] Faria NR, Mellan TA, Whittaker C, Claro IM, Candido DDS, Mishra S, et al. Genomics and epidemiology of the P.1 SARS-CoV-2 lineage in Manaus, Brazil. Science 2021;372(6544):815–21. doi: 10.1126/science.abh2644.

[7] Singh J, Rahman SA, Ehtesham NZ, Hira S, Hasnain SE. SARS-CoV-2 variants of concern are emerging in India. Nat Med 2021;27(7):1131–3. doi: 10.1038/s41591-021-01397-4.

[8] Hoffmann M, Arora P, Groß R, Seidel A, Hörnich BF, Hahn AS, et al. SARS-CoV-2 variants B.1.351 and P.1 escape from neutralizing antibodies. Cell 2021;184(9):2384–2393.e12. doi: 10.1016/j.cell.2021.03.036.

[9] Tian D, Sun Y, Zhou J, Ye Q. The global epidemic of the SARS-CoV-2 Delta variant, key spike mutations and immune escape. Front Immunol 2021;12:751778. doi: 10.3389/fimmu.2021.751778.

[10] Italian Ministry of Health. Aggiornamento sulla diffusione a livello globale delle nuove variant SARS-CoV-2, valutazione del rischio e misure di controllo. 2021. 0003787-31/01/2021-DGPRE-DGPRE-P.

[11] Gaiarsa S, Giardina F, Batisti Biffignandi G, Ferrari G, Piazza A, Tallarita M, et al. Comparative analysis of SARS-CoV-2 quasispecies in the upper and lower respiratory tract shows an ongoing evolution in the spike cleavage site. Virus Res 2022;315:198786. doi: 10.1016/j.virusres.2022.198786.

[12] Rapid spread of the SARS-CoV-2 Delta variant in some French regions, June 2021. Euro Surveill 2021;26(28):2100573. doi: 10.2807/1560-7917.ES.2021.26.28.2100573.

[13] Lindstrøm JC, Engebretsen S, Kristoffersen AB, Rø GØl, Palomares AD, Engø-Monsen K, et al. Increased transmissibility of the alpha SARS-CoV-2 variant: evidence from contact tracing data in Oslo, January to February 2021. Infect Dis (Lond) 2022;54(1):72–7. doi: 10.1080/23744235.2021.1977382.

[14] Lai A, Bergna A, Menzo S, Zehender G, Caucci S, Ghisetti V, et al. Circulating SARS-CoV-2 variants in Italy, October 2020-March 2021. Virol J 2021;18(1):168. doi: 10.1186/s12985-021-01638-5.

[15] Umair M, Ikram A, Salman M, Haider SA, Badar N. Rehman Z,et al. Genomic surveillance reveals the detection of SARS-CoV-2 Delta, Beta, and Gamma VOCs during the third wave in Pakistan. J Med Virol 2022;94(3):1115–29. doi: 10.1002/jmv.27429.

[16] Cassaniti I, Bergami F, Percivalle E, Gabanti E, Sammartino JC, Ferrari A, et al. Humoral and cell-mediated response against SARS-CoV-2 variants elicited by mRNA vaccine BNT162b2 in healthcare workers: a longitudinal observational study. Clin Microbiol Infect 2022;28(2):301.e1–8. doi: 10.1016/j.cmi.2021.09.016.

[17] Tegally H, Moir M, Everatt J, Giovanetti M, Scheepers C, Wilkinson E, et al. Emergence of SARS-CoV-2 Omicron lineages BA.4 and BA.5 in South Africa. Nat Med 2022;28(9):1785–90. doi: 10.1038/s41591-022-01911-2.

[18] Phan T, Boes S, McCullough M, Gribschaw J, Marsh JW, Harrison LH, et al. Emergence of SARS-CoV-2 Omicron BA.5 variant of concern in Western Pennsylvania, United States. J Med Virol 2022;94(10):4593–4. doi: 10.1002/jmv.27945.

[19] Li A, Yung A, Tran C, Boulet M. SARS-CoV-2 Omicron BA.5: riding the seventh wave in Central Canada. Influenza Other Respir Viruses 2022;16(6):1202–4. doi: 10.1111/irv.13046.

[20] Dao TL, Hoang VT, Colson P, Lagier JC, Million M, Raoult D, et al. SARS-CoV-2 infectivity and severity of COVID-19 according to SARS-CoV-2 variants: current evidence. J Clin Med 2021;10(12):2635. doi: 10.3390/jcm10122635.

[21] Nagy A, Pongor S, Győrffy B. Different mutations in SARS-CoV-2 associate with severe and mild outcome. Int J Antimicrob Agents 2021;57(2):106272. doi: 10.1016/j.ijantimicag.2020.106272.

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SUPPLEMENT ARTICLE

WILEY

SARS-CoV-2 infections in pediatric patients: A comparison of three pandemic waves

Francesca Rovida[1] | Federica Giardina[1] | Guglielmo Ferrari[1] | Stefania Paolucci[1] | Antonio Piralla[1] | Fausto Baldanti[1,2]

[1]Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

[2]Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy

Correspondence
Antonio Piralla, PhD Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Via Taramelli 5, Pavia 27100, Italy.
Email: a.piralla@smatteo.pv.it

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Abstract

The pediatric population seems to be at a lower risk of developing severe clinical symptoms of COVID-19. However, the clinical and epidemiological characteristics of COVID-19 in children are yet to be fully clarified. This retrospective observational study aimed to evaluate the frequency of pediatric laboratory-confirmed COVID-19 patients from February 2020 to April 2021. A total of 740 (5.1% of total) pediatric COVID-19 cases were observed during the study period. The peak of pediatric cases was observed in November 2020, with 239 cases. During the first wave of pandemics, the frequency of pediatric cases was 0.89% (49/5877 cases), ranging from 0.6% in February 2020 to 1.3% in April 2020. On the contrary, after the beginning of the second wave, the frequency of pediatric cases raised from 5.3% in September 2020 to 9.4%in February 2021, with an overall frequency of 8.2% (690/8416 cases). A different rate of SARS-CoV-2 circulation was observed among the pediatric population between the pandemic waves. During the second wave, two peaks of cases were observed. The last peak was associated with the spread of a more transmissive SARS-CoV-2 strain (VOC 202012/01).

KEYWORDS
COVID-19, epidemiology, pandemic, pediatric infection, SARS-CoV-2

1 | INTRODUCTION

Since coronavirus disease 2019 (COVID-19) was initially reported in China on December 30, 2019, SARS-CoV-2 spread worldwide.[1,2] As of April 12, 2021, 135 million confirmed infections and near to 3 million deaths have been reported worldwide.[3] At the beginning of the Pandemic, Lombardy was the Italian region most affected by the SARS-CoV-2 virus.[4] Generally, viral respiratory infections are primarily concentrated in the pediatric population. However, as early observed during the first pandemic wave, the pediatric population has not been mainly involved, and data on the effective burden of the pandemic in the pediatric population are very limited. Compared to adults, there are relatively few studies on pediatric COVID-19.[5–7] In particular, the clinical and epidemiological characteristics of COVID-19 in children aged 0–16 years are yet to be fully clarified. In the scenario of the rapid worldwide spread of SARS-CoV-2 infection, the rising number of pediatric cases is to be expected. This retrospective analysis aimed to evaluate the frequency of pediatric infection among COVID-19 cases diagnosed in our center, since the beginning of the Pandemic.

A total of 14353 COVID-19 cases have been detected at our Regional Reference Laboratory (Molecular Virology Unit,

Fondazione IRCCS Policlinico San Matteo Pavia, Italy) between February 20, 2020 and April 12, 2021. The presence of SARS-CoV-2 RNA was assessed using specific real-time RT-PCR as previously described.[8] Among COVID-19 cases, 740 (5.1% of total) were observed in patients <15 years, with 397 (53.6%) male and 343 (46.4%) female. As showed in Figure 1A, the peak of pediatric cases was observed in November 2020 with 239 cases. After December 2020, an overall decrease of cases was observed until a second peak observed in March 2021 with 138 cases. On the contrary, during the first wave of pandemic (February-June 2020), the peak of pediatric cases was observed in March 2020, with 28 cases

Key message

Overall, only 5% of COVID-19 cases accounted for patients <15 years. Two different rates of SARS-CoV-2 circulation were observed among the pediatric population between the pandemic waves. During the second wave, two peaks of cases were observed. The last peak was associated with the spread of a more transmissive SARS-CoV-2 strain (VOC 202012/01).

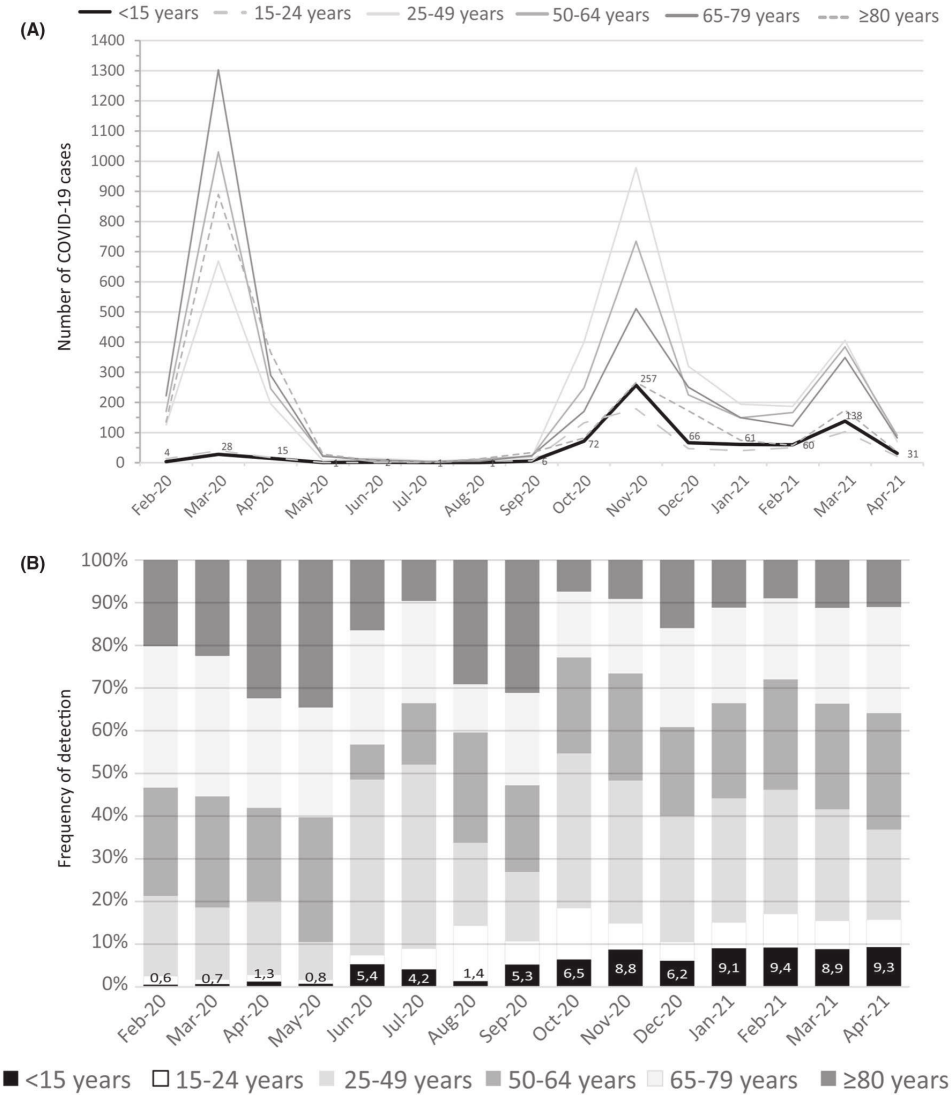

FIGURE 1 The number of COVID-19 cases according to age category between February 2020 and April 2021 (A). The number of pediatric cases (<15 years) is reported near the peak for each month. Frequency of SARS-CoV-2 cases detection according to age category (B). The percentage of pediatric cases (<15 years) is reported within the bars for each month

corresponding to the month with the most significant number of cases (n = 3960) observed in our hospital (Figure 1A). Between May 2020 and September 2020, less than 10 cases/month were observed, according to the reduced circulation of SARS-CoV-2 in Lombardy. During the first wave of pandemics, the frequency of pediatric cases was 0.89% (49/5877 cases), ranging from 0.6% in February 2020 to 1.3% in April 2020 (Figure 1B). On the contrary, after the beginning of the second wave, the frequency of pediatric cases raised from 5.3% in September 2020 to 9.4%in February 2021, with an overall frequency of 8.2% (690/8416 cases) (Figure 1B).

Despite the evidence of the wide circulation of other respiratory viruses (eg, influenza and respiratory syncytial virus) among the pediatric population in our study, only 5% of COVID-19 cases accounted for patients <15 years. In addition, two different rates of circulation were observed comparing first and second pandemic waves (<1% vs. 8.2%). This difference could be explained by the changing containing measures implemented during the lockdown on March-May 2020 compared to the second wave (September 2020 – April 2021). As previously observed, most pediatric patients were asymptomatic and can spread the infection to their family members, some of whom develop severe symptoms.[9] Thus, in this context, it is expected that the rates of circulation observed have been different.

In our analysis, three peaks of cases were observed. The first on March 2020 was supported by the uncontrolled circulation of SARS-CoV-2 probably present in our Region since January 2020.[10] The second peak was observed during the second wave started in September 2020, where a global increase in the number of newly reported cases was seen in the European and Eastern countries.[3] Lastly, after introducing the SARS-CoV-2 variant of concern (VOC) 202012/01 (alpha, lineage B.1.1.7) in January 2020 in Italy, a third peak of cases on March 2021 was observed. The occurrence of a third peak was supported by the circulation of VOC 202012/01 associated with an increased rate of transmission.[11]

In conclusion, different rates of SARS-CoV-2 circulation were observed among the pediatric population between the pandemic waves. During the second wave, two peaks of cases were observed. The last peak was associated with the spread of a more transmissive SARS-CoV-2 strain (VOC 202012/01).

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CONFLICT OF INTEREST

All authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Francesca Rovida: Conceptualization (equal); Supervision (lead); Writing-review & editing (equal). **Federica Giardina**: Methodology (equal), Writing-review & editing (equal). **Guglielmo Ferrari:** Methodology (equal), Writing-review & editing (equal). **Federica Novazzi**: Methodology (equal), Writing-review & editing (equal). **Stefania Paolucci**: Methodology (equal), Writing-review & editing (equal). **Antonio Piralla:** Conceptualization (equal); Supervision (lead); Writing-review & editing (equal). **Fausto Baldanti**: Conceptualization (equal); Supervision (equal); Writing-review & editing (equal).

ORCID

Francesca Rovida https://orcid.org/0000-0003-2345-2790
Federica Giardina https://orcid.org/0000-0001-6559-8099
Stefania Paolucci https://orcid.org/0000-0001-8447-8586
Antonio Piralla https://orcid.org/0000-0002-6062-2579
Fausto Baldanti https://orcid.org/0000-0002-3358-8969

REFERENCES

1. Dong E, Du H, Gardner L. An interactive web-based dashboard to track COVID-19 in real time. *Lancet Infect Dis*. 2020;20:533-534.
2. Wu F, Zhao S, Yu B, et al. A new coronavirus associated with human respiratory disease in China. *Nature*. 2020;579:265-269.
3. WHO Coronavirus (COVID-19) Dashboard (Accessed 15th April 2021). https://covid19.who.int
4. Riccardo F, Ajelli M, Andrianou XD, et al. Epidemiological characteristics of COVID-19 cases and estimates of the reproductive numbers 1 month into the epidemic, Italy, 28th January to 31st March 2020. *Euro Surveill*. 2020;25:2000790.
5. Wei M, Yuan J, Liu Y, et al. Novel coronavirus infection in hospitalized infants under 1 year of age in China. *JAMA*. 2020;323:1313-1314.
6. Dong Y, Mo X, Hu Y, et al. Epidemiology of COVID-19 among children in China. *Pediatrics*. 2020;145:e20200702.
7. Ma H, Hu J, Tian J, et al. A single-center, retrospective study of COVID-19 features in children: a descriptive investigation. *BMC Med*. 2020;18:123.
8. Rovida F, Cereda D, Novati S, et al. Low risk for SARS-CoV2 symptomatic infection and early complications in paediatric patients during the ongoing CoVID19 epidemics in Lombardy. *Clin Microbiol Infect*. 2020;26:1569-1571.
9. Castagnoli R, Votto M, Licari A, et al. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in children and adolescents: a systematic review. *JAMA Pediatr*. 2020;174:882-889.
10. Alteri C, Cento V, Piralla A, et al. Genomic epidemiology of SARS-CoV-2 reveals multiple lineages and early spread of SARS-CoV-2 infections in Lombardy. *Italy. Nat Commun*. 2021;12:434.
11. Davies NG, Abbott S, Barnard RC, et al. Estimated transmissibility and impact of SARS-CoV-2 lineage B.1.1.7 in England. *Science*. 2021;372.

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Comparative analysis of SARS-CoV-2 quasispecies in the upper and lower respiratory tract shows an ongoing evolution in the spike cleavage site

Stefano Gaiarsa [a,1], Federica Giardina [a,1], Gherard Batisti Biffignandi [b,1], Guglielmo Ferrari [a], Aurora Piazza [b], Monica Tallarita [a], Federica Novazzi [a], Claudio Bandi [c], Stefania Paolucci [a], Francesca Rovida [a,b], Giulia Campanini [a], Antonio Piralla [a,*], Fausto Baldanti [a,b]

[a] Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Via Tamelli 5, Pavia 27100, Italy
[b] Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy
[c] Department of Biosciences and Pediatric Clinical Research Center "Romeo ed Enrica Invernizzi", University of Milan, Milan, Italy

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ABSTRACT

Studies are needed to better understand the genomic evolution of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This study aimed to describe viral quasispecies population of upper and lower respiratory tract by next-generation sequencing in patients admitted to intensive care unit. A deep sequencing of the S gene of SARS-CoV-2 from 109 clinical specimens, sampled from the upper respiratory tract (URT) and lower respiratory tract (LRT) of 77 patients was performed. A higher incidence of non-synonymous mutations and indels was observed in the LRT among minority variants. This might be explained by the ability of the virus to invade cells without interacting with ACE2 (e.g. exploiting macrophage phagocytosis). Minority variants are highly concentrated around the gene portion encoding for the Spike cleavage site, with a higher incidence in the URT; four mutations are highly recurring among samples and were found associated with the URT. Interestingly, 55.8% of minority variants detected in this locus were T>G and G>T transversions. Results from this study evidenced the presence of selective pressure and suggest that an evolutionary process is still ongoing in one of the crucial sites of spike protein associated with the spillover to humans.

1. Introduction

Since coronavirus disease 2019 (COVID-19) was initially reported in China on 30th December 2019 (Dong et al., 2020; Wu et al., 2020), SARS-CoV-2 has been spreading worldwide. As of 8th March 2022, there have been 447 million confirmed infections and more than 6 million deaths have been reported worldwide (Dong et al., 2020, https://ourworldindata.org/coronavirus). The origin of SARS-CoV-2 is still debated but a hypothesis suggests the Malayan pangolins (*Manis javanica*) to be the possible intermediate host for the virus and recombination signals between pangolin, bat and human coronavirus sequences have been identified (Lam et al., 2020; Wong et al., 2020; Wu et al., 2020; Xiao et al., 2020). In fact, the SARS-CoV-2 genome sequence showed a high percentage of genomic identity (around 96%) with BatCoV-RaTG13 virus as well as (around 88%) with two other SARS-like bat viruses (Bat-SL-CoV-ZC45 and Bat-SL-CoV-ZXC21) (Zhou et al., 2020). Instead, the comparison with the SARS-CoV genome sequences showed an overall lower identity, approximately 79.6% (Gralinski and Menachery 2020; Zhou et al., 2020). Similar identity scores were observed when comparison analyzes were focused on the Spike (S) protein (around 75%) (Gralinski and Menachery 2020; Zhou et al., 2020). The S protein is the main determinant of viral tropism and is responsible for receptor binding and membrane fusion (Belouzard et al., 2012). For this reason, amino acid changes on this protein might have effects on infectivity, viral pathogenesis as well as transmissibility. It was initially reported that mutation D614G in the S protein, which has emerged and has become dominant, might have induced an enhancement of viral replication and viral fitness (Shi et al., 2020). Monitoring of emerging mutations, especially in the S protein, has been performed extensively with the establishment of Virus Evolution Expert Working Group (VEWG) by the WHO (World Health Organization WHO, 2021). The great effort on this concern is also highlighted by the huge number of SARS-CoV-2

* Corresponding author.
E-mail address: a.piralla@smatteo.pv.it (A. Piralla).
[1] These authors contributed equally to this work.

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sequences submitted on public repositories (e.g. GISAID). Initial report suggested a little viral diversity for SARS-CoV-2 (Karamitros et al., 2020; Simmonds, 2020), however, since December 2020 a process of positive selection with presumed advantages such as increased transmission rates has been documented for a series of variants of concern (VOCs) such as alpha, delta and omicron (Harvey et al., 2021; Tao et al., 2021; Volz et al., 2021). These VOCs have demonstrated a significant public health impact, with changes in the virus transmissibility and reduce the efficacy of vaccines (Harvey et al., 2021; Tao et al., 2021). Quasispecies is believed to be a strategy of virus evolution (Domingo and Perales, 2019) and although the viral kinetics of SARS-CoV-2 infection from the upper respiratory tract (URT) to the lower respiratory tract (LRT) have been gradually clarified more work is still needed to explore the inter-host and intra-host variations of SARS-CoV-2. Overall, studies aimed at investigating intra-host evolution or the dynamic of SARS-CoV-2 quasispecies have been mainly focused on samples collected from upper respiratory tract (URT) (Al Khatib et al., 2020; Jary et al., 2020; Pérez-Lago et al., 2021; Shen et al., 2020; Siqueira et al., 2020; Sun et al., 2021; To et al., 2020). In fact, the dynamic of SARS-CoV-2 population in the lower respiratory tract (LRT) of patients showing severe acute respiratory infections (SARIs) is poorly investigated. It would be important to elucidate the role of specific mutations in the progression of SARS-CoV-2 from upper to lower respiratory tract or identify specific mutational patterns associated with severe infection. In the present study, high-depth next-generation sequencing (NGS) of the S gene was performed in a set of 109 respiratory samples from URT ($n = 58$) and LRT ($n = 51$) in order to: (i) evaluate the genetic diversity in two different body compartments; (ii) identify minority variants potentially associated with progression from upper to lower respiratory tract on paired samples from patients admitted to intensive care unit (ICU) with severe infection.

2. Material and methods

2.1. Patients and samples

A total of 109 clinical specimens from URT (nasopharyngeal swabs; NPS) and LRT (bronchoalveolar lavage; BAL or broncho aspirate; Brasp) were collected and analyzed from 77 COVID-19-positive patients (Appendix Table S1). Respiratory samples were prospectively collected from patients hospitalized at ICUwith severe to critical COVID-19 disease and from patients with mild symptoms not requiring hospitalization according to the WHO clinical management of COVID-19 guide (World Health Organization WHOa, 2020). Among patients admitted to ICU with severe to critical COVID-19 infection, whenever possible, paired URT and LRT samples were collected. All specimens were collected from late February 2020 to January 2021 at the Microbiology and Virology Department of Fondazione IRCCS Policlinico San Matteo in Pavia as Regional Reference laboratory for COVID-19 diagnosis. The presence of SARS-CoV-2 RNA in respiratory specimens was assessed by using specific real-time reverse transcriptase–polymerase chain reaction (RT-PCR) targeting the RNA-dependent RNA polymerase and E genes, following the WHO guidelines and the Corman et colleagues' protocols (Corman et al., 2020; World Health Organization WHOb, 2020). Quantification cycle (Cq) values according to MIQE guidelines (Bustin et al., 2009), were used as a semiquantitative measure of SARS-CoV-2 viral load. The sequence investigation of patient samples was approved by the Ethics Committee of our institution (P_20200085574).

2.2. S gene amplification and sequencing

Total RNA was extracted using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions using a starting volume of 400 μL elute in a final volume of 60 μL.

The extracted RNA was subjected to a one-step RT-PCR using the SuperScript IV One-Step RT-PCR System (Thermo Fisher Scientific, USA). Two strategies were adopted: a "long PCR" for the amplification of the entire S gene (near 4000 bp) using the primer pairs SARS-2-S-F3 (tatcttggcaaaccacgcgaacaa) and SARS-2-S-R3 (acccttggagagtgctagttgccatctc) or using a semi-nested approach with the following two primers pairs: the first step with SARS-2-S-F3 and SARS-2-R6 (ttctgcaccaagtgacatagtgtaggca), followed by a second step with SARS-2-F6 (tcaggatgttaactgcacagaagtcc) and SARS-2-S-R3 (complete list of primers and their position are reported on Appendix Table S2). The thermal profile for the retro transcription was 55 °C for 10 min, followed by the "long PCR" with an initial denaturation/RT inactivation step at 98 °C for 2 min, the amplification for 42 cycles including the initial denaturation (98 °C for 10 s), the annealing step at 60 °C for 10 s, and the extension at 72 °C for 3 min; a final extension of 5 min at 72 °C. The semi-nested PCR was performed using the Platinum SuperFi DNA Polymerase with 5 μl of the first-step DNA and the following thermal program: 98 °C for 30 s, the amplification for 40 cycles including the initial denaturation (98 °C for 10 s), the annealing step at 60 °C for 10 s, and the extension at 72 °C for 2 min and 30 s. The proper PCR products were purified with AMPure Beads, with elution in TE buffer. Enriched DNA samples were used to prepare sequencing libraries with the Nextera XT kit. Sequencing was performed on an Illumina MiSeq machine, aiming for ~1,000,000 250 bp paired-end reads per sample.

2.3. In silico analysis of sequences

Quality control of sequencing reads was performed using the program FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads in each sample were filtered and trimmed for quality using fastp (Chen et al., 2018). In addition, 28 bases were cut from each end of all reads, to remove sequences generated from the semi-nested PCR primers. Filtered reads were aligned to the S gene of the Wuhan-hu-1 reference genome (NC_045512.2) (Wu et al., 2020) using bowtie2 (Langmead and Salzberg, 2012). Haplotype sequences for each sample were obtained from the alignment SAM files, using the software CliqueSNV (Knyazev et al., 2018). The hedgehog algorithm was used on the most abundant haplotype of each sample to classify the SARS-CoV-2 strains using only the S protein sequences (O'Toole et al., 2022). In parallel, alignment data was processed with samtools (Li et al., 2009) and bam-readcount (https://github.com/genome/bam-readcount) in order to calculate the number of occurrences of each nucleotide and indel in all positions of the reference. Only nucleotides and indels with at least 1% prevalence were considered in the following analyzes.

Python and R scripting (scripts are available from github link) was used to extract and classify all mutations using the following algorithm:

(a) For each position in all samples, the nucleotide or indel with the highest prevalence was called the "majority variant".
(b) All other bases with at least 1% prevalence were called "minority variants" (MVs).
(c) The correlation between the presence of MVs and the respiratory tract district of sampling was tested for all positions of the gene using the Fisher exact test ($p < 0.05$).
(d) MVs in each sample were counted and classified by mutation type (synonymous, non-synonymous or indel), gene sub-domain (Huang et al., 2020), and mutation pattern (from which majority base to which low prevalence base). The differential distribution of the number of MVs between URT and LRT samples was tested using the Wilcoxon rank sum test ($p < 0.05$). The test was repeated for all classes determined, weighting each count on the total number of MVs of the sample.

This allowed us to test their association with LRT or URT (Fisher exact test) and to measure the gene variability sampled in this work. Sequencing reads are available on the SRA database under BioProject ID PRJNA686083. The scripts generated to perform this project are available on GitHub at https://github.com/SteMIDIfactory/DeepSpike.

2.4. Statistical analyzes

Comparisons of Cq, number of minority variants and haplotypes were performed with Mann-Witney test for continuous unpaired variables and in paired respiratory samples with the Wilcoxon rank sum test for continuous paired variables. Correlations between two quantitative variables were measured by the Spearman correlation test. Fisher's exact test for categorical variables was used for analysing mutation frequencies between groups of patients. Descriptive statistics and linear regression lines were performed using Graph Pad Prism software (version 8.3.0).

3. Results

3.1. Patients population

A total of 77 patients were included in the study. Fifty-five (71.4%) of them had severe presentations and were admitted to the ICU, while 22 (28.6%) had mild infections not requiring hospitalization. Among 55 ICU patients, for 28 (50.9%) of them paired URT and LRT samples were available, for 19 (34.5%) patients only LRT samples were analyzed (two of them with two and one with three serially collected BAL, respectively) and for 8 (14.6%) patients only URT samples were available. Among 28 patients with paired URT and LRT samples, the LRT sample was collected at the same time for a great majority of paired samples (range -4 to 9 days of difference). From the 22 patients with mild disease only URT samples were collected and analyzed.

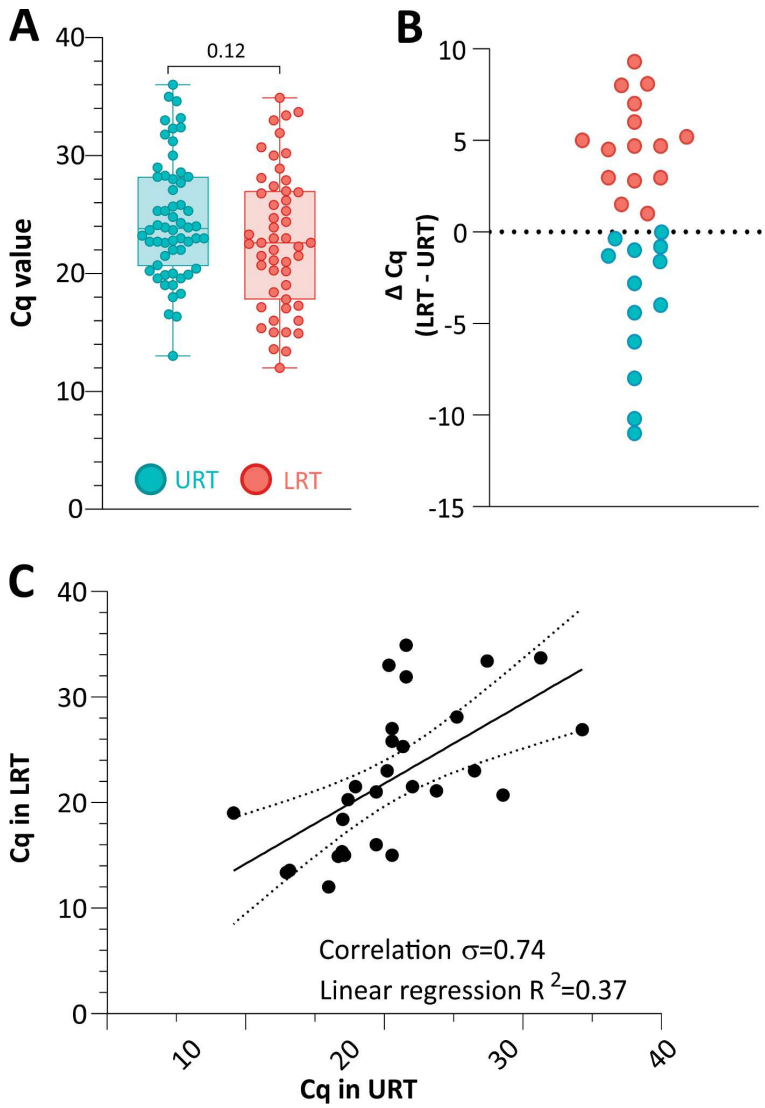

Fig. 1. (A) Distribution of Cq in the URT and LRT samples. (B, C) Differences and correlation plots for Cq values in 28 paired URT and LRT samples. The statistic Spearman's correlation coefficient and linear regression R^2 value are also reported.

3.2. Dataset description

A total of 109 samples were included in the NGS analyzes, of which 58 (53.2%) were collected from the URT and 51 (46.8%) from the LRT. The DNA of the S gene was enriched with PCR methods and deep short-read sequencing was performed. A total of 69279628 reads were obtained from sequencing, with an average of 635593 reads for sample (range 56214-3346036). Reads were mapped to the SARS-CoV-2 reference strain and the average depth obtained was 6923x (range 1301x-7954x). Reads mapping allowed to extract, classify, and count all genomic variants present in the samples with a prevalence of at least 1% of the sequencing depth (corresponding to ~10x depth in the samples with the worst read yield).

The hedgehog algorithm was used in order to classify the SARS-CoV-2 strains using only the S protein sequences. The great majority of SARS-CoV-2 included in this study belonged to A_1 lineage harbouring mainly the D614G change (84/109; 77.1%). Thirteen (11.9%) strains belonged to B.1.177_1 lineage (A222V, D614G), 7 (6.4%) to alpha VOC (lineage A_9; del69-70, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H), 4 (3.7%) strains to lineage B.1_14 (D614G, D839Y) and 1 (0.9%) strain to lineage B.1.177.52_1 (A222V, D614G, P1162R) (Supplementary Material Table S2)

3.3. Viral load and correlation between Cq and intra-host variability

The median viral load measured as Cq value observed in URT samples (23.8; range 13.0–36.0) was similar to those observed in the LRT samples (22.6; range 12.0-34.9; $p = 0.12$) (Fig. 1A). Similarly, among 28 paired samples no difference in the median Cq value was observed ($p = 0.83$). In order to describe the vial load variability on paired samples, we performed a correlation analysis and a plot on difference in Cq value between paired URT and LRT samples. In 13/28 (46.4%) paired samples the Ct value was lower in URT (higher viral load) as compared to LRT samples with a median ΔCq of 2.8 (range 0.04–11.0 ΔCq) (pink circles on Fig.1B), while in 15/28 (53.6%) paired samples, Ct value was lower (higher viral load) in LRT as compared to URT samples with a median

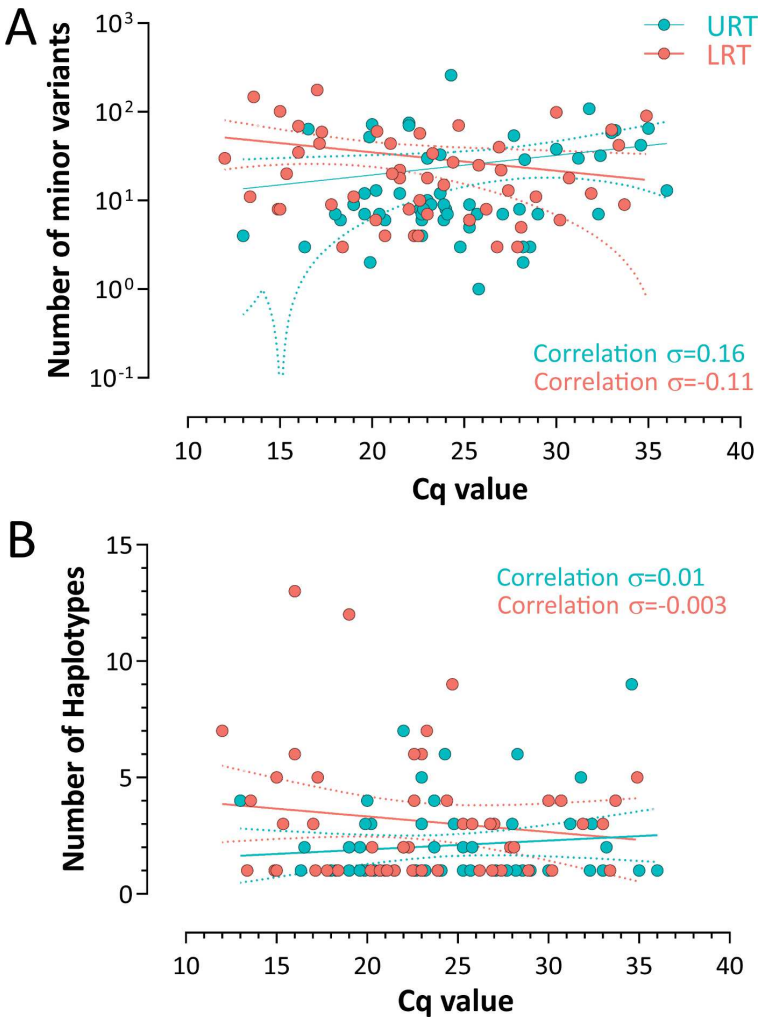

Fig. 2. Comparison of number of haplotypes and minority variants in upper vs lower respiratory tract samples. (A) Correlation between the number of minority variants and viral load expressed in cycle of quantification (Cq). (B) Correlation between the number of haplotypes and viral load expressed in Cq.

ΔCq of 4.7 (range 0.1 to 9.3 ΔCq) (light blue circles on Fig. 1B). In addition, an overall correlation was observed among paired samples (rho=0.74, Fig. 1C).

In general, viral replication has been associated with the intra-host diversification of viral population. For this reason, we compared the Cq values, as expression of the viral load, and the number of MVs and haplotypes observed. No evidence of correlation between Cq and number of MVs in both URT (σ=0.16) and LRT samples (σ = 0.11) was observed (Fig. 2A). Similar findings were observed in the comparison of Cq and the number of haplotypes in URT samples (σ = 0.01) as well as in the LRT (σ = -0.003) (Fig. 2B).

3.4. Haplotype and minority variant counts

The number of MVs is slightly higher in LRT (median 13.5; range 3–99) than in URT samples (median 8; range 0–263), but with no statistical significance (p = 0.07, Fig. 3A). Conversely, the number of haplotypes identified was significatively greater in LRT samples (median 2; range 1–13), compared to URT ones (median 1; range 1-9; p = 0.02, Fig. 3B). The ratio of non-synonymous (dN) to synonymous (dS) substitutions (dN/dS) was calculated. The median value observed in LRT samples (median 2.65, range 0–11) was greater than those observed in UTR samples (median 1.81, range 0–8; p = 0.02), suggesting a higher positive selective pressure in the lung environment (Fig. 3C).

With a more in-depth analysis, the weighted incidence of synonymous mutations was higher in the URT samples compared to LRT samples (p = 0.01, Fig. 3D), while, although not significant, a greater number of indels was observed in the LRT samples as compared to URT samples, (p = 0.10, Fig. 3D). Lastly, no difference was observed in the number of non-synonymous mutations. The analysis was repeated considering only deletions and frameshifting insertions; both of them had greater incidence in the LRT, without significant difference (p = 0.12 and p = 0.13, Fig. 3E).

The weighted incidence of MVs was calculated in all regions of the gene corresponding to the functional and structural domains of the protein. We observed that MVs in the N-terminal Domain (NTD) are more likely to occur in the LRT (p < 0.001). On the other hand, mutations in the Fusion Peptide (FP, p = 0.006) and in Subdomain 2 (SD2, p < 0.001) are more common in the URT (Fig. 4A). Furthermore, MVs in the region coding for the protein subunit S1 are more common in the LRT, while in subunit S2 we measured a significantly higher abundance in the URT (Fig. 4B). This result is expected as it is the reflection of the values observed in the functional domains.

Incidence of mutation patterns were tested as well, both counting the total events, and weighting them on the total number of MVs of each sample. Fig. 4C shows the weighted incidence of each mutation pattern in both respiratory tract compartments, while Appendix Fig. S3 shows the absolute one. Both analyzes show a higher presence of A>C and T>G mutations in the MVs of URT samples, while A>T, T>A, and T>C have a higher incidence in the LRT.

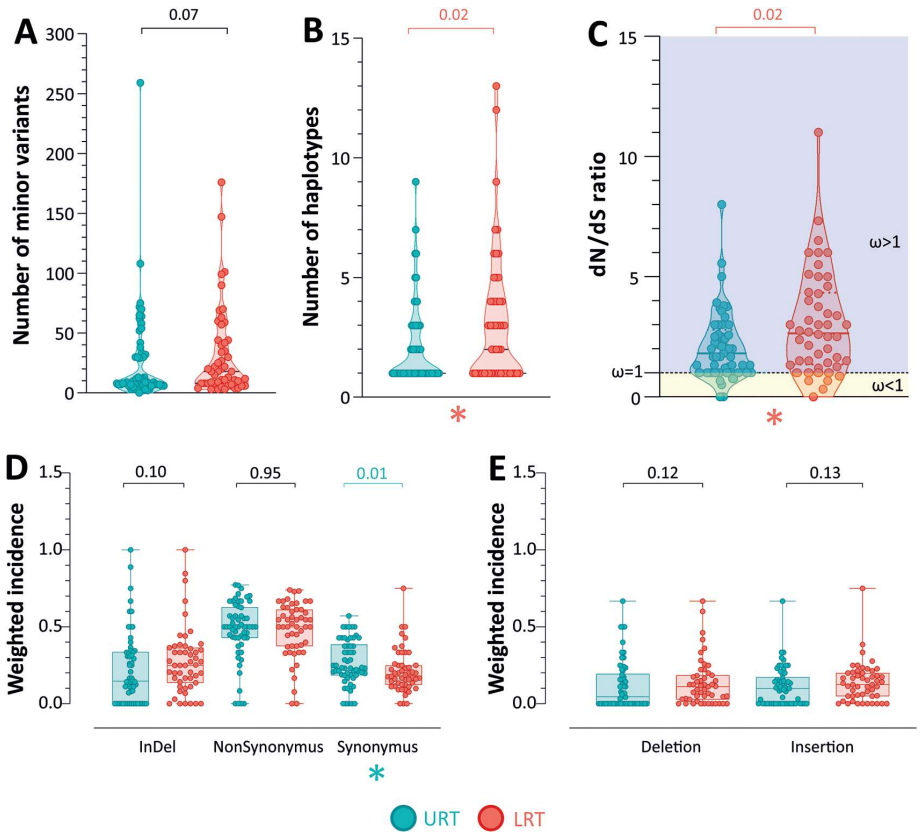

Fig. 3. Distribution of the number of (A) minority variants, (B) haplotypes and (C) dN/dS ratio in the URT and LRT samples. Values are represented as a boxplot with all points inscribed. (D) Distribution of the weighted incidence of synonymous, non-synonymous, and insertions and deletions (Indel) in the URT and the LRT samples. (E) Distribution of the weighted incidence of frameshifting insertions and deletions in the URT and the LRT. Values are weighted by dividing them by the total number of minority variants in the sample.

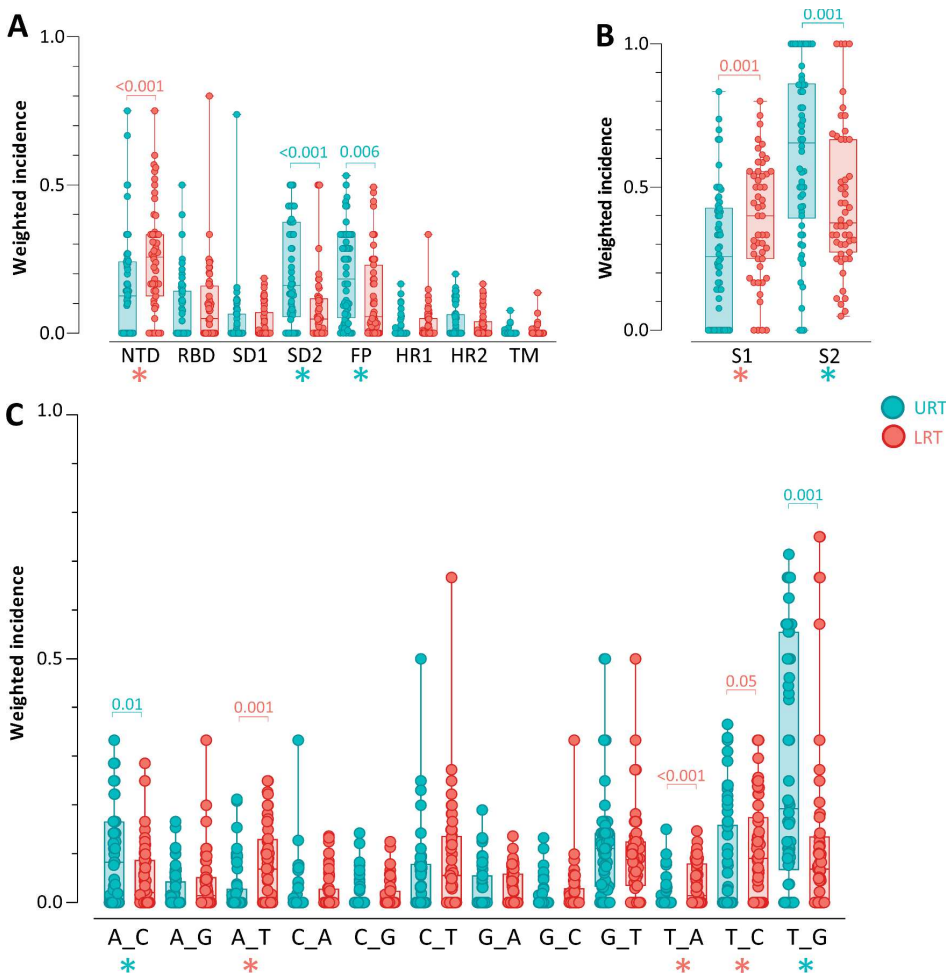

Fig. 4. (A) Distribution of the weighted incidence of minority variants in the S gene subdomains (NTD: N-Terminal Domain; RBD: Receptor-Binding Domain; SD1: Structural Domain 1; SD2: Structural Domain 2; FP: Fusion Peptide; HR1: Heptad Repeat 1; HR2: Heptad Repeat 2; TM: TransMembrane domain) in the URT and LRT samples. (B) Distribution of the weighted incidence of minority variants in the two S gene subunits in the URT and the LRT (S1: Subunit 1; S2: Subunit 2). (C) Distribution in the URT and in the LRT of the weighted incidence of minority variants, classified by mutation patterns. Values are weighted by dividing them by the total number of minority variants in the sample.

Lastly, we tested the correlation in all nucleotide positions between the presence of MVs (binary value) and the two respiratory tract districts (shown in Fig. 5A). Fig. 5B, instead, shows the incidence of MVs along the gene. Mutations are equally distributed on the sequence, with the exception of the area around the cleavage site between the two subunits of the gene. Here mutation sites are highly concentrated both in the upper and in the lower respiratory tract samples, especially in the former, as already seen in Fig. 5A. In addition, the presence of mutations is associated with the respiratory district in nine codons, five correlated with URT, four with LRT (see Table 1). Four of the MVs associated with URT are located around the cleavage site of the two subunits. In this area, we detected a high concentration of MV transversions between T andG: from position 2000 to position 2150, 248 out of 643 (38.6%) mutations are T>G and 11 (17.3%) are G>T (total = 359; 55.8%)..

4. Discussion

The evolution of SARS-CoV-2 was initially quite slow, when compared to other RNA viruses (van Dorp et al., 2020). Yet, its rapid global spread has allowed to record thousands of mutations in public databases; some of those were favourable and have emerged worldwide (Long et al., 2020). The emergence of VOCs was favored by more than 400 million of infections worldwide (Parra-Lucares et al., 2022) with a more significant number of mutations observed in S sequences as compared to other genomic regions (Yusof et al., 2021). Generally, mutations in viral structural such as S glycoprotein can play a crucial role in their virulence by possibly determining changes in their cellular tropism and the generation of antibody escape variants as reported for Delta and Omicron variants (Andrews et al., 2022; Dejnirattisai et al. 2022; Mlcochova et al., 2021). The emergence of these variants has been promoted by the viral quasispecies evolution and the severity of SARS-CoV-2 infection is driven by progression from URT to LRT (Ke et al., 2020). In this perspective, our study has investigated the genetic diversity in SARS-CoV-2 quasispecies focusing on structural S protein sequences in two body compartments in order to: (i) evaluate the genetic diversity in two different body compartments; (ii) identify minority

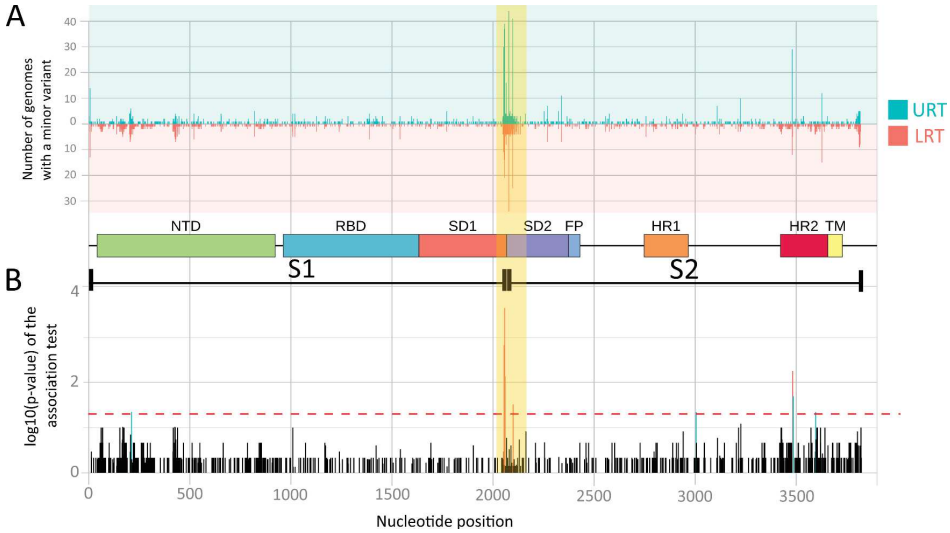

Fig. 5. Graphical distribution of changes along S protein gene. (A) Number of samples containing minority variants in each position. Two separate histograms are used for URT and LRT samples, which are indicated upside down for image clarity. (B) Correlation of the presence of minority variants with URT and LRT in each position of the gene. Bar height represents the log10 (*p*-value) of the Fisher exact test. In the middle, a scheme of the gene subdomains and subunits is used as separator. NTD: N-Terminal Domain; RBD: Receptor-Binding Domain; SD1: Structural Domain 1; SD2: Structural Domain 2; FP: Fusion Peptide; HR1: Heptad Repeat 1; HR2: Heptad Repeat 2; TM: TransMembrane domain; S1: Subunit 1; S2: Subunit 2.

Table 1
Description of the 9 Minority Variants (MVs) positions associated with either URT or LRT compared to the reference sequence (NC_045512.2). Global frequency is referred to the frequency of the mutation in the same amino acid in GISAID global database. Data are accessible at www.cov.lanl.gov .

Codon position	Amino acid	Reference	Mutation	Mutation type	Associated with
212	71	C(S)	A(Y)/T (F)	NotSyn	LRT
2055	685	T(R)	G(R)	Syn	URT
2058	686	T(S)	G(R)	NotSyn	URT
2060	687	T(V)	G(G)	NotSyn	URT
2100	700	T(G)	G(G)	Syn	URT
3005	1002	A(Q)	T(L) / -	NotSyn/ Del	LRT
3483	1161	A(S)	C(S)	Syn	URT
3485	1162	C(P)	T(L)/G (R)	NotSyn	LRT
3596	1199	A (D)	G(G)/T (G)	NotSyn	LRT

variants potentially associated with progression from upper to lower respiratory tract on paired samples from patients admitted to intensive care unit with severe infection.

Among SARS-CoV-2 evolution, G614 variant in S protein has become worldwide predominant since April 2020 and was associated with an increased fitness advantage (Korber at al., 2020). This finding was also confirmed by other studies that compared D614 and G614 variants and found that G614 was associated with increased replication in human lung epithelial cells (Shi et al., 2020). On the contrary, G614 variant was not associated with an increased disease severity and its role in pathogenesis has yet to be elucidated (Long et al., 2020; Shi et al., 2020). More than 60% of patients included in this study had severe infections developing severe pneumonia and requiring oxygen therapy. Mutations associated with these symptoms were explored by obtaining sequences from the LRT samples. All SARS-CoV-2 sequences generated in this study harboured the G614 variant, while the original D614 variant was found neither among majority nor among minority variants. Thus, we do not report any evidence of the G614 variant favouring the severe presentation. Moreover, no evidence of mutations on S gene associated with progression from upper to lower respiratory tract emerged from our analysis. This result was also observed in a series of paired samples and is consistent with the finding, previously reported by Rueca et al. in a lower number of patients (Rueca et al., 2020). Our data are in keeping with Wylezich et colleagues reporting no evidence of compartment-specific pattern of mutations between different respiratory compartments (Wylezich et al., 2021). This finding suggests us that disease severity could be mainly determined by host factor such as comorbidities, age and absence of pre-existing immunity (Al Khatib et al., 2020). In addition, deletions have been observed as MVs in a few samples but with a lower frequency than that observed in a recent publication (Andrés et al. 2020).

Overall, the S gene sequences originated in our study showed a greater variability (number of haplotypes) in LRT as compared to URT samples. This difference was unrelated to the viral load (measured as Ct) since comparable Ct values were observed in URT and LRT samples. Indeed, no correlation between viral load and viral diversity was observed and this is consistent with the finding previously reported by Siqueira et colleagues, who investigated quasispecies variation in cancer patients (Siqueira et al., 2020). The difference in viral population between URT and LRT could be explained by the hypothesis of an independent replication in the two respiratory districts also suggested by Wölfel et al. (2020). In addition, a higher positive selective pressure in the lung environment has been observed as compared to upper respiratory tract (dN/dS>1). Similar observation was reported by Sun et al suggesting that diversifying of the quasispecies mutants indicated potential independent virus replication in different tissues or organs (Sun et al. 2021). The great variability in the LRT samples resulted also in an increased number of frameshifting deletion and insertions. The presence of deleterious mutations could indicate a loss of function of the S protein in a fraction of the viral population. This subpopulation might be maintained thanks to replication and cell invasions events that do not imply the ACE2 receptor (e.g. within syncytia or in macrophages after

phagocytosis)(Abassi et al., 2020). However, this and other theories on how such mutations can influence viral replication in lung tissues might be elucidated and extensively investigated with additional studies.

Nucleotides changes arise during the virus replication and persistence, in particular A>G. These were shown to be related to the host editing mechanisms such as the APOBEC and ADARs (Carpenter et al., 2009; Di Giorgio et al., 2020). The A>G transition is caused by the deamination from Adenosine to Inosine (A>I) generated by the ADARs. Thus, the significant rate of T>C observed in the LRT both by Di Giorgio et al. (2020) and in this study agrees with the hypothesis that T>C in SARS-CoV-2 could also be related to the ADARs mechanism[32]. Although not associated with host editing mechanism, the T>G pattern in MVs is also of particular interest in this study, as they are the most prevalent pattern observed in the entire dataset and they were found associated with the URT.

Finally, the cleavage site between S1 and S2 of the S protein corresponds to one of the two genetic sites in which Andersen and colleagues found crucial mutations associated with the spillover to humans (Andersen et al., 2020). For this reason, we can hypothesize the presence of an evolutionary selective pressure in this site and that the driving force of this evolution might reside in the URT. Modifications (mutations or deletion) in the S1/S2 junction site has been associated with virus attenuation in hamsters (Wang et al., 2021). Alternatively, these observations might be explained by an absence of negative selection on random occurring mutations: this is a cleavage site; thus, SNPs affect the protein structure much less than in other sites. This last hypothesis explains the high density of mutations in the cleavage site (S1/S2 junction) both in URT and LRT samples. However, it does not explain the higher incidence in the URT and the presence of associated mutations. Moreover, other studies found specific low frequency mutations around the cleavage site, i.e. deletions that were associated with milder symptoms (Andrés et al., 2020; Lau et al., 2020). Whereas, in our study mutations are mainly T>G and G>T transversions in this site (359/643 MVs). Since such mutations are usually rare changes in nucleic acids, this observation underlines further the presence of a selective pressure. Interestingly, the T>G pattern was also identified as an inexplicable intra-host mutational signature in HPV (Zhu et al., 2020).

In conclusion, the results of the present study indicate that severe SARS-CoV-2 infections are not associated with a specific mutational pattern. However, a great variability was observed on viral population in LRT also associated with a positive selective pressure. How the difference may impact immune response escape, tissue tropism and pathogenicity is still to be elucidated. Moreover, we observed the evidence of possible ongoing evolution in one of the gene loci that were crucial for the spillover to humans. This highlights the importance of genomic surveillance to predict and avoid vaccine escape mutants.

CRediT authorship contribution statement

Stefano Gaiarsa: Writing – original draft, Software, Formal analysis, Data curation. **Federica Giardina:** Writing – original draft, Formal analysis, Investigation. **Gherard Batisti Biffignandi:** Methodology, Software, Formal analysis, Data curation. **Guglielmo Ferrari:** Investigation. **Aurora Piazza:** Validation, Investigation, Data curation. **Monica Tallarita:** Investigation. **Federica Novazzi:** Investigation. **Claudio Bandi:** Writing – review & editing. **Stefania Paolucci:** Resources, Visualization. **Francesca Rovida:** Investigation, Resources. **Giulia Campanini:** Writing – review & editing. **Antonio Piralla:** Conceptualization, Methodology, Writing – original draft, Project administration, Funding acquisition. **Fausto Baldanti:** Supervision, Project administration, Writing – review & editing.

Declaration of Competing of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2022.198786.

References

Abassi, Z., Knaney, Y., Karram, T., Heyman, S.N., 2020. The lung macrophage in SARS-CoV-2 infection: a friend or a foe? Front. Immunol. 11, 1312. https://doi.org/10.3389/fimmu.2020.01312.

Al Khatib, H.A., Benslimane, F.M., Elbashir, I.E., Coyle, P.V., Al Maslamani, M.A., Al-Khal, A., Al Thani, A.A., Yassine, H.M, 2020. Within-host diversity of SARS-CoV-2 in COVID-19 patients with variable disease severities. Front. Cell. Infect. Microbiol. 10, 575613 https://doi.org/10.3389/fcimb.2020.575613.

Andersen, K.G., Rambaut, A., Lipkin, W.I., Holmes, E.C., Garry, R.F., 2020. The proximal origin of SARS-CoV-2. Nat. Med. 26 (4), 450–452. https://doi.org/10.1038/s41591-020-0820-9.

Andrés, C., Garcia-Cehic, D., Gregori, J., Piñana, M., Rodriguez-Frias, F., Guerrero-Murillo, M., Esperalba, J., Rando, A., Goterris, L., Codina, M.G., Quer, S., Martín, M. C., Campins, M., Ferrer, R., Almirante, B., Esteban, J.I., Pumarola, T., Antón, A., Quer, J., 2020. Naturally occurring SARS-CoV-2 gene deletions close to the spike S1/S2 cleavage site in the viral quasispecies of COVID19 patients. Emerg. Microbes Infect. 9 (1), 1900–1911. https://doi.org/10.1080/22221751.2020.1806735.

Andrews, N., Stowe, J., Kirsebom, F., Toffa, S., Rickeard, T., Gallagher, E., Gower, C., Kall, M., Groves, N., O'Connell, A.M., Simons, D., Blomquist, P.B., Zaidi, A., Nash, S., Iwani Binti Abdul Aziz, N., Thelwall, S., Dabrera, G., Myers, R., Amirthalingam, G., Gharbia, S., Barrett, J.C., Elson, R., Ladhani, S.N., Ferguson, N., Zambon, M., Campbell, C.N.J., Brown, K., Hopkins, S., Chand, M., Ramsay, M., Lopez Bernal, J., 2022. Covid-19 vaccine effectiveness against the omicron (B.1.1.529) variant. N. Engl. J. Med. https://doi.org/10.1056/NEJMoa2119451, 10.1056/NEJMoa2119451. Advance online publication.

Belouzard, S., Millet, J.K., Licitra, B.N., Whittaker, G.R., 2012. Mechanisms of coronavirus cell entry mediated by the viral spike protein. Viruses 4 (6), 1011–1033. https://doi.org/10.3390/v4061011.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T, 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55 (4), 611–622. https://doi.org/10.1373/clinchem.2008.112797.

Carpenter, J.A., Keegan, L.P., Wilfert, L., O'Connell, M.A., Jiggins, F.M., 2009. Evidence for ADAR-induced hypermutation of the drosophila sigma virus (Rhabdoviridae). BMC Genet. 10, 75. https://doi.org/10.1186/1471-2156-10-75.

Chen, S., Zhou, Y., Chen, Y., Gu, J., 2018. Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34 (17), i884–i890. https://doi.org/10.1093/bioinformatics/bty560 (Oxford, England).

Corman, V.M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D.K., Bleicker, T., Brünink, S., Schneider, J., Schmidt, M.L., Mulders, D.G., Haagmans, B.L., van der Veer, B., van den Brink, S., Wijsman, L., Goderski, G., Romette, J.L., Ellis, J., Zambon, M., Peiris, M., Goossens, H., Reusken, C., Koopmans, M.P., Drosten, C., 2020. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. Bull. Eur. Mal. Transm. Eur. Commun. Dis. Bull. 25 (3), 2000045 https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045.

Dejnirattisai, W., Huo, J., Zhou, D., Zahradník, J., Supasa, P., Liu, C., Duyvesteyn, H.M. E., Ginn, H.M., Mentzer, A.J., Tuekprakhon, A., Nutalai, R., Wang, B., Dijokaite, A., Khan, S., Avinoam, O., Bahar, M., Skelly, D., Adele, S., Johnson, S.A., Amini, A., Ritter, T.G., Mason, C., Dold, C., Pan, D., Assadi, S., Bellass, A., Omo-Dare, N., Koeckerling, D., Flaxman, A., Jenkin, D., Aley, P.K., Voysey, M., Costa Clemens, S.A., Naveca, F.G., Nascimento, V., Nascimento, F., Fernandes da Costa, C., Resende, P.C., Pauvolid-Correa, A., Siqueira, M.M., Baillie, V., Serafin, N., Kwatra, G., Da Silva, K., Madhi, S.A., Nunes, M.C., Malik, T., Openshaw, P.J.M., Baillie, J.K., Semple, M.G., Townsend, A.R., Huang, K.A., Tan, T.K., Carroll, M.W., Klenerman, P., Barnes, E., Dunachie, S.J., Constantinides, B., Webster, H., Crook, D., Pollard, A.J., Lambe, T., , OPTIC Consortium, ISARIC4C Consortium, Paterson, N.G., Williams, M.A., Hall, D. R., Fry, E.E., Mongkolsapaya, J., Ren, J., Schreiber, G., Stuart, D.I., Screaton, G.R., 2022. SARS-CoV-2 omicron-B.1.1.529 leads to widespread escape from neutralizing antibody responses. Cell 185 (3), 467–484. https://doi.org/10.1016/j.cell.2021.12.046 e15Epub 2022 Jan 4. PMID: 35081335; PMCID: PMC8723827.

Di Giorgio, S., Martignano, F., Torcia, M.G., Mattiuz, G., Conticello, S.G., 2020. Evidence for host-dependent RNA editing in the transcriptome of SARS-CoV-2. Science Advances 6 (25), eabb5813. https://doi.org/10.1126/sciadv.abb5813.

Domingo, E., Perales, C., 2019. Viral quasispecies. PLoS Genet. 15 (10), e1008271 https://doi.org/10.1371/journal.pgen.1008271.

Dong, E., Du, H., Gardner, L., 2020. An interactive web-based dashboard to track COVID-19 in real time. Lancet. Infect. Dis. 20 (5), 533–534. https://doi.org/10.1016/S1473-3099(20)30120-1.

Gralinski, L.E., Menachery, V.D., 2020. Return of the coronavirus: 2019-nCoV. Viruses 12 (2), 135. https://doi.org/10.3390/v12020135.

Harvey, W.T., Carabelli, A.M., Jackson, B., Gupta, R.K., Thomson, E.C., Harrison, E.M., Ludden, C., Reeve, R., Rambaut, A., , COVID-19 Genomics UK (COG-UK) Consortium, Peacock, S.J., Robertson, D.L., 2021. SARS-CoV-2 variants, spike mutations and immune escape. Nature reviews. Microbiology 19 (7), 409–424. https://doi.org/10.1038/s41579-021-00573-0.

Huang, Y., Yang, C., Xu, X.F., Xu, W., Liu, S.W., 2020. Structural and functional properties of SARS-CoV-2 spike protein: potential antivirus drug development for COVID-19. Acta Pharmacol. Sin. 41 (9), 1141–1149. https://doi.org/10.1038/s41401-020-0485-4.

Jary, A., Leducq, V., Malet, I., Marot, S., Klement-Frutos, E., Teyssou, E., Soulié, C., Abdi, B., Wirden, M., Pourcher, V., Caumes, E., Calvez, V., Burrel, S., Marcelin, A.G., Boutolleau, D., 2020. Evolution of viral quasispecies during SARS-CoV-2 infection. Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. 26 (11), 1560.e1–1560.e4. https://doi.org/10.1016/j.cmi.2020.07.032.

Karamitros, T., Papadopoulou, G., Bousali, M., Mexias, A., Tsiodras, S., Mentis, A., 2020. SARS-CoV-2 exhibits intra-host genomic plasticity and low-frequency polymorphic quasispecies. J. Clin. Virol. 131, 104585 https://doi.org/10.1016/j.jcv.2020.104585.

Ke, R., Zitzmann, C., Ribeiro, R.M., et al., 2020. Kinetics of SARS-CoV-2 infection in the human upper and lower respiratory tracts and their relationship with infectiousness. medRxiv. https://doi.org/10.1101/2020.09.25.20201772, 2020.09.25.20201772.

Knyazev, S., Tsyvina, V., Shankar, A., Melnyk, A., Artyomenko, A., Malygina, T., Porozov, Y.B., Campbell, E.M., Switzer, W.M., Skums, P., Zelikovsky, A., Knyazev, S., 2018. CliqueSNV: an efficient noise reduction technique for accurate assembly of viral variants from NGS data. bioRXiv. https://doi.org/10.1101/264242.

Korber, B., Fischer, W.M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., Hengartner, N., Giorgi, E.E., Bhattacharya, T., Foley, B., Hastie, K.M., Parker, M.D., Partridge, D.G., Evans, C.M., Freeman, T.M., de Silva, T.I., , Sheffield COVID-19 Genomics Group, McDanal, C., Perez, L.G., Tang, H., Moon-Walker, A., Whelan, S.P., LaBranche, C.C., Saphire, E.O., Montefiori, D.C., 2020. Tracking changes in SARS-CoV-2 Spike: evidence that D614G increases infectivity of the COVID-19 virus. Cell 182 (4), 812–827. https://doi.org/10.1016/j.cell.2020.06.043 e19.

Lam, T.T., Jia, N., Zhang, Y.W., Shum, M.H., Jiang, J.F., Zhu, H.C., Tong, Y.G., Shi, Y.X., Ni, X.B., Liao, Y.S., Li, W.J., Jiang, B.G., Wei, W., Yuan, T.T., Zheng, K., Cui, X.M., Li, J., Pei, G.Q., Qiang, X., Cheung, W.Y., Li, L., Sun, F., Qin, S., Huang, J., Leung, G.M., Holmes, E.C., Hu, Y., Guan, Y., Cao, W.C., 2020. Identifying SARS-CoV-2-related coronaviruses in Malayan pangolins. Nature 583 (7815), 282–285. https://doi.org/10.1038/s41586-020-2169-0.

Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat. Methods 9 (4), 357–359. https://doi.org/10.1038/nmeth.1923.

Lau, S.Y., Wang, P., Mok, B.W., Zhang, A.J., Chu, H., Lee, A.C., Deng, S., Chen, P., Chan, K.H., Song, W., Chen, Z., To, K.K., Chan, J.F., Yuen, K.Y., Chen, H., 2020. Attenuated SARS-CoV-2 variants with deletions at the S1/S2 junction. Emerg. Microbes Infect. 9 (1), 837–842. https://doi.org/10.1080/22221751.2020.1756700.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., 2009. & 1000 Genome project data processing subgroup. The Sequence Alignment/Map format and SAMtools Bioinformatics 25 (16), 2078–2079 (Oxford, England)10.1093.

Long, S.W., Olsen, R.J., Christensen, P.A., Bernard, D.W., Davis, J.J., Shukla, M., Nguyen, M., Saavedra, M.O., Yerramilli, P., Pruitt, L., Subedi, S., Kuo, H.C., Hendrickson, H., Eskandari, G., Nguyen, H., Long, J.H., Kumaraswami, M., Goike, J., Boutz, D., Gollihar, J., McLellan, J.S., Chou, C.W., Javanmardi, K., Finkelstein, I.J., Musser, J.M., 2020. Molecular architecture of early dissemination and massive second wave of the SARS-CoV-2 virus in a major Metropolitan area. mBio 11 (6), e02707–e02720. https://doi.org/10.1128/mBio.02707-20.

Mlcochova, P., Kemp, S.A., Dhar, M.S., Papa, G., Meng, B., Ferreira, I., Datir, R., Collier, D.A., Albecka, A., Singh, S., Pandey, R., Brown, J., Zhou, J., Goonawardane, N., Mishra, S., Whittaker, C., Mellan, T., Marwal, R., Datta, M., Sengupta, S., Ponnusamy, K., Radhakrishnan, V.S., Abdullahi, A., Charles, O., Chattopadhyay, P., Devi, P., Caputo, D., Peacock, T., Wattal, C., Goel, N., Satwik, A., Vaishya, R., Agarwal, M., , Indian SARS-CoV-2 Genomics Consortium (INSACOG), Genotype to Phenotype Japan (G2P-Japan) Consortium, CITIID-NIHR BioResource COVID-19 Collaboration, Mavousian, A., Lee, J.H., Bassi, J., Silacci-Fegni, C., Saliba, C., Pinto, D., Irie, T., Yoshida, I., Hamilton, W.L., Sato, K., Bhatt, S., Flaxman, S., James, L.C., Corti, D., Piccoli, L., Barclay, W.S., Rakshit, P., Agrawal, A., Gupta, R.K., 2021. SARS-CoV-2 B.1.617.2 Delta variant replication and immune evasion. Nature, 599 (7883), 114–119. https://doi.org/10.1038/s41586-021-03944-y. Epub 2021 Sep 6. PMID: 34488225; PMCID: PMC8566220.

O'Toole, Á., Pybus, O.G., Abram, M.E., Kelly, E.J., Rambaut, A., 2022. Pango lineage designation and assignment using SARS-CoV-2 spike gene nucleotide sequences. BMC Genom. 23 (1), 121. https://doi.org/10.1186/s12864-022-08358-2.

Parra-Lucares, A., Segura, P., Rojas, V., Pumarino, C., Saint-Pierre, G., Toro, L., 2022. Emergence of SARS-CoV-2 variants in the world: how could this happen? Life 12 (2), 194. https://doi.org/10.3390/life12020194 (Basel, Switzerland).

Pérez-Lago, L., Aldámiz-Echevarría, T., García-Martínez, R., Pérez-Latorre, L., Herranz, M., Sola-Campoy, P.J., Suárez-González, J., Martínez-Laperche, C., Comas, I., González-Candelas, F., Catalán, P., Muñoz, P., García de Viedma, D., On Behalf Of Gregorio Marañón Microbiology-Id Covid Study Group, 2021. . Different within-host viral evolution dynamics in severely immunosuppressed cases with persistent SARS-CoV-2. Biomedicines 9 (7), 808. https://doi.org/10.3390/biomedicines9070808.

Rueca, M., Bartolini, B., Gruber, C., Piralla, A., Baldanti, F., Giombini, E., Messina, F., Marchioni, L., Ippolito, G., Di Caro, A., Capobianchi, M.R., 2020. Compartmentalized replication of SARS-Cov-2 in upper vs. lower respiratory tract assessed by whole genome quasispecies analysis. Microorganisms 8 (9), 1302. https://doi.org/10.3390/microorganisms8091302.

Shen, Z., Xiao, Y., Kang, L., Ma, W., Shi, L., Zhang, L., Zhou, Z., Yang, J., Zhong, J., Yang, D., Guo, L., Zhang, G., Li, H., Xu, Y., Chen, M., Gao, Z., Wang, J., Ren, L., Li, M., 2020. Genomic diversity of severe acute respiratory syndrome-coronavirus 2 in patients with coronavirus disease 2019. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 71 (15), 713–720. https://doi.org/10.1093/cid/ciaa203.

Shi, P.Y., Plante, J., Liu, Y., Liu, J., Xia, H., Johnson, B., Lokugamage, K., Zhang, X., Muruato, A., Zou, J., Fontes-Garfias, C., Mirchandani, D., Scharton, D., Kalveram, B., Bilello, J., Ku, Z., An, Z., Freiberg, A., Menachery, V., Xie, X., Weaver, S., 2020. Spike mutation D614G alters SARS-CoV-2 fitness and neutralization susceptibility. Res. Sq. 70482. https://doi.org/10.21203/rs.3.rs-70482/v1 rs.3.rs.

Simmonds, P., 2020. Rampant C→U hypermutation in the genomes of SARS-CoV-2 and other coronaviruses: causes and consequences for their short- and long-term evolutionary trajectories. mSphere 5 (3), e00408–e00420. https://doi.org/10.1128/mSphere.00408-20.

Siqueira, J.D., Goes, L.R., Alves, B.M., de Carvalho, P.S., Cicala, C., Arthos, J., Viola, J., de Melo, A.C., Soares, M.A., 2020. SARS-CoV-2 genomic and quasispecies analyses in cancer patients reveal relaxed intrahost virus evolution. bioRxiv Prepr. Serv. Biol. https://doi.org/10.1101/2020.08.26.267831, 2020.08.26.267831.

Sun, F., Wang, X., Tan, S., Dan, Y., Lu, Y., Zhang, J., Xu, J., Tan, Z., Xiang, X., Zhou, Y., He, W., Wan, X., Zhang, W., Chen, Y., Tan, W., Deng, G., 2021. SARS-CoV-2 quasispecies provides an advantage mutation pool for the epidemic variants. Microbiol. Spectr. 9 (1), e0026121 https://doi.org/10.1128/Spectrum.00261-21.

Tao, K., Tzou, P.L., Nouhin, J., Gupta, R.K., de Oliveira, T., Kosakovsky Pond, S.L., Fera, D., Shafer, R.W, 2021. The biological and clinical significance of emerging SARS-CoV-2 variants. Nat. Rev. Genet. 22 (12), 757–773. https://doi.org/10.1038/s41576-021-00408-x.

To, K.K., Tsang, O.T., Leung, W.S., Tam, A.R., Wu, T.C., Lung, D.C., Yip, C.C., Cai, J.P., Chan, J.M., Chik, T.S., Lau, D.P., Choi, C.Y., Chen, L.L., Chan, W.M., Chan, K.H., Ip, J.D., Ng, A.C., Poon, R.W., Luo, C.T., Cheng, V.C., Chan, J.F., Hung, I.F., Chen, Z., Chen, H., Yuen, K.Y., 2020. Temporal profiles of viral load in posterior oropharyngeal saliva samples and serum antibody responses during infection by SARS-CoV-2: an observational cohort study. Lancet Infect. Dis. 20 (5), 565–574. https://doi.org/10.1016/S1473-3099(20)30196-1.

van Dorp, L., Richard, D., Tan, C., Shaw, L.P., Acman, M., Balloux, F., 2020. No evidence for increased transmissibility from recurrent mutations in SARS-CoV-2. Nat. Commun. 11 (1), 5986. https://doi.org/10.1038/s41467-020-19818-2.

Volz, E., Mishra, S., Chand, M., Barrett, J.C., Johnson, R., Geidelberg, L., Hinsley, W.R., Laydon, D.J., Dabrera, G., O'Toole, Á., Amato, R., Ragonnet-Cronin, M., Harrison, I., Jackson, B., Ariani, C.V., Boyd, O., Loman, N.J., McCrone, J.T., Gonçalves, S., Jorgensen, D., Myers, R., Hill, V., Jackson, D.K., Gaythorpe, K., Groves, N., Sillitoe, J., Kwiatkowski, D.P., , COVID-19 Genomics UK (COG-UK) consortium, Flaxman, S., Ratmann, O., Bhatt, S., Hopkins, S., Gandy, A., Rambaut, A., Ferguson, N.M., 2021. Assessing transmissibility of SARS-CoV-2 lineage B.1.1.7 in England. Nature 593 (7858), 266–269. https://doi.org/10.1038/s41586-021-03470-x.

Wang, P., Lau, S.Y., Deng, S., Chen, P., Mok, B.W., Zhang, A.J., Lee, A.C., Chan, K.H., Tam, R.C., Xu, H., Zhou, R., Song, W., Liu, L., To, K.K., Chan, J.F., Chen, Z., Yuen, K.Y., Chen, H., 2021. Characterization of an attenuated SARS-CoV-2 variant with a deletion at the S1/S2 junction of the spike protein. Nat. Commun. 12 (1), 2790. https://doi.org/10.1038/s41467-021-23166-0.

World Health Organization (WHOa). 2020. Clinical management of severe acute respiratory infection when novel coronavirus (ʻ2019-nCoV)ʼ infection is suspected: interim guidance. World Health Organization. Available at: https://apps.who.int/iris/handle/10665/330893 [Accessed on 19 October 2020].

World Health Organization (WHOb). 2020. Diagnostic detection of 2019-nCoV by real-time RT-PCR. Available at: https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf. [Accessed 30 January 2020].

World Health Organization (WHO). 2021. Terms of Reference for the Technical Advisory Group on SARS-CoV-2 Virus Evolution (TAG-VE). COVID-19: Surveillance, case investigation and epidemiological protocols. Available at: https://www.who.int/publications/m/item/terms-of-reference-for-the-technical-advisory-group-on-sars-cov-2-virus-evolution-(tag-ve) [Accessed 1 December 2021].

Wölfel, R., Corman, V.M., Guggemos, W., Seilmaier, M., Zange, S., Müller, M.A., Niemeyer, D., Jones, T.C., Vollmar, P., Rothe, C., Hoelscher, M., Bleicker, T., Brünink, S., Schneider, J., Ehmann, R., Zwirglmaier, K., Drosten, C., Wendtner, C., 2020. Virological assessment of hospitalized patients with COVID-2019. Nature 581 (7809), 465–469. https://doi.org/10.1038/s41586-020-2196-x.

Wong, M.C., Javornik Cregeen, S.J., Ajami, N.J., Petrosino, J.F., 2020. Evidence of recombination in coronaviruses implicating pangolin origins of nCoV-2019. bioRxiv Prepr. Serv. Biol. https://doi.org/10.1101/2020.02.07.939207, 2020.02.07.939207.

Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Song, Z.G., Hu, Y., Tao, Z.W., Tian, J.H., Pei, Y.Y., Yuan, M.L., Zhang, Y.L., Dai, F.H., Liu, Y., Wang, Q.M., Zheng, J.J., Xu, L., Holmes, E.C., Zhang, Y.Z., 2020. A new coronavirus associated with human respiratory disease in China. Nature 579 (7798), 265–269. https://doi.org/10.1038/s41586-020-2008-3.

Wylezich, C., Schaller, T., Claus, R., Hirschbühl, K., Märkl, B., Kling, E., Spring, O., Höper, D., Schlegel, J., Beer, M., Dintner, S., 2021. Whole-genome analysis of SARS-CoV-2 samples indicate no tissue specific genetic adaptation of the virus in COVID-19 patients' upper and lower respiratory tract. Diagn. Microbiol. Infect. Dis. 101 (4), 115520 https://doi.org/10.1016/j.diagmicrobio.2021.115520.

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Molecular Epidemiology of Rhinovirus/Enterovirus and Their Role on Cause Severe and Prolonged Infection in Hospitalized Patients

Federica A. M. Giardina [1], Antonio Piralla [1,*], Guglielmo Ferrari [1], Federica Zavaglio [1], Irene Cassaniti [1] and Fausto Baldanti [1,2]

[1] Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy; federica.giardina01@universitadipavia.it (F.A.M.G.); guglielmo.ferrari01@universitadipavia.it (G.F.); fede.zavaglio90@gmail.com (F.Z.); i.cassaniti@smatteo.pv.it (I.C.); fausto.baldanti@unipv.it (F.B.)

[2] Department of Clinical-Surgical, Diagnostic and Pediatric Sciences, Università degli Studi di Pavia, 27100 Pavia, Italy

* Correspondence: a.piralla@smatteo.pv.it; Tel.: +39-038-250-2420

Abstract: Rhinovirus is one of the most common respiratory viruses, causing both upper and lower respiratory tract infections. It affects mainly children and could cause prolonged infections, especially in immunocompromised patients. Here we report our data on a 15-month surveillance of Rhinovirus seasonality and circulation in Lombardy Region, Italy. All rhinovirus/enterovirus-positive samples were amplified with RT-PCR for the VP4-VP2 region to assign the correct genotype. The median age of RV/EV-positive patients is 9 years, with a range of 0–96. RV-A and RV-C were detected in the majority of cases, while RV-B accounted for less than 10% of cases. An enterovirus species was detected in 6.45% of the cases. A total of 7% of the patients included in this study had a prolonged infection with a median duration of 62 days. All these patients were immunocompromised and most of them were pediatric with an RV-A infection. Two outbreaks were identified, mainly in the neonatal intensive care unit (NICU) and Oncohematology Department, caused by RV A89 and C43, respectively. Nearly 4.5% of the patients were admitted to the ICU requiring mechanical ventilation; all of which had preexisting comorbidities.

Keywords: respiratory infection; rhinovirus; enterovirus; prolonged infection

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1. Introduction

Human rhinoviruses (RVs) are small viruses belonging to the Enterovirus genus within the Picornaviridae family. At least more than 100 genotypes have been identified and recently a new species of RV, named RV-C, including more than 50 genotypes, was firstly discovered in 2006 [1]. RV infections are quite common and their circulation seems to be distributed into two yearly peaks; in early fall and spring [2]. In particular, RV-A and RV-C represent the species most frequently detected, while RV-B is less frequent in comparison.

Although RVs are considered the etiologic agents of the "common cold", RVs have been recently associated with a severe acute respiratory infection (SARI) in children, older people, and immunosuppressed subjects [3–5]. Clinical manifestations of RV-associated SARI are croup, bronchiolitis or community-acquired pneumonia (CAP) which often requires hospitalization and mechanical ventilation [6–8]. Among RV species, RV-C seems to be more frequently associated with severe infections [9] including asthma exacerbations in children and life-threatening conditions in infants [10,11]. Other studies suggest that RV-C is more likely to cause lower respiratory tract infections than other types of RVs in the pediatric population rather than in the adult population [12]. Furthermore, RVs are also implicated in nosocomial outbreaks, as observed in neonatal intensive care units [13,14].

In addition to RVs, enteroviruses (EVs) belonging to the same Picornaviridae family have been observed as emerging pathogens causing a wide range of clinical syndromes, ranging from mild to more severe clinical outcomes [15,16].

RV and EV shedding usually lasts less than 2 weeks in immunocompetent subjects [17], while prolonged RV infections have been mainly observed in those who are immunocompromised, such as patients undergoing chemotherapy or in a post-transplant phase [18].

This study aimed to investigate clinical and virological features of RV/EV infections providing the increasingly recognized role of these viruses as important disease-causing agents in order to describe their impact on short- and long-term morbidity.

2. Materials and Methods

2.1. Study Population

This study was conducted in a cohort of patients with a respiratory syndrome, both hospitalized and outpatients, all referring to the Fondazione I.R.C.C.S. Policlinico San Matteo hospital in Pavia, Italy. All respiratory samples (nasal swabs and bronchoalveolar lavages) were collected between 1 September 2017, and 31 December 2018, then analyzed for the presence of respiratory viruses. Patients with rhinitis, pharyngitis, and laryngitis were considered as affected by an upper respiratory tract infection (URTI), whereas patients with bronchitis, bronchiolitis, and pneumonia (characterized by cough, wheezing, and/or dyspnea) were classified as affected by a lower respiratory tract infection (LRTI). In addition to the suggestive clinical picture, all cases of pneumonia were radiologically confirmed. The term "episode" indicated a single respiratory syndrome, whose duration was defined by the presence of respiratory symptoms. Respiratory syndromes occurring in the same patient at least 3 weeks following the disappearance of respiratory symptoms of a previous episode were defined as a "separate episode" and were analyzed independently from the previous one. "Multiple picornavirus detection" stated the presence of different RV/EV strains or species during the same episode. Episodes were defined as "prolonged" if the same RV/EV type was detected in specimens collected at least 30 days apart. The study was conducted in accordance with the Declaration of Helsinki, and the protocol on respiratory virus epidemiology was approved by the Ethics Committee of our hospital (P-20180022616).

2.2. Molecular Analysis

Viral RNA was extracted on the QiaSymphony platform using a Virus Pathogens DSP Midi Kit (Qiagen, Heidelberg, Germany). Clinical specimens were tested for respiratory viruses using a panel which included RV/EV, human influenza type A and B (FluA and FluB), human coronaviruses (hCoVs), human metapneumovirus (hMPV), human respiratory syncytial virus (hRSV), human parainfluenza virus (hPiV) type 1–4 and human adenoviruses (hAdV) [19]. Real-time RT-PCR reactions were performed on Rotor-Gene Q with a Quantifast® Pathogen PCR+IC Kit (Qiagen, Heidelberg, Germany), according to the manufacturer's instructions. RV/EV-positive samples were amplified in a nested PCR targeting the VP4-VP2 region of the viral genome, according to Wisdom et al. [20], with a modified protocol. In detail, the first amplification was performed using the AgPath-ID One-Step RT-PCR kit (Ambion, Austin, TX, USA), according to the manufacturer's instructions. Primers used in the first amplification were OS458 (5′CCGGCCCCTGAATGYGGCTAA3′) and OAS1125 (5′ACATRTTYTSNCCAAANAYDCCCAT3′). Thermal profile was as follows: retrotranscription was conducted at 50 °C for 30 min and initial PCR activation at 95 °C for 10 min, then 50 cycles at 95 °C for the 30 s, 58 °C for 30 s and 72 °C for 1 min. The final step was at 72 °C for 5 min. Nested amplification was performed using AmpliTaqGold® with GeneAmp® (Life Technologies, Livingston, NJ, USA) according to the manufacturer's instructions with primers IS547 (5′ACCRACTACTTTGGGTGTCCGTG3′) and IAS1087 (5′TCWGGHARYTTCCAMCACCANCC3′). The thermal profile was: 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min. The final step of the reaction was at 72 °C for 5 min.

An alternative protocol targeting the EV's VP1 protein was used whenever the direct typing PCR resulted negative as described by Nix and colleagues [21]. The sequencing reaction was performed using internal primers on an ABI Prism Genetic Analyzer and sequences obtained were analyzed on Sequencer software.

2.3. Phylogenetic Analysis

Nucleotide sequences were aligned using the ClustalW method (we applied the ClustalW method for the nucleotide sequences' alignment) and a phylogenetic tree was constructed using the neighbor-joining method and the kimura-2-parameter to simultaneously estimate the distance among the sequences with MEGA software (version 5.05) [22]. Bootstrap values included 1000 replicates. RV/EV type assignment was defined by the nearest reference strains observed in the phylogenetic tree (The RV/EV type was assigned taking into account the nearest reference strain/s observed in the phylogenetic tree).

2.4. Statistical Analysis

Comparisons of the continuous unpaired variables were performed with the Mann–Whitney test. Additionally, we carried out descriptive statistics and statistical comparisons using the Graph Pad Prism software (version 8.3.0).

3. Results

3.1. Samples

A total of 3310 respiratory specimens were collected from hospitalized patients and outpatients and then analyzed during the study period. As shown by the blue line in Figure 1, the highest number of specimens was collected in December 2017 and January 2018, at the beginning of the influenza viruses' circulation. Instead, the lowest number of specimens was collected and tested during the summer period in 2018. Of the 3310 specimens collected, 257 (7.6%) referred to 201 patients (8.9% of the total), were positive for RV/EV (177 nasal swabs, 45 nasopharyngeal swabs, and 35 bronchoalveolar lavages). A total of 127/201 (63.2%) patients were admitted to different departments of our hospital, including Infectious Disease Dept., Pediatrics Dept., Oncohematology Dept., ICU or NICU Dept. A total of 66/201 (32.8%) were instead outpatients. Regarding the remaining 8 (4%) RV/EV positive cases, specimens were sent to our hospital from other health institutes in the Lombardy Region. The median age of RV/EV-positive patients was 9 years old (range 10 days–96 years), including 117 males (58.2%) and 84 females (41.8%). Of the total, 188/201 (93.5%) patients had one single RV/EV episode, 11 (5.5%) had 2 different RVRV/EV episodes, and only 2 (1%) patients had 3 RV/EV episodes. Among 216 episodes of RV infection, 184/216 (85.2%) were URTI and 32/216 (14.8%) were LRTI.

Table 1 shows the demographic and virological features of the patients included in this study. In the 3.053 RV/EV negative specimens, hRSV was detected in 274 samples (8.3%), FluB in 126 (3.8%), FluA in 106 (3.2%), hPiV type 1/3 and hAdV in 65 samples each (2.0%), hMPV in 37 (1.1%) and hCoV type OC43/HKU1 in 24 (0.7%). hPiV type 2/4 and hCoV 229E/NL63 represented less than 0.5% of the total number of cases and were detected in 13, 4 and 5 specimens, respectively. Finally, 2334 (73.2%) samples resulted negative for the respiratory viruses included in the panel used.

3.2. The Peak of Viral Load and Prolonged Infection

The peak of the RV/EV load was between 10^3 and 10^5 copies/mL in 51.9% of the episodes (112/216), $>10^5$ copies/mL in 53/216 (24.5%) and lower than 10^3 copies/mL in the remaining 51/216 (23.6%) episodes.

Overall, the median duration of RV/EV episodes was 15 days (range 4–316 days). A total of 11 (5.5%) patients had a prolonged RV/EV infection (>30 days) with a median duration of 75 days (range 30–316 days). All of these patients were immunocompromised due to their age (<30 days old), ongoing chemotherapy or post-transplant immunosuppressive therapy.

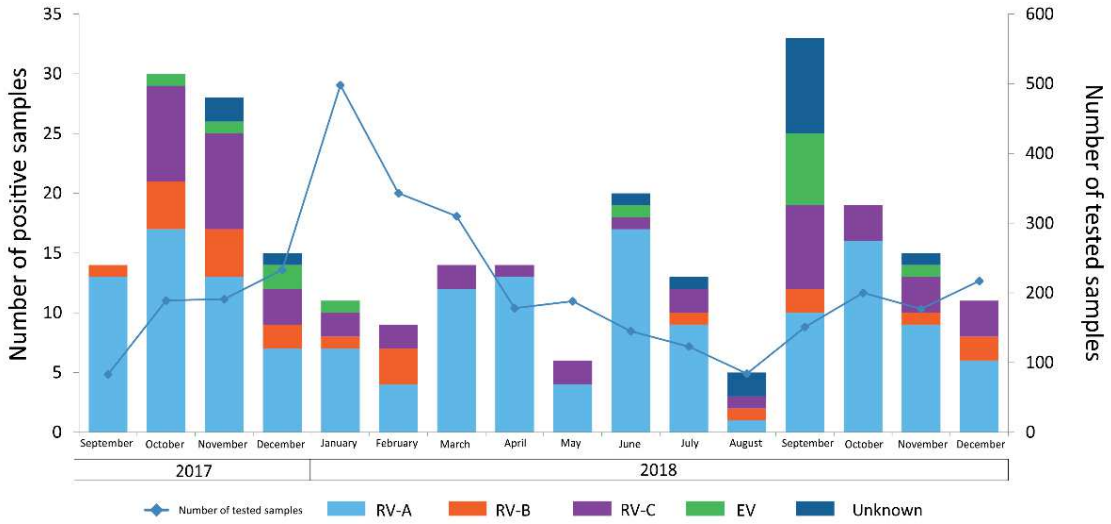

Figure 1. Monthly distribution of cases included in this study. The blue line represents the total number of respiratory specimens collected and tested during the study period. The bars correspond to the RV/EV positive cases with RV-A reported in light blue, RV-B in orange, RV-C in violet and EV cases in green. Blue bars represent those cases for which typing was not possible due to a very low viral load.

Table 1. Demographic and virological features of all RV/EV episodes with successful typing.

Categories		RV Species				*p*-Value [a]
		RV-A (127)	RV-B (20)	RV-C (44)	EV (12)	
Gender	Male	77 (60.6%)	10 (50.0%)	28 (63.6%)	5 (41.7%)	0.57
	Female	50 (39.4%)	10 (50.0%)	16 (36.4%)	7 (58.3%)	
Age	<1 year	26 (20.5%)	4 (20.0%)	17 (38.6%)	3 (25.0%)	0.02
	1–5 years	24 (18.9%)	7 (35.0%)	13 (29.5%)	2 (16.7%)	
	5–15 years	20 (15.7%)	4 (20.0%)	0	0	
	16–65 years	43 (33.9%)	4 (20.0%)	10 (22.7%)	5 (41.7%)	
	>65 years	14 (11.0%)	1 (5.0%)	4 (9.1%)	2 (16.7%)	
Hospitalization	ICU Dept.	5 (3.9%)	3 (15.0%)	2 (4.5%)	0	0.10
	NICU Dept.	19 (15.0%)	2 (10.0%)	13 (29.5%)	2 (16.7%)	
	Infectious Diseases Dept.	4 (3.1%)	0	2 (2.5%)	2 (16.7%)	
	Other Depts.	96 (75.6%)	14 (70%)	27 (61.4%)	6 (50%)	
	Unknown	3 (2.4%)	1 (5.0%)	0	2 (16.7%)	
Immuno status	Immunocompromised	57 (44.9%)	6 (30.0%)	20 (45.5%)	5 (41.7%)	0.67
	Immunocompetent	50 (39.4%)	11 (55%)	16 (36.4%)	3 (25%)	
	Unknown	20 (15.7%)	3 (15%)	8 (18.2%)	4 (33.3%)	
Viral Load	$<10^3$ copies/ml	25 (19.7%)	5 (25%)	12 (27.3%)	1 (8.3%)	0.46
	10^3–10^5 copies/mL	68 (53.5%)	13 (65.0%)	22 (50.0%)	10 (83.3%)	
	$>10^5$ copies/mL	34 (26.8%)	2 (10.0%)	10 (22.7%)	1 (8.3%)	
Coinfections	No coinfections	106 (83.5%)	12 (60%)	36 (81.8%)	10 (83.3%)	0.17
	Coinfections	21 (16.5%)	8 (40.0%)	8 (18.2%)	2 (16.7%)	
	hADV	0	2 (10%)	2 (4.5%)	0	
	hCMV	6 (4.7%)	1 (5%)	1 (2.3%)	1 (8.3%)	
	hCOVs	1 (0.8%)	0	0	0	
	hMPV	1 (0.8%)	0	1 (2.3%)	0	
	hPIVs	2 (1.6%)	3 (15.0%)	0	0	
	hRSV	11 (8.7%)	2 (10.0%)	4 (9.1%)	1 (8.3%)	

Table 1. *Cont.*

Categories		RV Species				*p*-Value [a]
		RV-A (127)	RV-B (20)	RV-C (44)	EV (12)	
Genotypes	N. of detected genotypes	40	9	20	5	NA
	Unknown	7	6	4	1	NA
	Most detected genotype	A49	B35	C3	D68	NA

[a] *p*-value was calculated for comparison between RV-A, RV-B, and RV-C; NA, not applicable.

3.3. Typing and Coinfections

Out of 216 RV/EV episodes, 127 (58.3%) were caused by RV-A, 44 (19.9%) by RV-C and 20 (9.8%) by RV-B, and the remaining 12 (6.0%) episodes were caused by EV (Figure 2).

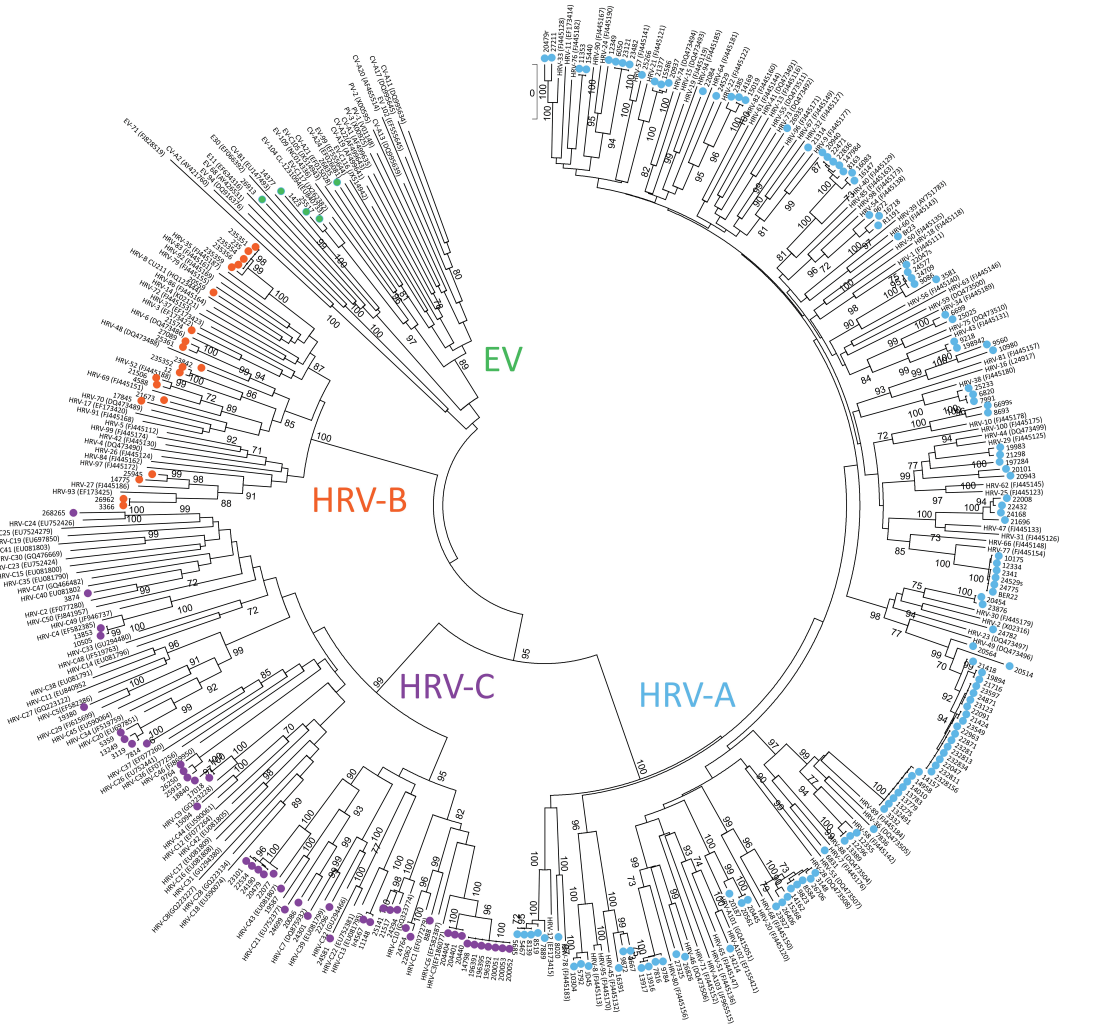

Figure 2. Shows the phylogenetic tree of the RV/EV cases in this study, based on the VP4-VP2 sequences obtained (n = 196). RV-A strains are reported with light blue circles, RV-B with orange circles, RV-C with violet circles and EV cases with green circles. EV-D68 sequences are not reported here due to the different sequences (partial VP1) analyzed.

In regard to the RV-A positive cases, 40 different genotypes were detected, 8 genotypes for RV-B and 18 for RV-C. For each RV species, the most detected genotypes were A49, B35 and C3, respectively. Concerning the EV-associated episodes, an EV-D68 was detected in seven of them, an EV-C104 was detected in two, and one single identification was obtained for EV-C117, CV-A21, and CV-B4. Among the 216 cases of infection, one subject was simultaneously infected by RV-A and EV-D68. In 14 RV/EV episodes typing could not be performed due to a very low viral load.

The median age of RV-A positive pediatric patients was 22 months, 29 months for RV-B, and 7 months for RV-C. Mann–Whitney tests showed that the median age between RV-A/B and RV-A/C positive pediatric patients was significantly different ($p < 0.05$), and that RV-A strains were observed in older people (16–65 and >65 years) as compared to RV-B and RV-C (Table 1). In 175/216 episodes (81.0%), RV/EV was the only virus detected, however, in 41/216 (19.0%) RV/EV was detected with at least one other respiratory virus. In detail, hRSV was detected in 24/41 (58.5%) coinfections, hAdV in 4/41 (9.8%), hPiV3 in 4/41 (9.8%), hMPV in 2/41 (4.9%), hPiV4 in 2/41 (4.97%) and finally, RV/EV was detected with two other viruses (hPiV and hCoV) in only one sample. Given the retrospective nature of this study, bacterial and fungal coinfections were not investigated.

3.4. Hospital Outbreaks

During the study period, at least two nosocomial outbreaks were observed in our hospital. The first outbreak occurred in October 2017 in a neonatal intensive care unit (NICU), with four patients infected by RV-C43 in a 30-day period. The other outbreak occurred in the same NICU in June 2018 where four patients were infected by RV-A89 during the same period (12 days).

3.5. EV Episodes Associated with Severe Infection

A total of 13 (4.5% of all the positive patients) RV/EV-positive patients were admitted to the ICU with a severe respiratory acute infection (SARI). The median age of the ICU patients was 54 years (range 5–66 years) including five patients with age less than 11 years. In all the cases, mechanical ventilation was needed. Eleven out of 13 patients (84.6%) had preexisting comorbidities including chronic respiratory diseases, and hematologic malignancies such as acute lymphoblastic leukemia, lymphoma and myelodysplastic syndrome. RV/EV was detected in bronchoalveolar lavage in 8/13 (61.5%) patients, nasal swab in 3/13 (23%) and in both samples in two patients. In six BALs, RV was detected with a viral load between 10^4–10^5 copies/mL, while in five BALs, the viral load was lower than 10^4 copies/mL. In one BAL, the RV/EV viral load was higher than 10^6 copies/mL. In seven patients, RV/EV was the only respiratory pathogen detected including four RV-A two RV-B, and one RV-C. On the contrary, in six patients, RV/EV (including three RV-A and three RV-B) was simultaneously detected with CMV (three patients), hAdV (one patient), hPiV3 (one patient), and hPiV4 (one patient). Bacterial/fungal coinfections were detected in two patients, one with *S. pneumoniae* (also positive for CMV) and one with both *S. pneumoniae* and *P. aeruginosa.*

4. Discussion

Influenza viruses and RSV are well-known respiratory pathogens, while RV/EV are increasingly recognized pathogens also responsible for SARI. In our study, 8.9% of all the patients referring to our hospital with acute respiratory syndromes had an RV/EV infection. Nearly 60% of them were pediatric (age < 16 years) and this finding is in agreement with recent studies investigating the epidemiology of respiratory infections. Those studies show that most RV/EV-positive patients were children under 10 years of age or less [23] and a difference in the median age in children infected by different RV genotypes has been observed [24–26]. Most of the episodes of RV infections observed in this study were caused by RV genotypes A and C, while RV-B accounts for about 10% of the total episodes. As previously described in other studies, comparable distribution

of RV species was observed worldwide, with a high incidence of RV-A and RV-C often present in equal or similar proportions [27,28]. The most frequently detected viruses in coinfections were RSV and AdV, while influenza viruses were never detected in coinfections with RV/EV. This finding is in agreement with many other studies investigating respiratory viruses' epidemiology, in which the viruses most frequently detected in coinfections with RV/EV were AdV and RSV together with human bocavirus [26,29]. In our study, the rate of coinfection with at least one other virus is 23.4%. Other studies reported frequencies of viral coinfection ranging from 9 [30] to 47% [31].

During our study period, two intra-hospital outbreaks were observed in October 2017 and June 2018. Molecular epidemiology of RV/EV has allowed us to identify several outbreaks in neonatal settings, including patients requiring mechanical ventilation [14,32,33]. Since RVs spread via aerosolization or direct contact with an infected person, intra-hospital outbreaks could be caused by contaminated surfaces or staff members during viral shedding following symptoms resolution, as has been assumed by Reese and colleagues [13].

In immunosuppressed patients, RV/EV shedding has been observed for a prolonged period, and this occurrence seems to be correlated with an early phase post-transplant [17,34]. This prolonged infection has been sustained by active viral excretion that can last several months [35].

In our study, twelve cases of prolonged RV/EV infection were observed. The great majority of them were observed in patients undergoing chemotherapy or post-transplant therapy, or in newborns. In most cases, the clinical picture associated with this prolonged infection was mild, but few severe cases were also observed. A similar scenario was also described in patients with prolonged RV infection after lung transplant; most of whom were asymptomatic [36].

Although RV has been considered the causative agent of the common cold, in our study 4.5% of RV/EV-positive patients had pneumonia and were admitted to the ICU. This finding has been recently investigated in the contest of other respiratory viruses. In 2015, Jain and colleagues reported that RV was the virus most frequently detected among ICU adult patients in the U.S., while in pediatric patients, RV was the second after hRSV, even if they were detected with a similar percentage [37,38]. Similar results were reported in Europe [39,40] as well as in Asia [41]. All these studies underline the need to consider RV/EV as the causative agent of severe respiratory infections. Sometimes severe RV/EV infections are also observed in patients with pre-existing comorbidities [17,38,42].

Nearly 60% of severe RV/EV infections were diagnosed based on LRT samples: this finding highlights the importance of adequate sampling collection. In fact, when both URT and LRT samples of the same patient were analyzed for the presence of RV/EV, it was detected only in LRT samples [19,43,44].

This study has several limitations. It is retrospective, and little data about patients' clinical conditions are available. Information regarding bacterial and fungal coinfection was available only for severe cases and they were not investigated in the general population.

5. Conclusions

RVs/EVs circulate throughout the year, causing upper respiratory tract infections in immunocompetent subjects. However, they could cause prolonged and severe infections requiring ICU admission in high-risk patients such as the older or immunocompromised populations. For this reason, RV/EV infection should be systematically monitored. The clinical impact of RV/EV infections is not limited only to the common cold, and these viruses should be considered as highly significant respiratory pathogens.

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References

1. Lamson, D.; Renwick, N.; Kapoor, V.; Liu, Z.; Palacios, G.; Ju, J.; Dean, A.; St George, K.; Briese, T.; Lipkin, W.I. MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in New York State during 2004–2005. *J. Infect. Dis.* **2006**, *194*, 1398–1402. [CrossRef]
2. Monto, A.S. The seasonality of rhinovirus infections and its implications for clinical recognition. *Clin. Ther.* **2002**, *24*, 1987–1997. [CrossRef]
3. Zacharie, S.; Vabret, A.; Guillois, B.; Dupont, C.; Brouard, J. Rhinovirus: Des pathogènes sous-estimés chez les patients en période néonatale. *Arch. Pediatr.* **2017**, *24*, 825–832. (In French) [CrossRef]
4. Drysdale, S.B.; Mejias, A.; Ramilo, O. Rhinovirus—Not just the common cold. *J. Infect.* **2017**, *74* (Suppl. S1), S41–S46. [CrossRef]
5. Aydin Köker, S.; Demirağ, B.; Tahta, N.; Bayram, N.; Oymak, Y.; Karapinar, T.H.; Gözmen, S.; Düzgöl, M.; Erçan Bozyer, H.; Vergin, C.; et al. A 3-Year Retrospective Study of the Epidemiology of Acute Respiratory Viral Infections in Pediatric Patients with Cancer Undergoing Chemotherapy. *J. Pediatr. Hematol. Oncol.* **2019**, *41*, e242–e246. [CrossRef]
6. Iwane, M.K.; Prill, M.M.; Lu, X.; Miller, E.K.; Edwards, K.M.; Hall, C.B.; Griffin, M.R.; Staat, M.A.; Anderson, L.J.; Williams, J.V.; et al. Human rhinovirus species associated with hospitalizations for acute respiratory illness in young US children. *J. Infect. Dis.* **2011**, *204*, 1702–1710. [CrossRef]
7. Louie, J.K.; Roy-Burman, A.; Guardia-Labar, L.; Boston, E.J.; Kiang, D.; Padilla, T.; Yagi, S.; Messenger, S.; Petru, A.M.; Glaser, C.A.; et al. Rhinovirus associated with severe lower respiratory tract infections in children. *Pediatr. Infect. Dis. J.* **2009**, *28*, 337–339. [CrossRef]
8. Sangil, A.; Calbo, E.; Robles, A.; Benet, S.; Viladot, M.E.; Pascual, V.; Cuchí, E.; Pérez, J.; Barreiro, B.; Sánchez, B.; et al. Aetiology of community-acquired pneumonia among adults in an H1N1 pandemic year: The role of respiratory viruses. *Eur. J. Clin. Microbiol. Infect. Dis.* **2012**, *31*, 2765–2772. [CrossRef]
9. Gern, J.E. The ABCs of rhinoviruses, wheezing, and asthma. *J. Virol.* **2010**, *84*, 7418–7426. [CrossRef]
10. Khetsuriani, N.; Lu, X.; Teague, W.G.; Kazerouni, N.; Anderson, L.J.; Erdman, D.D. Novel human rhinoviruses and exacerbation of asthma in children. *Emerg. Infect. Dis.* **2008**, *14*, 1793–1796. [CrossRef]
11. Calvo, C.; Garcia, M.L.; Pozo, F.; Reyes, N.; Pérez-Breña, P.; Casas, I. Role of rhinovirus C in apparently life-threatening events in infants, Spain. *Emerg. Infect. Dis.* **2009**, *15*, 1506–1508. [CrossRef]
12. Piralla, A.; Rovida, F.; Campanini, G.; Rognoni, V.; Marchi, A.; Locatelli, F.; Gerna, G. Clinical severity and molecular typing of human rhinovirus C strains during a fall outbreak affecting hospitalized patients. *J. Clin. Virol.* **2009**, *45*, 311–317. [CrossRef]
13. Reese, S.M.; Thompson, M.; Price, C.S.; Young, H.L. Evidence of nosocomial transmission of human rhinovirus in a neonatal intensive care unit. *Am. J. Infect. Control* **2016**, *44*, 355–357. [CrossRef]
14. Marcone, D.N.; Carballal, G.; Irañeta, M.; Rubies, Y.; Vidaurreta, S.M.; Echavarría, M. Nosocomial Transmission and Genetic Diversity of Rhinovirus in a Neonatal Intensive Care Unit. *J. Pediatr.* **2018**, *193*, 252–255.e1. [CrossRef]
15. Oberste, M.S.; Maher, K.; Schnurr, D.; Flemister, M.R.; Lovchik, J.C.; Peters, H.; Sessions, W.; Kirk, C.; Chatterjee, N.; Fuller, S.; et al. Enterovirus 68 is associated with respiratory illness and shares biological features with both the enteroviruses and the rhinoviruses. *J. Gen. Virol.* **2004**, *85 Pt 9*, 2577–2584. [CrossRef]
16. Piralla, A.; Principi, N.; Ruggiero, L.; Girello, A.; Giardina, F.; De Sando, E.; Caimmi, S.; Bianchini, S.; Marseglia, G.L.; Lunghi, G.; et al. Enterovirus-D68 (EV-D68) in pediatric patients with respiratory infection: The circulation of a new B3 clade in Italy. *J. Clin. Virol.* **2018**, *99–100*, 91–96. [CrossRef]
17. Piralla, A.; Lilleri, D.; Sarasini, A.; Marchi, A.; Zecca, M.; Stronati, M.; Baldanti, F.; Gerna, G. Human rhinovirus and human respiratory enterovirus (EV68 and EV104) infections in hospitalized patients in Italy, 2008–2009. *Diagn. Microbiol. Infect. Dis.* **2012**, *73*, 162–167. [CrossRef]

18. Engelmann, I.; Dewilde, A.; Lazrek, M.; Batteux, M.; Hamissi, A.; Yakoub-Agha, I.; Hober, D. In Vivo Persistence of Human Rhinoviruses in Immunosuppressed Patients. *PLoS ONE* **2017**, *12*, e0170774, Erratum in *PLoS ONE* **2017**, *12*, e0181296. [CrossRef]
19. Piralla, A.; Pariani, E.; Rovida, F.; Campanini, G.; Muzzi, A.; Emmi, V.; Iotti, G.A.; Pesenti, A.; Conaldi, P.G.; Zanetti, A.; et al. Severe Influenza A Task Force. Segregation of virulent influenza A(H1N1) variants in the lower respiratory tract of critically ill patients during the 2010–2011 seasonal epidemic. *PLoS ONE* **2011**, *6*, e28332. [CrossRef]
20. Wisdom, A.; Leitch, E.C.; Gaunt, E.; Harvala, H.; Simmonds, P. Screening respiratory samples for detection of human rhinoviruses (HRVs) and enteroviruses: Comprehensive VP4-VP2 typing reveals high incidence and genetic diversity of HRV species, C. *J. Clin. Microbiol.* **2009**, *47*, 3958–3967. [CrossRef]
21. Nix, W.A.; Oberste, M.S.; Pallansch, M.A. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. *J. Clin. Microbiol.* **2006**, *44*, 2698–2704. [CrossRef]
22. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739. [CrossRef]
23. Kenmoe, S.; Sadeuh-Mba, S.A.; Vernet, M.A.; Penlap Beng, V.; Vabret, A.; Njouom, R. Molecular epidemiology of Enteroviruses and Rhinoviruses in patients with acute respiratory infections in Yaounde, Cameroon. *Influenza Other Respir. Viruses* **2021**, *15*, 641–650. [CrossRef]
24. van der Linden, L.; Bruning, A.H.; Thomas, X.V.; Minnaar, R.P.; Rebers, S.P.; Schinkel, J.; de Jong, M.D.; Pajkrt, D.; Wolthers, K.C. A molecular epidemiological perspective of rhinovirus types circulating in Amsterdam from 2007 to 2012. *Clin. Microbiol. Infect.* **2016**, *22*, 1002.e9–1002.e14. [CrossRef]
25. Kamau, E.; Onyango, C.O.; Otieno, G.P.; Kiyuka, P.K.; Agoti, C.N.; Medley, G.F.; Cane, P.A.; Nokes, D.J.; Munywoki, P.K. An Intensive, Active Surveillance Reveals Continuous Invasion and High Diversity of Rhinovirus in Households. *J. Infect. Dis.* **2019**, *219*, 1049–1057. [CrossRef]
26. Hung, H.M.; Yang, S.L.; Chen, C.J.; Chiu, C.H.; Kuo, C.Y.; Huang, K.A.; Lin, T.Y.; Hsieh, Y.C.; Gong, Y.N.; Tsao, K.C.; et al. Molecular epidemiology and clinical features of rhinovirus infections among hospitalized patients in a medical center in Taiwan. *J. Microbiol. Immunol. Infect.* **2019**, *52*, 233–241. [CrossRef]
27. Esposito, S.; Daleno, C.; Tagliabue, C.; Scala, A.; Tenconi, R.; Borzani, I.; Fossali, E.; Pelucchi, C.; Piralla, A.; Principi, N. Impact of rhinoviruses on pediatric community-acquired pneumonia. *Eur. J. Clin. Microbiol. Infect. Dis.* **2012**, *31*, 1637–1645. [CrossRef]
28. Adam, D.C.; Chen, X.; Scotch, M.; MacIntyre, C.R.; Dwyer, D.; Kok, J. The Molecular Epidemiology and Clinical Phylogenetics of Rhinoviruses Among Paediatric Cases in Sydney, Australia. *Int. J. Infect. Dis.* **2021**, *110*, 69–74. [CrossRef]
29. Bruning, A.H.L.; Thomas, X.V.; van der Linden, L.; Wildenbeest, J.G.; Minnaar, R.P.; Jansen, R.R.; de Jong, M.D.; Sterk, P.J.; van der Schee, M.P.; Wolthers, K.C.; et al. Clinical, virological and epidemiological characteristics of rhinovirus infections in early childhood: A comparison between non-hospitalised and hospitalised children. *J. Clin. Virol.* **2015**, *73*, 120–126. [CrossRef]
30. Zlateva, K.T.; van Rijn, A.L.; Simmonds, P.; Coenjaerts, F.E.J.; van Loon, A.M.; Verheij, T.J.M.; de Vries, J.J.C.; Little, P.; Butler, C.C.; van Zwet, E.W.; et al. GRACE Study Group. Molecular epidemiology and clinical impact of rhinovirus infections in adults during three epidemic seasons in 11 European countries (2007–2010). *Thorax* **2020**, *75*, 882–890. [CrossRef]
31. Zhao, Y.; Shen, J.; Wu, B.; Liu, G.; Lu, R.; Tan, W. Genotypic Diversity and Epidemiology of Human Rhinovirus Among Children with Severe Acute Respiratory Tract Infection in Shanghai, 2013–2015. *Front. Microbiol.* **2018**, *9*, 1836. [CrossRef]
32. El Idrissi, K.R.; Isabel, S.; Carbonneau, J.; Lafond, M.; Quach, C.; Caya, C.; Fontela, P.S.; Beltempo, M.; Boivin, G.; Lefebvre, M.A.; et al. Molecular and epidemiologic investigation of a rhinovirus outbreak in a neonatal intensive care unit. *Infect. Control Hosp. Epidemiol.* **2019**, *40*, 245–247. [CrossRef]
33. Reid, A.B.; Anderson, T.L.; Cooley, L.; Williamson, J.; Mcgregor, A.R. An outbreak of human rhinovirus species C infections in a neonatal intensive care unit. *Pediatr. Infect. Dis. J.* **2011**, *30*, 1095–1096. [CrossRef]
34. Gerna, G.; Piralla, A.; Rovida, F.; Rognoni, V.; Marchi, A.; Locatelli, F.; Meloni, F. Correlation of rhinovirus load in the respiratory tract and clinical symptoms in hospitalized immunocompetent and immunocompromised patients. *J. Med. Virol.* **2009**, *81*, 1498–1507. [CrossRef]
35. Tapparel, C.; Cordey, S.; Junier, T.; Farinelli, L.; Van Belle, S.; Soccal, P.M.; Aubert, J.D.; Zdobnov, E.; Kaiser, L. Rhinovirus genome variation during chronic upper and lower respiratory tract infections. *PLoS ONE* **2011**, *6*, e21163. [CrossRef]
36. Ammerman, E.; Sweet, S.C.; Storch, G.A.; Buller, R.S.; Mason, S.; Conrad, C.; Hayes, D., Jr.; Faro, A.; Goldfarb, S.B.; Melicoff, E.; et al. Epidemiology and persistence of rhinovirus in pediatric lung transplantation. *Transpl. Infect. Dis.* **2020**, *22*, e13422. [CrossRef]
37. Jain, S.; Self, W.H.; Wunderink, R.G.; Fakhran, S.; Balk, R.; Bramley, A.M.; Reed, C.; Grijalva, C.G.; Anderson, E.J.; Courtney, D.M.; et al. Community-Acquired Pneumonia Requiring Hospitalization among, U.S. Adults. *N. Engl. J. Med.* **2015**, *373*, 415–427. [CrossRef]
38. Jain, S.; Williams, D.J.; Arnold, S.R.; Ampofo, K.; Bramley, A.M.; Reed, C.; Stockmann, C.; Anderson, E.J.; Grijalva, C.G.; Self, W.H.; et al. Community-acquired pneumonia requiring hospitalization among, U.S. children. *N. Engl. J. Med.* **2015**, *372*, 835–845. [CrossRef]
39. Piralla, A.; Mariani, B.; Rovida, F.; Baldanti, F. Frequency of respiratory viruses among patients admitted to 26 Intensive Care Units in seven consecutive winter-spring seasons (2009–2016) in Northern Italy. *J. Clin. Virol.* **2017**, *92*, 48–51. [CrossRef]

40. Visseaux, B.; Burdet, C.; Voiriot, G.; Lescure, F.X.; Chougar, T.; Brugière, O.; Crestani, B.; Casalino, E.; Charpentier, C.; Descamps, D.; et al. Prevalence of respiratory viruses among adults, by season, age, respiratory tract region and type of medical unit in Paris, France, from 2011 to 2016. *PLoS ONE* **2017**, *12*, e0180888. [CrossRef]
41. Choi, S.H.; Hong, S.B.; Ko, G.B.; Lee, Y.; Park, H.J.; Park, S.Y.; Moon, S.M.; Cho, O.H.; Park, K.H.; Chong, Y.P.; et al. Viral infection in patients with severe pneumonia requiring intensive care unit admission. *Am. J. Respir. Crit. Care Med.* **2012**, *186*, 325–332. [CrossRef]
42. Wiemken, T.; Peyrani, P.; Bryant, K.; Kelley, R.R.; Summersgill, J.; Arnold, F.; Carrico, R.; McKinney, W.P.; Jonsson, C.; Carrico, K.; et al. Incidence of respiratory viruses in patients with community-acquired pneumonia admitted to the intensive care unit: Results from the Severe Influenza Pneumonia Surveillance (SIPS) project. *Eur. J. Clin. Microbiol. Infect. Dis.* **2013**, *32*, 705–710. [CrossRef]
43. Piralla, A.; Baldanti, F.; Gerna, G. Phylogenetic patterns of human respiratory picornavirus species, including the newly identified group C rhinoviruses, during a 1-year surveillance of a hospitalized patient population in Italy. *J. Clin. Microbiol.* **2011**, *49*, 373–376. [CrossRef]
44. Baldanti, F.; Campanini, G.; Piralla, A.; Rovida, F.; Braschi, A.; Mojoli, F.; Iotti, G.; Belliato, M.; Conaldi, P.G.; Arcadipane, A.; et al. Severe outcome of influenza A/H1N1/09v infection associated with 222G/N polymorphisms in the haemagglutinin: A multicentre study. *Clin. Microbiol. Infect.* **2011**, *17*, 1166–1169. [CrossRef]

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RESEARCH ARTICLE

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Molecular characterization of emerging Echovirus 11 (E11) shed light on the recombinant origin of a variant associated with severe hepatitis in neonates

Antonio Piralla[1] | Federica Giardina[2] | Guglielmo Ferrari[1] | Stefano Gaiarsa[1] | Greta Romano[1] | Laura Pellegrinelli[3] | Cristina Galli[3] | Arlinda Seiti[3] | Sandro Binda[3] | Antonino Maria Guglielmo Pitrolo[1] | Angelo Genoni[4,5] | Francesca Drago Ferrante[5] | Federica Novazzi[4,5] | Nicasio Mancini[4,5] | Francesca Rovida[1,2] | Elena Pariani[3] | Fausto Baldanti[1,2]

[1]Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

[2]Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy

[3]Department of Biomedical Sciences for Health, University of Milan, Milan, Italy

[4]Laboratory of Medical Microbiology and Virology, Department of Medicine and Technical Innovation, University of Insubria, Varese, Italy

[5]Laboratory of Medical Microbiology and Virology, University Hospital of Varese, Varese, Italy

Correspondence

Antonio Piralla, Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy.
Email: antonio.piralla@unipv.it

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Abstract

Echovirus 11 (E11) has gained attention owing to its association with severe neonatal infections. Due to the limited data available, the World Health Organization (WHO) considers public health risk to the general population to be low. The present study investigated the genetic variation and molecular evolution of E11 genomes collected from May to December 2023. Whole genome sequencing (WGS) was performed for 16 E11 strains. Phylogenetic analysis on WG showed how all Italian strains belonged to genogroup D5, similarly to other E11 strains recently reported in France and Germany all together aggregated into separate clusters. A cluster-specific recombination pattern was also identified using phylogenetic analysis of different genome regions. Echovirus 6 was identified as the major recombinant virus in $3C^{pro}$ and $3D^{pol}$ regions. The molecular clock analysis revealed that the recombination event probably occurred in June 2018 (95% HPD interval: Jan 2016–Jan 2020). Shannon entropy analyses, within P1 region, showed how 11 amino acids exhibited relatively high entropy. Five of them were exposed on the canyon region which is responsible for receptor binding with the neonatal Fc receptor. The present study showed the recombinant origin of a new lineage of E11 associated with severe neonatal infections.

KEYWORDS

Echovirus 11 (E-11), enteroviruses, hepatitis, neonatal infection, recombinant strain

1 | INTRODUCTION

An upsurge of severe neonatal cases and fatalities linked to a novel variant of Echovirus 11 (E11) was documented in France, Italy and China.[1–3] World Health Organization (WHO) has gained attention owing to its association with severe neonatal infections since 2022.[4,5] Other cases have been reported occurring in European countries such as Spain, Sweden, and UK.[4,5] However, the prevention and control of E11 variants have been hampered by limited background data on the virus circulation and genetic variance. Additionally, the World Health Organization (WHO), having evaluated the limited data available, considers the public health risk to the general population to be low. Considering that non-polio enterovirus (NPEV) infections are not notifiable infectious diseases in Italy, the circulation of E11 in Lombardy (Northern Italy), where the first two cases of E11 neonatal infection were reported, has been implemented in the period April-December 2023. In detail, an event-based surveillance and a hospital-based surveillance considering inpatients exhibiting respiratory or neurological symptoms have showed a total of 20 additional cases.[6] Recently, a wastewater-based surveillance (WBS) performed in Sicily (a region in Southern Italy) between June 2022 and June 2023, showed that the new E11 lineage has circulated in this region during early 2023.[7] This report together with European data showed a silent unrecognized circulation of this new E11 variant.

The emergence of the novel E11 lineage has been related to recombination events, which apparently allowed this variant strain to infect humans more successfully.[1] However, a complete analysis exploring the parental genomes, genetic variability, and recombinant origin of this emerging variant is still missing. In addition, the increasing pathogenetic role of this variant needs to be fully elucidated with in-vitro models. In the present study, the genetic variation and molecular evolution of the E11 complete genomes through the collection of strains among our surveillances and E11 sequences published in the GenBank database have been investigated.

2 | MATERIAL AND METHODS

2.1 | Sample collection

A total of 16 E11 strains used in this study were from E11-positive patients identified in an event-based surveillance and a hospital-based surveillance considering inpatients exhibiting respiratory or neurological symptoms from May to December 2023. Our analysis aims to describe and examine the circulation of E11 in Lombardy (Northern Italy) as well as include WGS of the first two cases of E11 neonatal infection previously reported in Italy.[2,6] All these samples were analysed in two Lombardy's regional reference laboratories (Microbiology and Virology department, Fondazione IRCCS Policlinico San Matteo, Pavia, and Department of Biomedical Sciences for Health, University of Milan, Milan) as previously reported[2,6] and additional information of clinical samples were reported in Table S1.

2.2 | Next-generation sequencing by metagenomic approach

Total RNA was extracted directly from clinical samples using the QIAsymphony® instrument with QIAsymphony® DSP Virus/Pathogen Midi Kit (Complex 400 protocol (QIAGEN) or QIAamp Viral RNA Mini kit (QIAGEN) by means of an automated extractor (QIAcube, QIAGEN) according to the manufacturer's instructions. RNA was treated with TURBO DNase (Thermo Fisher Scientific, Waltham, MA) at 37°C for 20 min and then purified by RNA Clean and Concentrator-5 Kit (Zymo Research). RNA was used for the assessment of sequencing independent single primer amplification protocol (SISPA) with some modifications reported by Lorusso et al.[8] Libraries were prepared using Nextera DNA Flex Library Prep (Illumina Inc., San Diego, CA) according to the manufacturer's protocol. Sequencing was performed on the MiSeq (Illumina Inc., San Diego, CA) by MiSeq Reagent v2 (300-cycle). The obtained FastaQ were analysed with the CZ ID metagenomic pipeline.[9] The E11 virus consensus sequences were obtained by mapping to the reference with the highest coverage breadth and depth, obtained through the metagenomic pipeline. Accession number of sequences originated in the study are PP498690-PP498703.

2.3 | Phylogenetic analysis and recombinant analysis

All available E11 genomes ($n = 100$ strains) were downloaded from GenBank and used together with the 16 E11 strains originated in this study. Alignment was performed using MAFFT 7.475.[10] Maximum likelihood (ML) trees were constructed in IQ-TREE5[11] with a substitution model chosen according to BIC within the IQ-TREE internal pipeline with 1000 bootstrap replicates. DNA similarity searches of P1, P2, and P3 coding region sequences were performed separately using the NCBI WWW-BLAST (basic local alignment search tool) server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) on the GenBank DNA database (BLAST+ v. 2.15.0). Sequences with >85% similarity were considered prospective parental sequences and retrieved from GenBank. A data set of 15 enterovirus strains (listed in Figure 2 and Table S2) were used for recombination signal screening using SimPlot software (version 3.5.1).[12] A similarity and boot-scanning analysis were performed using a 400-nucleotide sliding window and shifting of 10-nucleotides.

2.4 | Phylodynamic analysis of recombinant genome region

NCBI BLASTn program (https://blast.ncbi.nlm.nih.gov) was used to perform multiple sequence alignments of E-11 new lineage in the $3C^{pro}$-$3D^{pol}$ genome region. Consensus sequences were inspected for nucleotide identity percentage ranging from 85.0% to 100% with a total of 63 sequences used to perform phylogenetic analysis. The

phylodynamic analyses workflow was showed in Figure S1. In detail, MEGA version 11[13] was used to perform the alignment using implemented MUSCLE algorithm and to build the phylogenetic tree based on a Neighbor-joining method with 1,000 bootstrap replicates and a mean nucleotide genetic distance (p-distance). MEGA version 11 and ModelFinder from IQ-TREE v.2.2.2.6[14] were used to select the best nucleotide substitution model (GTR + G + I). The temporal signal of the sequences was investigated using TempEst v.1.5.3[15] to confirm the presence of sufficient genetic change between sampling times ($R^2 = 0.82$, Correlation coefficient = 0.9). A Markov Chain Monte Carlo (MCMC) sampling method, implemented in BEAST v1.10.4,[16] a Bayesian statistical framework, was used to perform phylogenetic analysis and the years of samples collection (retrieved from GenBank) were used to calibrate the molecular clock.

The analyses were performed using an uncorrelated lognormal clock with a constant size model of demographic history. The program default priors on the substitution model (GTR + G) parameters were used in these analyses. Parameter estimates were obtained from MCMC run of 2×10^8 generation and a sampling frequency of 10^3. The performance of the transition kernel was inspected, and the acceptance ratio was greater than 0.234. The posterior distribution for each parameter was visualized with Tracer v.1.7.2,[16] a MCMC trace analysis tool which also estimated the Effective Sample Size (ESS) (i.e., measurement of the number of effectively independent samples in each run) of the parameters sampled from the MCMC. The analysis was considered to have converged and reached stability after the burn-in period when ESS was higher than 200. Maximum clade credibility tree was estimated with TreeAnnotator v1.10.4[16] removing the first 10% of trees as burn-in. Statistical support for the nodes in topology was assessed by a posterior probability (pp) value. The tree was visualized with FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree).

2.5 | Genome mutations analysis

The presence of conserved non-synonymous mutations between the two putative donor genomes E11 (OQ966171) and E6 (OR840838) and the new lineage sequences ($n = 37$ E11 strains) was assessed using the Snipit pipeline (https://github.com/aineniamh/snipit),[17] which was modified to select non-synonymous mutations and to work with amino acid notation.

2.6 | Shannon entropy analysis for measuring diversity

Amino acid variability of the P1 capsid precursor protein (VP4-VP2-VP3-VP1) was assessed using Shannon entropy on the E11 sequences data set ($n = 116$) used for phylogenetic analysis (described above). Shannon entropy was assessed for all 861 amino acids of P1 using an online analysis tool (available at https://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_one.html),[18] with extremely variable amino acid sites defined as those with entropy values > 0.6. The ChimeraX program[19] was used to visualize the distinct distributions of significant sites based on the 6LA6 model from the Protein Data Bank (PDB) database showing the concealed surface area between the FcRn receptor and capsid proteins (https://www.rcsb.org/structure/6la6).

3 | RESULTS

3.1 | Whole-genome sequence analysis of E11 strains

A total of 16 E11 WGS strains were performed in the present study and included in the analyses. A large open reading frame (ORF) encoding a potential polyprotein precursor of 2195 aa was cleaved into P1 (VP4, VP2, VP3, and VP1), P2 ($2A^{pro}$, 2B, and 2C), and P3 (3A, 3B, $3C^{pro}$, and $3D^{pol}$) regions with 861 aa, 578 aa, and 756 aa, respectively. All available E11 sequences ($n = 100$) retrieved from GenBank were included in a data set used for phylogenetic analysis. The phylogenetic tree showed that all investigated strains belonged to genogroup D5,, similar to other E11 strains recently reported in France and Germany aaccording to Savolainen-Kopra et al., 2009.[20] All aggregated into a separate lineage, including 37 E11 strains (Figure 1A). Within this new lineage, the average nucleotide genetic identity was 98.7% (range 97.9%–100%). Furthermore, nucleotide identity comparisons of WGS demonstrated that the E11 strain, which belonged to a new lineage, had an average of nucleotide genetic identity of 85.1% (range 81.9%–94.2%) compared to other E11 reference strains available in the GenBank database ($n = 79$ strains). A cluster-specific recombination pattern was also identified using phylogenetic analysis of the P1, P2, and P3 genome regions, and was analysed separately (data not showed). In the P1 and P2 capsid coding regions, the new lineage strains clustered together with the strain PMB_230005716902_FRA_2023 (OQ969171, Figure 1B). In the tree based on the P3 sequences, the new lineage strains clustered outside the E11 tree as an outgroup (data not showed). BLAST analysis of the P3 region sequence showed a high nucleotide identity >95% with the Echovirus 6 strain EV6_Fr22_-MAR9310 (OR840838 published in GenBank on December 18, 2023) suggesting the occurrence of one putative recombination event.

3.2 | Recombination and evolutionary analyses

BLAST results combined with the phylogenetic trees reported in Figure 1 showed that E6 was identified as the major putative parent for $3C^{pro}$ and $3D^{pol}$ regions (Figure 1C). SimPlot software was used to determine the recombination site position using all the 37 E11 strains, belonged to the new lineage, as query sequences (Figure 2A). The SimPlot results confirmed that the E11 strains had the highest similarity with the E11 (OQ969171) prototype strain in the P1, P2 and 3A-3B regions. Whereas in the $3C^{pro}$ and $3D^{pol}$ regions, the

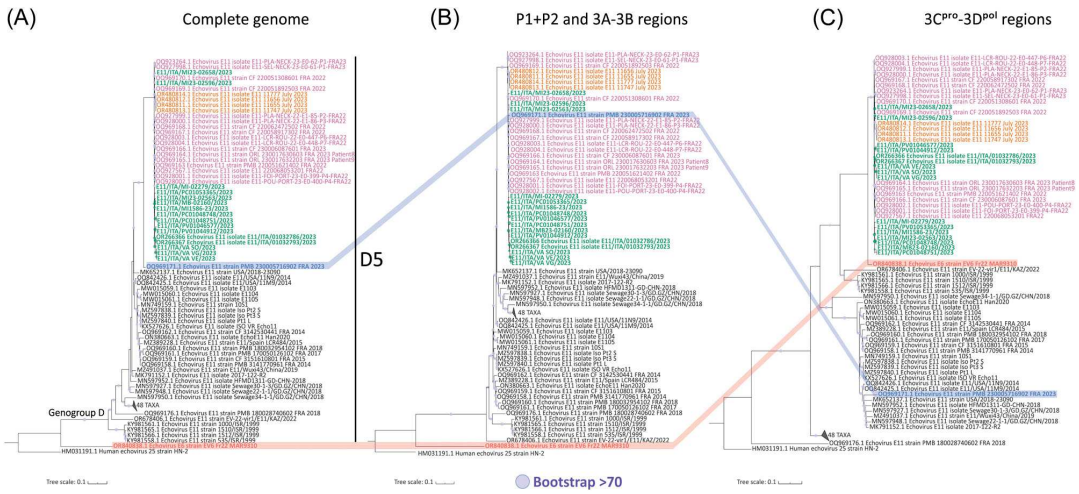

FIGURE 1 ML Phylogenetic trees constructed based on (A) complete genome (B) P1-P2 and 3A-3B (C) $3C^{pro}$-$3D^{pol}$ genome regions. Italian E11 strains ($n = 16$) are colored in green and their most related enterovirus sequences are reported in cerulean (E11, OQ696171) and coral (E6; OR840838), respectively. All available E11 strains belonged to new lineage ($n = 17$ from France[1] reported in purple and $n = 4$ from Germany reported in orange) are included. Scale bars represent the replacement of each site per year.

highest similarity score was observed with the E6 strain (OR840838). The phylogenetic trees established with the selected sequences (Figure 1B and C) were consistent with the results of SimPlot (Figure 2A) and BootScanning (Figure 2B), which confirmed the results of the recombination analysis.

To establish the timing of the recombination event, a phylodynamic analysis was performed using an alignment of multiple sequences of the $3C^{pro}$ and $3D^{pol}$ regions based on similarities obtained from BLAST analysis. The topology inferred by Beast package using the best fit models was used to describe the phylogenetic relationships that better characterized the samples of E11 new lineage strains and E6 as the parental genome of the $3C^{pro}$ and $3D^{pol}$ regions. The Bayesian molecular clock analysis was performed to infer the MCC tree and presented in Figure 3. The Bayesian analysis estimated a mean evolutionary substitution rate of 6.32×10^{-3} subs/site/years (95% HPD interval: 4.31×10^{-3}–8.57×10^{-3}). The molecular clock analysis revealed that the recombination event probably occurred in June 2018 (95% HPD interval: Jan 2016–Jan 2020; Figure 3), also assuming the common ancestor for E11 (new lineage) and E6 strains (OR840838) in the $3C^{pro}$ and $3D^{pol}$ regions.

3.3 | Genetic variance of genome

To identify crucial amino acid mutation sites that may affect the virulence of the variants, the modified Snipit script (https://github.com/aineniamh/snipit) was used to visualize the relative changes of each amino acid site as compared to the most related strains. A total of 102/2195 (4.6%) amino acid positions were observed to have at least one change in the coding sequences alignment as compared to parental genomes (E11, OQ696171 and E6, OR840838) (Figure 4). Of these, 40/102 (39.2%) changes occurred within the P1 region (structural proteins), 28/102 (57.5%) within the P2 region, 8/102 (7.8%) within the 3A-3B region and 26/102 (25.5%) in the recombinant $3C^{pro}$ and $3D^{pol}$ regions. Among the changes of the P1 region, 21/40 (52.5%) are located within the VP1 protein but none of these changes were in the BC- and DE-loop. A total of 11 amino acid changes were fixed in all the E11 strains belonged to the new lineage.

An alignment of P1 sequences was also examined using the Shannon entropy online analysis tool. A total of 11 amino acids (VP2, 136 and 138; VP3, 35, 64, 135, and 234; VP1, 92, 144, 235, 262 and 268) exhibited relatively high entropy values (higher than 0.60), suggesting a notable degree of amino acid diversity (Figure 5A). Cryo-electron microscopic structure of E11 and FcRn was available and used to point out results of entropy analysis (Figure 5B). Seven amino acids selected were exposed on the surface of the capsid on a canyon region responsible for receptor binding and colored in white in the 3D model (Figure 5B).

4 | DISCUSSION

The evolution history of E11 was well characterized since the 2004 by the phylogenetic analysis on E-11 isolates identifying several genogroups named A, B, C, and D1–D5.[21] The monitoring of E11 evolution confirmed the prevalence of genogroup D5 with distinct strains in the last 15 years.[20,22] Since summer 2022, a divergent lineage of E11 belonging to genogroup D5 has recently been associated with an increased number of hepatitis episodes in

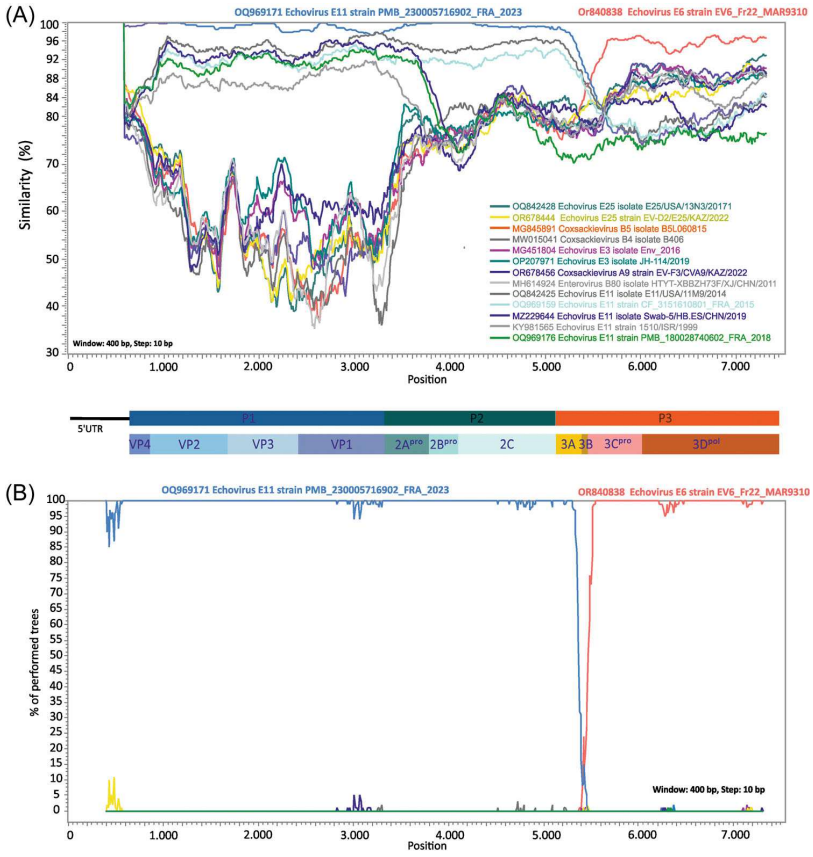

FIGURE 2 Recombination analyses of the E11 new lineage strains used as query ($n = 37$; $n = 16$ Italian, $n = 17$ from France[1] and $n = 4$ from Germany) with other EV-B strains. (A) Similarity plots and (B) boot scanning analyses of strains with potential parents.

neonates in Europe.[4] Since 2018, severe neonatal infections and mortality associated with genogroup D5 of E11 have been reported in Taiwan, and in the Hubei, and Guangdong provinces of China.[23,24] In addition, this scenario has also been described in a retrospective study in China reporting data from an E11 outbreak occurred in 2019.[3] In this report, severe infections defined as hepatic dysfunction or liver failure were observed in 30 out of 105 (28.6%) neonates.[3] However, a recently commentary of this study raises a concern about the results of those and potential new studies, if genomic data will be used to draw conclusions on association of E11 and unexpected clinical picture.[25]

The main medical needs emerged from a general point of view is the lack of notifiable status for enterovirus infections in many EU countries, thus there may be more cases than are currently known. This observation is directly linked to the unrecognized clinical presentations of the great majority of enterovirus infections. Data from previous studies suggest that more than 90% of patients with E11 infection are asymptomatic or present with mild fever.[26] Additionally, some enteroviruses may remain undetected for years before suddenly reappearing.[27] The lack of a notifiable disease status has resulted in low awareness among healthcare providers, possibly leading to an underdiagnosis of emerging enterovirus variants. An additional value could be obtained through wastewater surveillance, which can provide an early warning of viral spread in communities and offer crucial information about virus circulation and prevalence, as currently utilized for poliovirus.[28] In this perspective, an increased detection rate of E11 belonging to the new lineage in wastewater samples since August 2022 in the Sicily region (Southern Italy) has recently been reported.[7] These findings suggest that WBS is an important tool for enterovirus surveillance to promptly detect the emergence or re-emergence of variants that warrant public health control measures.

Phylogenetic analysis of WGS showed the spread of a monophyletic lineage including E11 strains identified in Italy, France, and Germany.[1,2,4] However, many other EU countries, such as Croatia, Spain, Sweden, and the UK have reported E11 cases during the 2022-2023 period but still no sequences are currently available.[4] Despite the limited public health impact assigned by the WHO to this

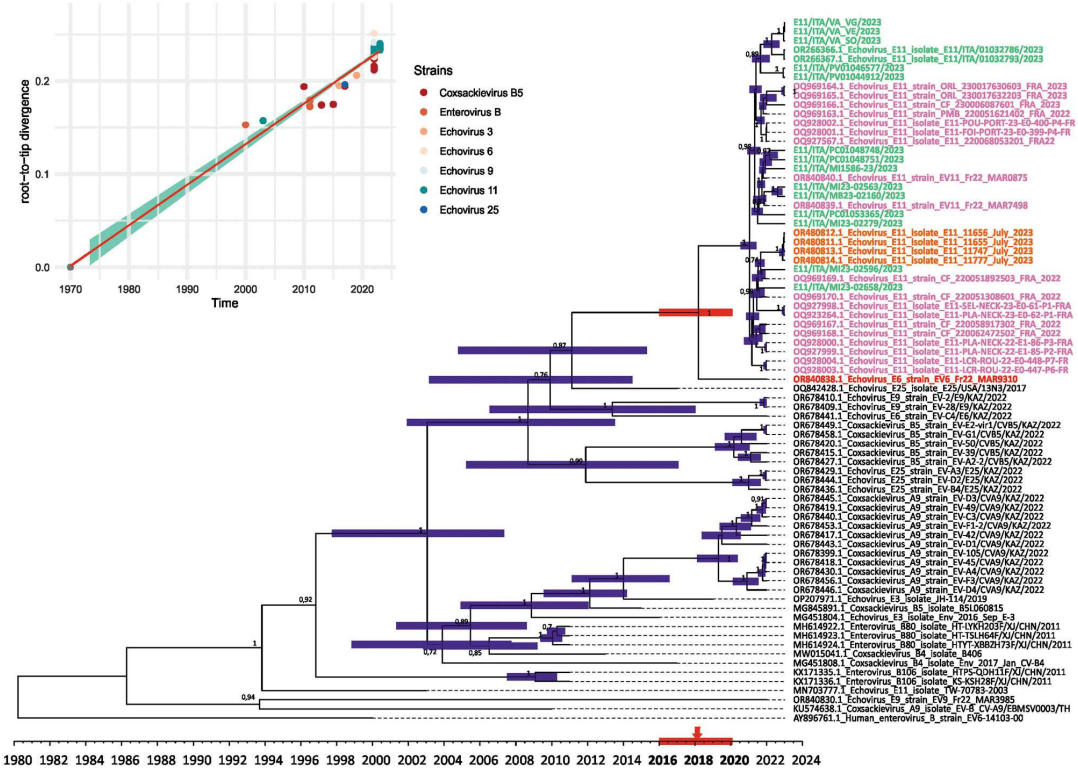

FIGURE 3 The MCC phylogenetic tree was generated using the MCMC method based on $3C^{pro}$-$3D^{pol}$ nucleotide sequences of Enterovirus strains with blast nucleotide identity results >85%. The blue bars indicate the 95% highest posterior density (HPD) for ancestor estimates. The HPD estimation of recombinant event is reported with a red bar. The *x*-axis is the time scale (years).

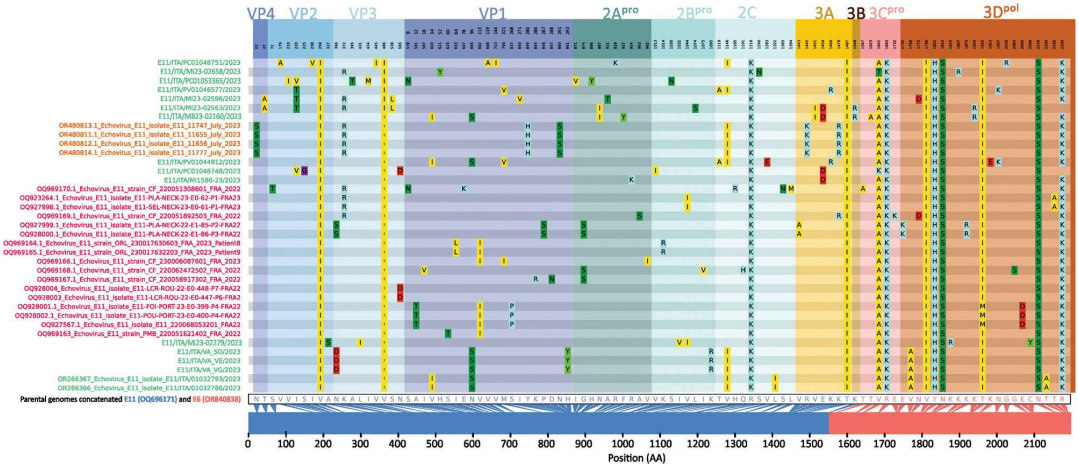

FIGURE 4 The figure was rendered using the *Snipit* tool and modified pipeline for amino acid visualization. (https://github.com/aineniamh/snipit). Italian E11 strains are colored in sea green. All available E11 strains belonged to new lineage (n = 17 from France[1] reported in purple and n = 4 from Germany reported in orange) are included.

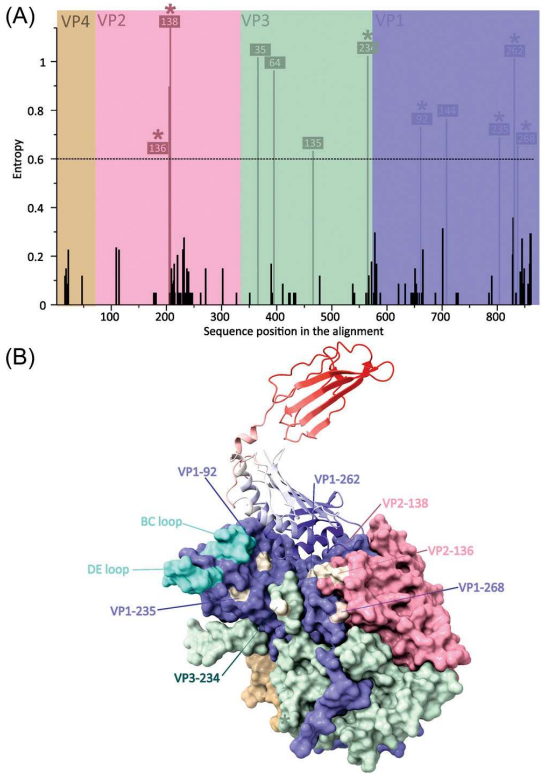

FIGURE 5 Analysis of the genetic variance of all available E11 sequences ($n = 116$). (A) Entropy value analysis of all 861 amino acids of the P1 region with a threshold value of 0.6. Position of amino acids with value > 0.6 are reported with a box. (B) 6LA6 as a model to illustrate the distribution of each amino acid site in E11 virus particles. The proteins are colored by chains: VP1 (blue), VP2 (rose gold), VP3 (green), VP4 (yellow). The potential interaction between amino acid sites and the FcRn receptor is also showed in the model. BC and DE loops are highlighted in cyan. Amino acids selected by entropy analysis and exposed at the surface of the 3D model and colored in white and reported with an asterisk in the panel A.

new E11 lineage, a consolidated surveillance system needs to be further implemented.[4] A phylogenetic incongruence between structural and nonstructural genes was observed suggesting that recombination events occurred by Grapin et al.[1] However, similarity as well as bootscan analyses presented in the French study were performed without the parental genome. In our study, the recombinant origin hypothesized by Grapin et al[1] was proved because on December 18th, 2023 was deposited in the public GenBank database a WGS of E6 strain (OR840838, Echovirus E6 strain EV6_Fr22_-MAR9310), identified in France in 2022. Therefore, further analyses using different approaches such as phylogenesis and algorithms on sequence similarity and detecting recombination (Bootscanning) revealed that E6 was the parental donor of the $3C^{pro}$ and $3D^{pol}$ genome regions for the new E11 lineage. Recombination is a well-known mechanism for enteroviruses evolution as previously observed for E11 as well.[29–31] However, none of these reports showed recombination in the $3C^{pro}$ and $3D^{pol}$ regions. More precise identification of the individual recombination events in E11 was achieved using time-correlated tree analysis and superimposition of branching points in the $3C^{pro}$ and $3D^{pol}$ tree, also previously used for EV-71.[32] The tMRCA of the new lineage of E11 and E6 viruses was dated between 2016 and 2020, suggesting a silent circulation of E11 until its emergence in 2022. This finding is also supported by the evolutionary rate calculated for E11, which is congruent with NPEV substitution rates estimated by others.[32,33]

Amino acid changes in structural proteins may affect viral virulence, as well described for NPEV.[34] Sequence comparison of the P1 region in the present study identified several positions with a significant Shanonn entropy not only located in the VP1 region. A series of seven (VP2, 136 and 138; VP3, 234; VP1, 92, 235, 262 and 268) amino acids with increasing entropy are neighboring to the "canyon" of the receptor-binding regions inside the monomeric structure of the capsid. This is a key area governing the binding of the FcRn receptor.[35] These mutations occurring at this specific site might affect the binding and the uncoating process of E11 and, thus, increase its transmission ability. None of the selected positions were situated in the critical binding regions for neutralizing antibodies that correspond to the BC or DE loop within the VP1 protein. Mutations in these epitopes have been associated with the virus ability to evade the immune system.[36] Although several specific amino acid mutations were observed in the present study, their significance requires further investigation. The impact of recombination on the virulence or pathogenesis of the new E11 lineage is currently undefined and needs further experimental investigation. In many cases other factors other than the genetic backbone of E11 could have driven the severity of these infections such as premature birth, lack of maternal immunity and the young age.

In conclusion, the present study showed the recombinant origin of a new lineage of E11 associated with severe neonatal infections. Further studies aiming at elucidating the increased pathogenicity of E11 variant are needed to better correlate genetic information with unexpected clinical presentations. WGS of enteroviruses is needed to evaluate the presence of recombinant strains and to better evaluate the phylodynamic and phylogeography in the context of molecular epidemiology of emerging enterovirus variants.

AUTHOR CONTRIBUTIONS

Antonio Piralla wrote the manuscript and design the study and experiments. Federica Giardina, performed most of the experiments with help from Guglielmo Ferrari, Antonino Maria Guglielmo Pitrolo, Laura Pellegrinelli, Cristina Galli, Arlinda Seiti, Angelo Genoni, Francesca Drago Ferrante, and Federica Novazzi. Stefano Gaiarsa, Greta Romano performed bioinformatic analysis; Sandro Binda, Nicasio Mancini, and Francesca Rovida contributed to the discussion and provided reagents., Elena Pariani, Fausto Baldanti supervised the overall study and reviewed the manuscript and provided resources. All of authors helped with data analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article. The data that support the findings of this study are available in the supplementary material of this article. Sequence data generated in this study have been submitted to GenBank under accession numbers PP498690-PP498703.

ETHICS STATEMENT

Ethical review and approval were waived for this study, considering that the study was conducted on referred samples as part of the routine activities of the Italian influenza and other respiratory virus surveillance network (InfluNet & RespirVirNet) and the routine management and treatment of patients.

ORCID

Antonio Piralla http://orcid.org/0000-0002-6062-2579
Laura Pellegrinelli http://orcid.org/0000-0003-2344-6032
Francesca Drago Ferrante http://orcid.org/0000-0003-4950-0703
Fausto Baldanti http://orcid.org/0000-0002-3358-8969

REFERENCES

1. Grapin M, Mirand A, Pinquier D, et al. Severe and fatal neonatal infections linked to a new variant of echovirus 11, France, July 2022 to April 2023. *Euro Surveill*. 2023;28(22):2300253.
2. Piralla A, Borghesi A, Di Comite A, et al. Fulminant Echovirus 11 hepatitis in male non-identical twins in northern Italy, April 2023. *Euro Surveill*. 2023;28(24):2300289.
3. Wang P, Xu Y, Liu M, et al. Risk factors and early markers for echovirus type 11 associated haemorrhage-hepatitis syndrome in neonates, a retrospective cohort study. *Front Pediatr*. 2023;11:1063558.
4. World Health Organization. Enterovirus-Echovirus 11 Infection - the European Region. WHO. Accessed February 27, 2024. https://www.who.int/emergencies/disease-outbreak-news/item/2023-DON474
5. Word Health Organization. *Enterovirus Infection - France. WHO*, 2024. Accessed February 27, 2024. https://www.who.int/emergencies/disease-outbreak-news/item/2023-DON469
6. Pellegrinelli L, Galli C, Giardina F, et al. Increased circulation of echovirus 11 in the general population and hospital patients as elicited by the non-polio enterovirus laboratory-based sentinel surveillance in northern Italy, 2023. *Int J Infect Dis*. 2024;142:106998. doi:10.1016/j.ijid.2024.106998
7. Giammanco GM, Filizzolo C, Pizzo M, et al. Detection of Echovirus 11 lineage 1 in wastewater samples in Sicily. *Sci Total Environ*. 2024;918:170519. doi:10.1016/j.scitotenv.2024.170519
8. Lorusso A, Calistri P, Mercante MT, et al. A "One-Health" approach for diagnosis and molecular characterization of SARS-CoV-2 in Italy. *One Health*. 2020;10:100135.
9. Kalantar KL, Carvalho T, de Bourcy CFA, et al. IDseq-An open source cloud-based pipeline and analysis service for metagenomic pathogen detection and monitoring. *Gigascience*. 2020;9(10):giaa111.
10. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol*. 2013;30(4):772-780. doi:10.1093/molbev/mst010
11. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol*. 2015;32(1):268-274.
12. Lole KS, Bollinger RC, Paranjape RS, et al. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J Virol*. 1999;73:152-160.
13. Tamura K, Stecher G, Kumar S. MEGA11: molecular evolutionary genetics analysis version 11. *Mol Biol Evol*. 2021;38:3022-3027.
14. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods*. 2017;14(6):587-589.
15. Rambaut A, Lam TT, Max Carvalho L, Pybus OG. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). *Virus Evol*. 2016;2(1):vew007.
16. Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol*. 2018;4(1):vey016.
17. O'Toole Á: *Aineniamh/Snipit*. 2021. https://github.com/aineniamh/snipit
18. Ming D, Cohn JD, Wall ME. Fast dynamics perturbation analysis for prediction of protein functional sites. *BMC Struct Biol*. 2008;8:5. doi:10.1186/1472-6807-8-5
19. Meng EC, Goddard TD, Pettersen EF, et al. UCSF ChimeraX: tools for structure building and analysis. *Prot Sci*. 2023;32:e4792.
20. Savolainen-Kopra C, Al-Hello H, Paananen A, et al. Molecular epidemiology and dual serotype specificity detection of echovirus 11 strains in Finland. *Virus Res*. 2009;139(1):32-38. doi:10.1016/j.virusres.2008.10.003
21. Oberste MS, Maher K, Pallansch MA. Evidence for frequent recombination within species human enterovirus B based on complete genomic sequences of all thirty-seven serotypes. *J Virol*. 2004;78(2):855-867. doi:10.1128/jvi.78.2.855-867.2004
22. Al-Hello H, Paananen A, Eskelinen M, et al. An enterovirus strain isolated from diabetic child belongs to a genetic subcluster of echovirus 11, but is also neutralised with monotypic antisera to coxsackievirus A9. *J Gen Virol*. 2008;89(Pt 8):1949-1959. doi:10.1099/vir.0.83474-0
23. Ho S-Y, Chiu C-H, Huang Y-C, et al. Investigation and successful control of an echovirus 11 outbreak in neonatal intensive care units. *Pediatr Neonatol*. 2020;61:180-187.
24. Lu J, Kang M, Zeng H, et al. Tracking echovirus eleven outbreaks in Guangdong, China: a metatranscriptomic, phylogenetic, and epidemiological study. *Virus Evol*. 2020;6:veaa029.
25. Al-Hello H, Blomqvist S, Savolainen-Kopra C. Commentary: risk factors and early markers for echovirus type 11 associated haemorrhage-hepatitis syndrome in neonates, a retrospective cohort study. *Front Pediatr*. 2024;12:1338097. doi:10.3389/fped.2024.1338097
26. Chuang YY, Huang YC. Enteroviral infection in neonates. *J Microbiol Immunol Infect*. 2019;52(6):851-857. doi:10.1016/j.jmii.2019.08.018
27. Forero EL, Knoester M, Gard L, et al. Changes in enterovirus epidemiology after easing of lockdown measures. *J Clin Virol*. 2023;169:105617.

28. World Health Organization. Global Polio Eradication Initiative—Guidelines on Environmental Surveillance for Detection of Polioviruses. 2015a. Accessed February 12, 2024. http://polioeradication.org/wp-content/uploads/2016/07/GPLN_GuidelinesES_April2015.pdf
29. Chevaliez S, Szendröi A, Caro V, et al. Molecular comparison of echovirus 11 strains circulating in Europe during an epidemic of multisystem hemorrhagic disease of infants indicates that evolution generally occurs by recombination. *Virology*. 2004;325(1):56-70.
30. Gong YN, Yang SL, Chen YC, Liu YC, Huang YC, Tsao KC. Novel intertypic recombination of Echovirus 11 in the Enterovirus species B. *J Med Virol*. 2024;96(1):e29323. doi:10.1002/jmv.29323
31. Rueca M, Lanini S, Giombini E, et al. Detection of recombinant breakpoint in the genome of human enterovirus E11 strain associated with a fatal nosocomial outbreak. *Virol J*. 2022;19(1):97. doi:10.1186/s12985-022-01821-2
32. McWilliam Leitch EC, Cabrerizo M, Cardosa J, et al. The association of recombination events in the founding and emergence of subgenogroup evolutionary lineages of human enterovirus 71. *J Virol*. 2012;86(5):2676-2685. doi:10.1128/JVI.06065-11
33. Lukashev AN, Vakulenko YA. Molecular evolution of types in non-polio enteroviruses. *J Gen Virol*. 2017;98(12):2968-2981. doi:10.1099/jgv.0.000966
34. Bek EJ, McMinn PC. Recent advances in research on human enterovirus 71. *Future Virol*. 2010;5:453-468.
35. Molecular and structural basis of Echovirus 11 infection by using the dual-receptor system of CD55 and FcRn. *CSB*, 65(1):67-79.
36. Zhang Y, Li J, Li Q. Immune evasion of enteroviruses under innate immune monitoring. *Front Microbiol*. 2018;9:1866. doi:10.3389/fmicb.2018.01866

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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On the lookout for influenza viruses in Italy during the 2021-2022 season: Along came A(H3N2) viruses with a new phylogenetic makeup of their hemagglutinin

Cristina Galli [a,1], Laura Pellegrinelli [a,1], Federica Giardina [b], Guglielmo Ferrari [b], Sara Colonia Uceda Renteria [c], Federica Novazzi [d,e], Elisa Masi [f], Elisabetta Pagani [f], Giulia Piccirilli [g], Maria Vittoria Mauro [h], Sandro Binda [a], Benedetta Corvaro [i], Claudia Tiberio [j], Eleonora Lalle [k], Fabrizio Maggi [k], Cristina Russo [l], Stefania Ranno [l], Elisa Vian [m], Elena Pariani [a,2,*], Fausto Baldanti [b,n,2], Antonio Piralla [b,2], on behalf of the AMCLI-GLIViRe working group

[a] Department of Biomedical Sciences for Health, University of Milan, Milan, Italy
[b] Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
[c] Virology Unit, Clinical Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy
[d] Ospedale di Circolo e Fondazione Macchi, ASST Sette Laghi, Varese, Italy
[e] University of Insubria, Varese, Italy
[f] Laboratorio Aziendale di Microbiologia e Virologia, Hospital of Bolzano (SABES-ASDAA), Bolzano-Bozen, Italy
[g] Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy
[h] Microbiology & Virology Unit, Annunziata Hub Hospital, Azienda Ospedaliera di Cosenza, Cosenza, Italy
[i] Virology Laboratory, Azienda Ospedaliera Ospedali Riuniti di Ancona, Ancona, Italy
[j] Microbiology and Virology, Cotugno Hospital AORN dei Colli, Naples, Italy
[k] Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani, Rome, Italy
[l] Department of Diagnostic and Laboratory Medicine, Unit of Microbiology and Diagnostic Immunology, Bambino Gesù Children Hospital IRCCS, Rome, Italy
[m] Microbiology Unit, Azienda ULSS2 Marca Trevigiana, Treviso, Italy
[n] Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy

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ABSTRACT

Aims: To assess influenza viruses (IVs) circulation and to evaluate A(H3N2) molecular evolution during the 2021-2022 season in Italy.

Materials and methods: 12,393 respiratory specimens (nasopharyngeal swabs or broncho-alveolar lavages) collected from in/outpatients with influenza illness in the period spanning from January 1, 2022 (week 2022-01) to May 31, 2022 (week 2022-22) were analysed to identify IV genome and were molecularly characterized by 12 laboratories throughout Italy. A(H3N2) evolution was studied by conducting an in-depth phylogenetic analysis of the hemagglutinin (HA) gene sequences. The predicted vaccine efficacy (pVE) of vaccine strain against circulating A(H3N2) viruses was estimated using the sequence-based $P_{epitope}$ model.

Results: The overall IV-positive rate was 7.2% (894/12,393), all were type A IVs. Almost all influenza A viruses (846/894; 94.6%) were H3N2 that circulated in Italy with a clear epidemic trend, with 10% positivity rate threshold crossed for six consecutive weeks from week 2022-11 to week 2022-16. According to the phylogenetic analysis of a subset of A(H3N2) strains (n=161), the study HA sequences were distributed into five different genetic clusters, all of them belonging to the clade 3C.2a, sub-clade 3C.2a1 and the genetic subgroup 3C.2a1b.2a.2. The selective pressure analysis of A(H3N2) sequences showed evidence of diversifying selection particularly in the amino acid position 156. The comparison between the predicted amino acid sequence of the 2021-2022 vaccine strain (A/Cambodia/e0826360/2020) and the study strains revealed 65 mutations in 59 HA

* Correspondig author.
E-mail address: elena.pariani@unimi.it (E. Pariani).
[1] These authors contributed equally.
[2] Co-senior authors.

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amino acid positions, including the substitution H156S and Y159N in antigenic site B, within major antigenic sites adjacent to the receptor-binding site, suggesting the presence of drifted strains. According to the sequence-based $P_{epitope}$ model, antigenic site B was the dominant antigenic site and the p(VE) against circulating A(H3N2) viruses was estimated to be -28.9%.

Discussion and conclusion: After a long period of very low IV activity since public health control measures have been introduced to face COVID-19 pandemic, along came A(H3N2) with a new phylogenetic makeup. Although the delayed 2021-2022 influenza season in Italy was characterized by a significant reduction of the width of the epidemic curve and in the intensity of the influenza activity compared to historical data, a marked genetic diversity of the HA of circulating A(H3N2) strains was observed. The identification of the H156S and Y159N substitutions within the main antigenic sites of most HA sequences also suggested the circulation of drifted variants with respect to the 2021-2022 vaccine strain. Molecular surveillance plays a critical role in the influenza surveillance architecture and it has to be strengthened also at local level to timely assess vaccine effectiveness and detect novel strains with potential impact on public health.

1. Introduction

Seasonal influenza viruses evolve to evade pre-existing immunity and gain competitive advantage through surface protein mutations which yield new antigenic variants (Petrova and Russell, 2018 Jan) that cause annual epidemics on average accounting for infections in 5–15% of the global population and up to 650,000 deaths a year (Word Health Organization (OMS) 2022). Three main features contribute to the rapid evolution of influenza viruses: large populations, short generation times, and high mutation rates (Shao et al., 2017 Aug). Influenza viruses undergo antigenic drift by mutation in the hemagglutinin (HA) gene, which encodes the main protein target for immune responses. Accumulation of these mutations can result in the emergence of antigenically distinct groups if certain amino acid substitutions are introduced into the HA glycoprotein (Petrova and Russell, 2018 Jan, Weis et al., 1988 Jun). The globular head of HA includes the receptor binding site (RBS) (Weis et al., 1988 Jun) that – although usually conserved - may be exposed to mutations that evade antibody recognition (Thyagarajan and Bloom, 2014 Jul, Lee and Wilson, 2015). The pace of antigenic selection varies over time for influenza A virus (IAV) subtypes and influenza B virus (IBV) lineages mainly due to population-level fluctuations in immune pressure, thus confounding vaccine strain selection, which relies on the anticipation of antigenic evolution (Barrat-Charlaix et al., 2021 06 25). Among human IAVs, H3N2 subtypes are those with the highest mutation rate; after their introduction into the human population in 1968, they started circulating displaying a rapid turn-over of the viral population, with the appearance of new antigenic variants every 2-5 years, usually generating epidemics characterized by high morbidity and mortality, and reducing influenza vaccine efficacy (Allen and Ross, 2018). The constant evolution to evade host immune pressure is achieved through the addition of N-glycosylation sites, antigenic drift, and charged amino acid substitutions near the RBS (Petrova and Russell, 2018 Jan, Allen and Ross, 2018). Specifically, the emergence of new H3N2 variants has been associated with the accumulation of amino acid substitutions at five antigenic sites (designated as A-E and incorporating more than 100 amino acid positions) on the globular head of H3. The substitution of a single amino acid in only one of seven specific amino acid positions adjacent to the RBS may cause major antigenic changes during the evolution of IAVs (Koel et al., 2013 Nov).

Population density and regional interconnectedness play an important role in maintaining viral populations (Ebranati et al., 2015, Russell et al., 2008 Apr 18, Bahl et al., 2011 Nov 29). However, the genetic and antigenic diversity of seasonal influenza has been severely impacted since the onset of the COVID-19 pandemic in March 2020. Since then, most countries have seen historically low seasonal influenza virus circulation (Sullivan et al., 2020, Olsen et al., 2020 Sep 18, Istituto Superiore di Sanità (ISS) 2022) attributable to non-pharmaceutical interventions (NPIs), such as travel restrictions, social distancing, school and workplace closures, mask wearing, and enhanced hygiene. NPIs have similarly disrupted the circulation of other common respiratory viruses such as respiratory syncytial virus and human metapneumovirus (Baker et al., 2020, Adenaiye et al., 2022 Aug 24, Leung et al., 2020, Tang et al., 2021 Jul, Gomez et al., 2021) by limiting opportunities for reintroduction and local transmission. As the use of NPIs to limit COVID-19 pandemic has been gradually declining and international travels have been returning to pre-pandemic levels, a resurgence of influenza virus circulation with an increased severity (due to reduced population immunity over the last couple of years) are expected.

The objectives of this study were i) to describe IVs detection and distribution during the 2021-2022 season in Italy, and ii) to conduct an in-depth phylogenetic analysis of the HA gene of influenza A(H3N2) viruses identified in Italy during the 2021-2022 influenza season in order to evaluate the evolution of these viruses after a long period of very low activity.

2. Materials and methods

2.2. Clinical samples and IAVs/IBVs detection and subtyping methods

Respiratory specimens (nasopharyngeal swabs or broncho-alveolar lavages) collected from in/outpatients with influenza illness in the period spanning from January 1, 2022 (week 2022-01) to May 31, 2022 (week 2022-22) were analysed to detect IV genome by 12 laboratories located in 8 Italian regions belonging to 4 macro-areas (according to NUTS classification (NUTS 2022)): North-West (Lombardy), North-East (Emilia Romagna, Trentino Alto-Adige, Veneto), Centre (Lazio, Marche), and South (Campania, Calabria). Laboratory names and their location by region and macro-area are detailed in Table 1.

Respiratory samples were collected from outpatients with the symptoms of influenza-like illness (ILI) or from hospitalised patients with symptoms ranging from mild to severe respiratory syndromes such as, acute respiratory infection (ARI), severe acute respiratory infection (SARI) and acute respiratory distress syndrome (ARDS). Clinical samples were analysed by means of specific real-time PCR assays according to protocols of each participating laboratory. The methods used by each laboratory are detailed in Table 1.

2.3. A(H3N2) influenza viruses sequencing and phylogenetic analysis

A representative subgroup of influenza A(H3N2) positive samples were molecularly characterised by means of the sequence analysis of the complete HA gene (nt. 1-1663). A one-step RT-PCR was performed from 15 µl of extracted RNA in a final reaction volume of 60 µl by using the kit SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Thermofisher) in order to obtain the entire influenza A virus genome. The amplification of the complete HA gene was carried out by an in-house nested-PCR split into two reaction mixtures in order to amplify two overlapped fragments of 970 nt. (nt. 1-969) and 813 nt. (nt. 851-1663) (Galli et al., 2020). The HA amplicons were purified and sequenced with both forward and reverse primers by means of the Sanger method.

All HA nucleotide sequences were obtained directly from clinical

Table 1
Methods used for molecular detection of IVs by each GLIViRe center.

	Laboratory	Region (Macro-area)	IAVs/IBVs RNA detection method	IAVs subtyping method
1	Department of Biomedical Sciences for Health, University of Milan, Milan, Italy	Lombardy (North-West)	Home-made (Word Health Organization (WHO) 2011)	Home-made [(Word Health Organization (WHO) 2011, Centers for Disease Control and Prevention (CDC) 2022)]
2	Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy	Lombardy (North-West)	Home-made (Centers for Disease Control and Prevention (CDC) 2022)	Home-made (Word Health Organization 2022)
3	Virology Unit, Clinical Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy	Lombardy (North-West)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene); Alinity mResp-4-Plex assay (Abbott)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)
4	Ospedale di Circolo e Fondazione Macchi, ASST Sette Laghi, Varese, Italy	Lombardy (North-West)	Alinity M Resp-4-Plex AMP Kit (Abbott)	Home-made [(Word Health Organization (WHO) 2011, Centers for Disease Control and Prevention (CDC) 2022)]
5	Laboratorio Aziendale di Microbiologia e Virologia, Hospital of Bolzano (SABES-ASDAA), Bolzano-Bozen, Italy	Trentino Alto-Adige (North-East)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)
6	Microbiology Unit, Azienda ULSS2 Marca Trevigiana, Treviso, Italy	Veneto (North-East)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)
7	Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy	Emilia Romagna (North-East)	Simplexa Flu A/B & RSV Direct Kit (Diasorin)	Home-made (The European Centre for Disease Prevention and Control (ECDC) 2022)
8	Virology Laboratory, Azienda Ospedaliera Ospedali Riuniti di Ancona, Ancona, Italy	Marche (Centre)	Alinity M-Resp-4-Plex AMP Kit (Abbott)	Home-made [(Word Health Organization (WHO) 2011, Centers for Disease Control and Prevention (CDC) 2022)]
9	Department of Diagnostic and Laboratory Medicine, Unit of Microbiology and Diagnostic Immunology, Bambino Gesù Children Hospital IRCCS, Rome, Italy	Lazio (Centre)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)
10	Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani, Rome, Italy	Lazio (Centre)	Home-made (Centers for Disease Control and Prevention (CDC) 2022)	Home-made (Word Health Organization 2022)
11	Microbiology and Virology, Cotugno Hospital AORN dei Colli, Naples, Italy	Campania (South)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)
12	Microbiology & Virology Unit, Annunziata Hub Hospital, Azienda Ospedaliera di Cosenza, Cosenza, Italy.	Calabria (South)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)

specimens and submitted to GISAID database (GISAID 2022) under the accession numbers provided in Supplementary Table 1. Sequences were aligned with reference sequences, retrieved from the online repository GISAID (GISAID 2022), by using ClustalW program implemented in BioEdit software (Hall, 1999). The alignment was used to construct the phylogenetic tree by means of the Neighbor-Joining method and the Kimura 2-parameter model with the bioinformatic programme MEGA6 (Tamura et al., 2013 Dec). A bootstrap analysis with 1000 replicates was conducted and bootstrap values ≥70% were considered significant.

Mean nucleotide identities and mean amino acid similarities were calculated by using the Sequence Identity Matrix tool of BioEdit software (Hall, 1999) for intra-group sequence analysis and between study and reference sequences, including the vaccine reference strain of Northern hemisphere for 2021-2022 influenza season (A/Cambodia/e0826360/2020_egg-derived; EPI_ISL_806547).The mean values are expressed as crude rate with the respective range. The genetic distance among sequences of the same genetic group was calculated by means of the *p-distance* model using MEGA6 program (Tamura et al., 2013 Dec) and it was expressed as mean value and the respective standard deviation (DS).

The predicted amino acid HA sequences were obtained by the Toggle translation tool implemented in BioEdit (Hall, 1999) and amino acid residues were numbered according to the H3 numbering (Lindstrom et al., 1996). Predicted amino acid sequences of the study strains were compared with that of the vaccine strain of Northern hemisphere for 2021-2022 influenza season (A/Cambodia/e0826360/2020; EPI_ISL_806547) to identify amino acid changes, focusing on mutations within the 5 HA antigenic sites of A(H3N2) strains (Wiley and Skehel, 1987), particularly at major antigenic sites within the RBS (Yang et al., 2015 Mar).

By comparing the predicted amino acid sequences of the study and vaccine strains, the predicted vaccine efficacy (pVE) against circulating A(H3N2) viruses was estimated by using a sequence-based model, named $P_{epitope}$ model, as previously described by others (Bonomo and Deem, 2018 Sep, Gupta et al., 2006 May, Bonomo et al., 2019 May 27). $P_{epitope}$ is a mathematical model that allows to measure the antigenic distance between the predominant circulating strains and the vaccine virus by considering the amino acid substitutions observed within the residues of the five A(H3N2) antigenic sites. The antigenic site with the highest $P_{epitope}$ value is considered as the dominant antigenic site and is used to estimate the pVE applying the following formula: $(-3.32 \times P_{epitope(dominant\ antigenic\ site)} + 0.66) \times 100\%$. When there is a perfect match between circulating strain and vaccine strain, the $P_{epitope}$ is null and the pVE is 66% (maximum pVE that could be estimated by this sequence-based model) (Bonomo and Deem, 2018 Sep). A negative value of pVE suggests a suboptimal vaccine efficacy against the circulating strains.

2.4. Selective pressure analysis

In order to evaluate the HA evolution due to immunological pressure, a series of probabilistic models of codon substitution were used. In detail, tests for positive selection were conducted using single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), the mixed-effects model of evolution (MEME), fast unconstrained Bayesian approximation (FUBAR), adaptive Branch-Site Random Effects

Likelihood (aBSREL), and Branch-site Unrestricted Statistical Test for Episodic Diversification (BUSTED) methods on the Datamonkey 2.0 server (Weaver et al., 2018 Mar 01). To avoid an excessive false-positive rate, sites with SLAC, FEL, MEME and aBSREL p-values <0.05 and a FUBAR posterior probability >0.95 were accepted as candidates for selection.

2.5. Statistical analysis

Statistical analysis was performed using the Open Source Epidemiologic Statistics for Public Health OpenEpi, version 3.03 (Dean et al., 2022). The frequency of positive samples was expressed as a crude proportion, with the corresponding 95% confidence interval (95% CI) calculated by the Mid-P exact test, assuming a normal distribution. The inter-quartile range (IQR) was computed as the difference between the first and third quartiles of the age distribution. The positivity rate was calculated as the number of laboratory-confirmed infections out of the total number of samples. Proportions between groups were compared using the Mid-P exact test based on binomial distribution. For continuous variables, the paired t-test was used.

We estimated influenza seasonal characteristics, including season onset (or start), duration, peak and offset (or end) applying the RS10 method (Midgley et al., 2017), which defines the start of epidemic season as the first 2 consecutive weeks when virus detection exceeds 10% of virus-positivity rate.

A p-value <0.05 was considered significant (two-tailed test).

3. Results

IVs detection and distribution during the 2021-2022 season in Italy

Overall, 12,393 respiratory specimens were tested for IVs detection. Of these, 894 tested positive to IVs, resulting in an overall positivity rate of 7.2%. IV-positivity rate by center ranged from 1.1% to 14.6% in samples collected in hospital setting and it was 17.3% in an outpatient care setting. IV positivity rates by macro-area are summarized in Table 2. The IVs positivity rate was 7.8% in the centers of North-West Italy, 7.7% in those of North-East Italy, and 1.6% in participating centers of Central and Southern Italy.

All the 894 IV-positive samples were IAVs: 94.6% (846/894) of those were H3N2 and 5.4% (48/894) belonged to the H1N1 subtype (Table 2).

During the study period (from week 2022-01 to week 2022-22), A (H3N2) detection in respiratory samples had a clear epidemic trend, crossing the 10% positivity rate threshold for six consecutive weeks (from week 2022-11 to week 2022-16). In fact, A(H3N2) epidemic wave started in week 2022-11, peaked in week 2022-13 and ended in week 2022-16 (Fig. 1). During the peak, the overall positivity rate reached 20% (117/578). Temporal distribution showed a geographical pattern from North-West to North-East Italy. Considering results by center in each Italian macro-area, A(H3N2) epidemic was evident in centers of both North-West and North-East Italy, whereas A(H3N2) started to be detected from week 2022-08 and 2022-09 in centers from Southern and Central Italy, respectively, never crossing the 10% positivity rate threshold (Fig. 2).

Table 2
Influenza virus positivity rate by type/subtype and by macro-area.

Macro-area	IVs positivity rate %	IAVs positivity rate %	IBVs positivity rate %	A(H3N2) positivity rate %	A(H1N1) positivity rate %
North-West Italy	7.8%	7.8%	0%	99.6%	0.4%
North-East Italy	7.7%	7.7%	0%	99.5%	0.5%
Central Italy	1.6%	1.6%	0%	97.9%	2.1%
Southern Italy	1.6%	1.6%	0%	100%	0%
Total	7.2%	7.2%	0%	94.6%	5.4%

3.1. Phylogenetic analysis of A(H3N2) IVs

Overall, 161 A(H3N2) circulating strains were molecularly characterised by sequencing and their HA sequences were phylogenetically analysed. The H3N2 viruses considered in this study were identified in 161 individuals (median age: 9 years; IQR: 29 years; range: 0-93 years; 52% males); 110/161 (68%) were inpatients from hospital settings and 51/161 (32%) were outpatients from ambulatory care settings.

The study strains showed a mean nucleotide identity of 99.3% (range: 98.2%-100%) and a mean amino acid similarity of 99.2% (range: 97.5%-100%). As shown in Fig. 3, all A(H3N2) strains of this study belonged to the clade 3C.2a, sub-clade 3C.2a1 sharing a mean nucleotide identity of 97% (range: 96.6%-97.3%) and a mean amino acid similarity of 95.7% (range: 95.3%-96.1%) to the reference strain A/Singapore/INFIMH-16-0019/2016.

Study sequences were furtherly characterised by the amino acid substitutions E62G (in antigenic site E), K92R (in antigenic site E), P194L (in antigenic site B) and H311Q (in antigenic site C) in HA1 and E150G in HA2, defining the genetic group 3C.2a1b (bootstrap 99%) and sharing a mean nucleotide identity of 97.5% (range: 97.2%-97.9%) and a mean amino acid similarity of 95.9% (range: 95.5%-96.3%) to the reference strain A/Netherlands/10260/2018. The 3C.2a1b genetic group also includes the 2021-2022 vaccine reference strain A/Cambodia/e0826360/2020. However, while the vaccine strain belonged to the genetic subgroup *2a.1*, the A(H3N2) strains of this study were characterised by the amino acid mutations Y159N, K160I, L164Q and D190N (all in the antigenic site B of HA1), and segregated into the genetic subgroup *2a.2*, displaying a mean nucleotide identity of 99.1% (range: 98.7%-99.5%) and a mean amino acid similarity of 98.9% (range: 98.5%-99.3%) to the reference strain A/Bangladesh/4005/2020. In more detail, study sequences showed a mean intra-group *p-distance* of 0.006 (standard deviation, SD=0) and they were furtherly distributed in 5 different clusters. By comparing the study sequences with the A/Bangladesh/4005/2020 reference strain, our sequences segregated into different clusters (described below), each of them characterized by specific amino acid mutations. Cluster I (4/161 sequences; 2.5%), characterized by the amino acid substitutions F79V and I140K (in antigenic site A) in HA1, showed a mean intra-group nucleotide identity of 99.7% (range: 99.5% -99.9%) and a mean intra-group amino acid similarity of 99.6% (range: 99.3%-100%). Cluster II (3/161 sequences; 1.9%), characterized by the amino acid mutations H56Y, S205F, A212T (in antigenic site D) and S270T in HA1, showed a mean intra-group nucleotide identity of 99.8% (range: 99.7% -99.9%) and a mean intra-group amino acid similarity of 99.8% (range: 99.7%-100%).

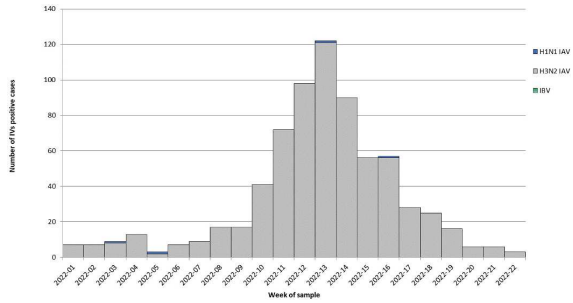

Fig. 1. Influenza viruses positive samples by type/subtype and by week of sample collection.

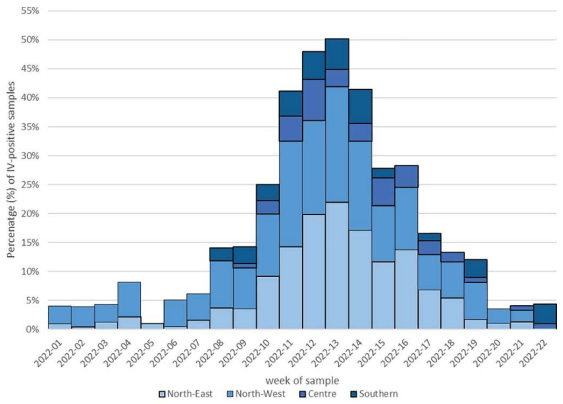

Fig. 2. Temporal distribution of IV-positivity rate by week (from week 2022-01 to week 2022-22) and by macro-area.

Cluster III (21/161 sequences; 13%), characterized by the amino acid substitutions D53N (in antigenic site C) and I192F (in antigenic site B) in HA1 and N49S in HA2, showed a mean intra-group nucleotide identity of 99.6% (range: 99.2%-100%) and a mean intra-group amino acid similarity of 99.6% (range: 98.9%-100%). Cluster IV (5/161 sequences; 3.1%), characterized by the amino acid mutations I25V, G78D/N, R201K (in antigenic site D) and S219Y (in antigenic site D) in HA1, showed a mean intra-group nucleotide identity of 99.8% (range: 99.7%-100%) and a mean intra-group amino acid similarity of 99.8% (range: 99.5%-100%). Cluster V (128/161 sequences; 79.5%), characterized by the amino acid substitutions D104G and K276R (in antigenic site C) in HA1, showed a mean intra-group nucleotide identity and amino acid similarity of 99.7% (range: 98.9%-100%). Finally, no significant difference was observed in the HA sequences distribution by type of setting (ambulatory or hospital) and by geographical area.

3.2. Comparison between study and vaccine strains and predicted vaccine efficacy

The 161 A(H3N2) HA sequences of this study showed a mean nucleotide identity of 98.4% (range: 97.9%-98.7%) and a mean amino acid similarity of 97.5% (range: 97.1%-97.9%) to the A(H3N2) vaccine strain of the Northern hemisphere for the 2021-2022 influenza season, A/Cambodia/e0826360/2020(H3N2).

The comparison between the predicted amino acid sequences of A (H3N2) study viruses and the 2021-2022 vaccine reference strain A/Cambodia/e0826360/2020 revealed 65 mutations in 59 HA amino acid positions; particularly, 9/65 (14%) amino acid substitutions were observed in >80% of study sequences, and all of them were within an antigenic site (antigenic site B: 7/9, 78%; C: 1/9, 11%; D: 1/9, 11%). The list of mutations by amino acid position is presented in Supplementary Table 2. Overall, 54% (35/65) of amino acid mutations was observed within an antigenic site (designated as A-E). All of the five antigenic sites had at least one mutated amino acid position. Particularly, 55% of the substitutions are located in the antigenic sites D (29%, 10/35) and B (26%, 9/35). 17% (6/35) of the mutations are in the antigenic site A, 17% (6/35) in the antigenic site C, and 11% in the antigenic site E.

Among the 7 major antigenic sites (amino acid positions: 145, 155, 156, 158, 159, 189 and 193) adjacent to the RBS, two amino acid positions were characterised by a substitution: H156S shared by 154/161 (96%) study sequences belonging to cluster III-V, and Y159N in all the

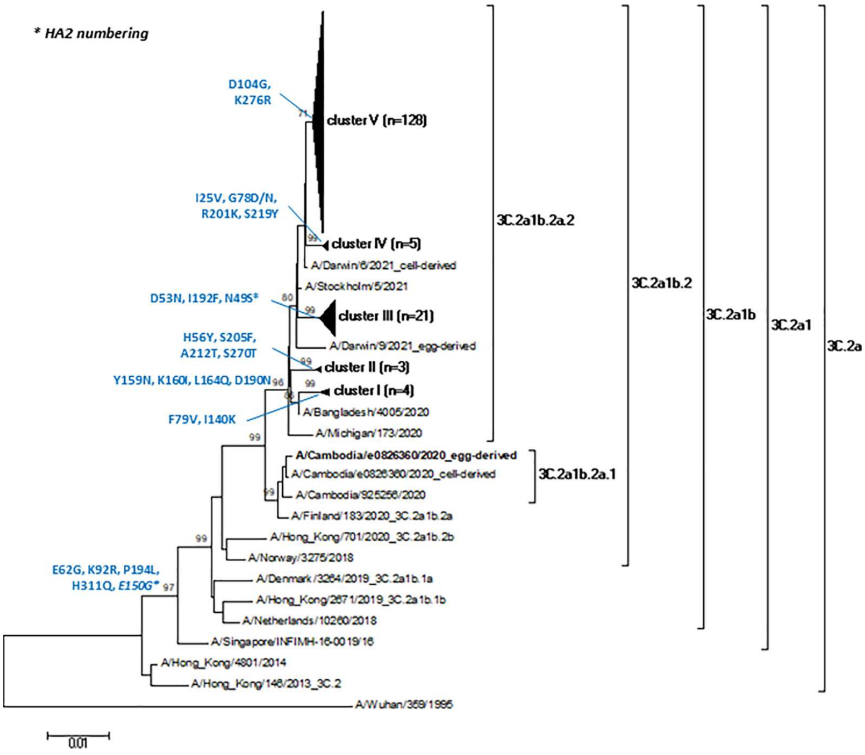

Fig. 3. Phylogenetic tree of the 161 HA nucleotide sequences (1479 nt.) of A (H3N2) strains identified in this study. The vaccine A(H3N2) strain of the Northern hemisphere for 2021-2022 influenza season (A/Cambodia/e0826360/2020) is in bold. For reasons of clarity, interior branches representing the study sequence clusters are compressed into elongated triangles, whose height is proportional to the number of taxa condensed and whose width is proportional to the maximum distance between taxa. Amino acid substitutions characterising the main branches are detailed close to each node. Only bootstrap values >70% are displayed.

study strains (161/161; 100%).

The comparison between the predicted amino acid sequence of the 2021-2022 vaccine reference strain A/Cambodia/e0826360/2020 (genetic subgroup 3C.2a1b.2a.1) and the circulating A(H3N2) strains of this study (genetic subgroup 3C.2a1b.2a.2) showed the following changes in 100% HA study sequences: N171K in antigenic site D and Y159N, K160I, L164Q, R186D, D190N and P198S in antigenic site B (Supplementary Table 2). According to the $P_{epitope}$ model, the antigenic site B was the dominant antigenic site, revealing a $P_{epitope}$ value of 0.286. The pVE of vaccine strain against the A(H3N2) viruses circulating during the 2021-2022 influenza season was estimated to be -28.9%.

3.3. Selective pressure analysis

Overall, one site in position 156 was identified as being under diversifying selection by site-specific analyses in the HA of study A (H3N2) IVs alignment by at least three of the methods used (SLAC, FEL, REL, FUBAR and MEME) (Table 3). The aBSREL analyses showed evidence of episodic diversifying selection for a branch of tree including 6 strains, with Y159N, T160I, L164Q, N171K, S186D, D190N, P198S changes (A/Bolzano/24/2022, A/Varese/04/2022, A/PoliclinicoMilano/22/2022, A/Milano/04/2022, A/Milano/21/2022, and A/Milano/63/2022) as compared to the vaccine strain. In three of them (A/Bolzano/24/2022, A/Varese/04/2022, A/Policlinico_Milano/22/2022), additional changes were observed (E50K, F79V and I140), while A/Milano/04/2022, A/Milano/21/2022, and A/Milano/63/2022 were characterized by the presence of other additional changes (H56Y, S205F, A212T and S270T). On these branches, BUSTED analyses evidenced that at least one site on at least one test branch experienced diversifying selection (LRT, p-value=0.033 p-value $\leq$0.05).

4. Discussion

Following the worldwide abrupt halt of influenza circulation caused by the emergence and widespread of SARS-CoV-2, scientists have been worrying about increased IVs activity and new viral phylogenetic makeup. In fact, COVID-19 pandemic restrictions such as lockdowns, school closure, facemask use and social distancing helped keep respiratory viruses at bay so much that influenza largely disappeared until early 2022 [(Istituto Superiore di Sanità (ISS) 2022, The European Centre for Disease Prevention and Control (ECDC) 2022, Word Health Organization (WHO) 2022)]. Data from the Southern Hemisphere and in particular from the Australia's Department of Health and Aged Care have showed an unusual influenza activity in 2022, spiking and dropping earlier than usual with the laboratory-confirmed influenza rate higher than the five-year average (Australian Influenza 2022).

Our study aimed at describing IVs distribution during the 2021-2022 season in Italy conducting an in-depth phylogenetic analysis of the HA gene of A(H3N2) influenza viruses. According to our findings, even if there was a clear evidence of influenza epidemic in 2022 in Italy, with the epidemic threshold of 10% positivity crossed for six consecutive weeks, the overall influenza positivity rate was 7.2%, significantly lower than that observed during the pre-COVID-19 seasons [(Istituto Superiore di Sanità (ISS) 2022, The European Centre for Disease Prevention and Control (ECDC) 2022, The European Centre for Disease Prevention and Control (ECDC) 2022, Pellegrinelli et al., 2022)]. In fact, according to the ECDC annual reports, a percentage of influenza virus positive specimens ranging between 40% (2016-2017) and 49% (2017-2018) was observed in the framework of ILI and ARI sentinel consultations from 2016–2017 to 2018-2019 influenza season [(The European Centre for Disease Prevention and Control (ECDC) 2022, The European Centre for Disease Prevention and Control (ECDC) 2022, The European Centre for Disease Prevention and Control (ECDC) 2022)]. The percentage of respiratory samples positive for influenza went across the 10% threshold, indicating the beginning of the epidemic, in week 2022-11, later than observed in previous seasons when the epidemic usually started between week 48 and week 52 [(Istituto Superiore di Sanità (ISS) 2022, The European Centre for Disease Prevention and Control (ECDC) 2022, The European Centre for Disease Prevention and Control (ECDC) 2022, Pellegrinelli et al., 2022, The European Centre for Disease Prevention and Control (ECDC) 2022)]. The percentage of influenza virus positive specimens at its peak (week 2022-13) was 20%, much lower than what observed during the 2018-2019 season when the percentage of influenza viruses positive samples reached up to 62% (The European Centre for Disease Prevention and Control (ECDC) 2022). In Italy, the temporal distribution did not show a clear geographical pattern, probably in consideration to the low number of influenza viruses detected in Central and Southern Italian macro-area (influenza positive rate of 1.6%).

Overall, our data underlined that the 2021-2022 influenza season in Italy was characterized by a significant reduction of the width of the epidemic curve (6 weeks versus 12-19 weeks observed between 2014-2015 to 2018-2019 season; [(Istituto Superiore di Sanità (ISS) 2022, Pellegrinelli et al., 2022)]) and in the intensity of the influenza activity compared to previous seasons that can be undoubtedly related to the emergence of SARS-CoV-2, the actions introduced to control the COVID-19 pandemic and the increased number of testing.

As observed in Europe and in Southern hemisphere countries (Australian Influenza 2022, The European Centre for Disease Prevention and Control (ECDC) March 2022), no influenza B viruses were identified in Italy and among type A influenza viruses, H3N2 subtype largely dominated over H1N1.

To investigate the molecular and evolutionary characteristics of the influenza A(H3N2) viruses circulating during the 2021-2022 season, a phylogenetic analysis of the HA gene sequences of nearly 20% of A (H3N2) viruses detected during the study was conducted. All A(H3N2) study strains belonged to the clade 3C.2a, sub-clade 3C.2a1 and the genetic group 3C.2a1b, sharing a high mean nucleotide identity (99.3%) and a high mean amino acid similarity (99.2%). Our results mirror the European data of influenza virus molecular characterisation during the 2021-2022 season [(The European Centre for Disease Prevention and Control (ECDC) 2022, Istituto Superiore di Sanità 2022)]. Despite belonging to the same phylogenetic branch, the HA sequence analysis of study A(H3N2) strains pointed out the constant tendency of influenza viruses to evolve. In fact, A(H3N2) viruses in the clade 3C.2a were dominant since the 2014-2015 influenza season with the 3C.2a1b viruses predominating over the course of the 2019-2020 season (The Francis Crick Institute - Worldwide Influenza Centre lab 2022). However, the A(H3N2) strains of this study, as observed for the majority of the 2021-2022 strains in other Northern hemisphere countries [(The European Centre for Disease Prevention and Control (ECDC) 2022, Istituto Superiore di Sanità 2022)], segregated into the genetic subgroup 3C.2a1b.2a.2, circulating since October 2020 and named during the 2021-2022 season (The Francis Crick Institute - Worldwide Influenza Centre lab 2022). The 3C.2a1b.2a.2 viruses result in the loss of the glycosylation site at residues 158-160 in HA1, and the acquisition of

Table 3
Selected sites of HA for A(H3N2) IV strains identified in this study.

Methods	H3 codons or tree branch Diversifying selection	Negative selection
SLAC	None	10, 24, 239
FEL	53, **156**, 192, 201, 219, 378	None
FUBAR	none	10, 24, 35, 56, 66, 65. 90, 108, 142, 214, 239, 321, 403, 436, 470, 496
MEME	**156**	None
aBSREL	Branch with 6 strains[a] (**H156**, D186, D225).	None

Positions selected by at least 3 methods are reported in bold.
[a] A/Bolzano/24/2022, A/Varese/04/2022, A/Policlinico_Milano/22/2022, A/Milano/04/2022, A/Milano/21/2022, and A/Milano/63/2022

which had been a defining feature of clade 3C.2a viruses (e.g. A/Hong Kong/4801/2014). Moreover, the 161 HA study sequences were further distributed into five different genetic clusters, each of them marked by specific amino acid substitutions; this genetic diversity confirms the influenza viruses' continuous ability to mutate. This observation was also emphasized by the fact that the selective pressure analysis showed evidence that some amino acid positions (particularly amino acid 156) are under diversifying selection, therefore resulting more prone to evolve.

Finally, from the comparison between the HA sequences of the A (H3N2) strains of this study and the vaccine strain of the Northern hemisphere for the 2021-2022 season (A/Cambodia/e0826360/2020), a mean nucleotide identity of 98.4% and a mean amino acid similarity of 97.5% were observed. Although the vaccine strain A/Cambodia/e0826360/2020 belongs to the same genetic group (3C.2a1b) of our study sequences, it differs from them for the genetic subgroup (2a.1 for vaccine strain vs. 2a.2 for study sequences). Our analysis of the predicted amino acid sequences of the HA gene of study A(H3N2) strains revealed numerous amino acid substitutions (65 mutations in 59 sites) compared to the HA sequence of the 2021-2022 vaccine strain. In particular, most of the study sequences had the H156S (96%) and Y159N (100%) mutations, which are located within the major antigenic sites of the receptor binding site. The receptor binding site is generally conserved, but it may also be exposed to selective pressure which determines the introduction of new mutations in order to evade the antibody recognition (Allen and Ross, 2018). The amino acid substitutions in the major antigenic sites (particularly positions 145, 155, 156, 158, 159, 189 and 193) are mutations that, more than others, lead to antigenic changes (Koel et al., 2013 Nov). Thus, the presence of H156S and Y159N mutations in the study sequences suggests the circulation of HA drifted A(H3N2) strains compared to the 2021-2022 vaccine strain; this means that circulating strains could be able to evade the recognition of vaccine-induced antibodies. Overall, more than half (54%) of HA amino acid substitutions was observed within an antigenic site and, among them, 55% were located in the antigenic site D (29%) and B (26%). According to the $P_{epitope}$ model (Bonomo and Deem, 2018 Sep), the predicted vaccine efficacy of the 2021-2022 vaccine strain against the circulating A(H3N2) viruses was estimated to be -28.9%. $P_{epitope}$ is a mathematical model that accounts for immunological diversity, modularity, and hierarchy during human antibody recognition of influenza antigens, previously described by Bonomo et al. (Bonomo et al., 2019 May 27). This is a sequence-based model which allows to estimate the antigenic distance between the A(H3N2) predominant circulating strains and the vaccine virus and it can be used only to estimate the vaccine efficacy and not the vaccine effectiveness. Therefore, the $P_{epitope}$ model cannot replace the test-negative design studies that remain the gold standard to estimate the vaccine effectiveness (Chua et al., 2020 Jan). The pVE estimated in our study by the $P_{epitope}$ model suggests a suboptimal vaccine efficacy against A(H3N2) circulating in the 2021-2022 influenza season. Although the pVE calculated in this study is the result of a sequence-based analysis only, a suboptimal vaccine effectiveness against circulating A(H3N2) has also been reported by interim analyses of 2021-2022 seasonal influenza vaccine effectiveness through observational studies conducted in the US (Chung et al., 2022 Mar 11), in Denmark (Emborg et al., 2022, Kim et al., 2022), and in Canada (Emborg et al., 2022, Kim et al., 2022). Vaccine effectiveness estimates from these studies have suggested that the low vaccine effectiveness could be due to the circulation of A(H3N2) drifted variants. Surely, a limitation of our study is that no data on the antigenic characteristics of circulating viruses were available, therefore the interpretation of our results is limited to genotypic characteristics of the viruses, and does not consider any phenotypic alterations. However, considering the antigenic analyses of circulating influenza viruses provided by the WHO (The Francis Crick Institute - Worldwide Influenza Centre lab 2022), we can complement our HA gene sequence analysis and speculate that the circulating strains identified in this study are antigenically different from the A(H3N2) vaccine strain, so much that, for the following influenza season, the vaccine composition was updated and the A(H3N2) vaccine strain was changed.

This study has some limitations in terms of heterogeneity in sampled populations among the centres and, partially, in methods used to detect influenza viruses in respiratory samples.

5. Conclusion

As influenza viruses have the potential to emerge with new phylogenetic makeup and in consideration that this study has uncovered the introduction of A(H3N2) HA drifted variants after a long period of very low influenza activity in Italy, it is critical to further strengthen molecular surveillance at local level to promptly assess vaccine effectiveness and detect any novel strains with potential impact on public health.

Working group: Others GLIViRe members and collaborators are listed as follow: Alessandra Pierangeli, Guido Antonelli (Virology Laboratory, Department of Molecular Medicine, "Sapienza" University, Rome, Italy); Francesca Rovida (Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy and Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo); Massimo Oggioni (Virology Unit, Clinical Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy); Tiziana Lazzarotto (Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Italy; Section of Microbiology, Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Italy); Eva Caterina Borgatti (Section of Microbiology, Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Italy); Stefano Menzo (Department of Biomedical Sciences and Public Health, Università Politecnica delle Marche, Ancona, Italy and Virology Laboratory, Azienda Ospedaliera Ospedali Riuniti di Ancona, Ancona, Italy); Luana Coltella (Department of Diagnostic and Laboratory Medicine, Unit of Microbiology and Diagnostic Immunology, Bambino Gesù Children Hospital IRCCS, Rome, Italy); Guido Scalia, Concetta Ilenia Palermo (U.O.C. Laboratory Analysis Unit, A.O.U. 'Policlinico-Vittorio Emanuele', University of Catania, Catania, Italy) Ferreri Monica Lucia (UOC Laboratorio Analisi G.B. Grassi - ASL Roma 3), Angelo Paolo Genoni (Ospedale di Circolo e Fondazione Macchi, ASST Sette Laghi, Varese, Italy).

Others GLIViRe members and collaborators

Alessandra Pierangeli, Guido Antonelli (Virology Laboratory, Department of Molecular Medicine, "Sapienza" University, Rome, Italy); Elizabeth Iskandar (Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy), Francesca Rovida (Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy and Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo); Tiziana Lazzarotto (Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Italy; Section of Microbiology, Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Italy); Eva Caterina Borgatti (Section of Microbiology, Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Italy); Stefano Menzo (Department of Biomedical Sciences and Public Health, Università Politecnica delle Marche, Ancona, Italy and Virology Laboratory, Azienda Ospedaliera Ospedali Riuniti di Ancona, Ancona, Italy); Luana Coltella (Department of Diagnostic and Laboratory Medicine, Unit of Microbiology and Diagnostic Immunology, Bambino Gesù Children Hospital IRCCS, Rome, Italy); Guido Scalia, Concetta Ilenia Palermo (U.O.C. Laboratory Analysis Unit, A.O.U. 'Policlinico-Vittorio Emanuele', University of Catania, Catania, Italy) Ferreri Monica Lucia (UOC Laboratorio Analisi G.B. Grassi - ASL Roma 3), Angelo Paolo Genoni (Ospedale di Circolo e Fondazione Macchi, ASST Sette Laghi, Varese, Italy).

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CRediT authorship contribution statement

Cristina Galli: Data curation, Formal analysis, Writing – original draft. **Laura Pellegrinelli:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Federica Giardina:** Methodology, Writing – review & editing. **Guglielmo Ferrari:** Methodology, Writing – review & editing. **Sara Colonia Uceda Renteria:** Investigation, Writing – review & editing. **Federica Novazzi:** Investigation, Writing – review & editing. **Elisa Masi:** Investigation, Writing – review & editing. **Elisabetta Pagani:** Validation, Writing – review & editing. **Giulia Piccirilli:** Investigation, Writing – review & editing. **Maria Vittoria Mauro:** Investigation, Writing – review & editing. **Sandro Binda:** Validation, Writing – review & editing. **Benedetta Corvaro:** Investigation, Writing – review & editing. **Claudia Tiberio:** Investigation, Writing – review & editing. **Eleonora Lalle:** Investigation, Writing – review & editing. **Fabrizio Maggi:** Validation, Writing – review & editing. **Cristina Russo:** Investigation, Writing – review & editing. **Stefania Ranno:** Investigation, Writing – review & editing. **Elisa Vian:** Investigation, Writing – review & editing. **Elena Pariani:** Conceptualization, Validation, Writing – original draft, Writing – review & editing. **Fausto Baldanti:** Conceptualization, Supervision, Writing – review & editing. **Antonio Piralla:** Conceptualization, Writing – original draft, Writing – review & editing.

Conflict of interest

Nothing to declare.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2022.199033.

References

Petrova, VN, Russell, CA., 2018 Jan. The evolution of seasonal influenza viruses. Nat. Rev. Microbiol. 16 (1), 47–60.
Word Health Organization (OMS). Influenza seasonal. https://www.who.int/health-topics/influenza-seasonal#tab=tab_1. Last Accessed: 29/11/2022.
Shao, W, Li, X, Goraya, MU, et al., 2017 Aug. Evolution of influenza A virus by mutation and Re-assortment. Int. J. Mol. Sci. 18 (8), E1650.
Weis, W, Brown, JH, Cusack, S, et al., 1988 Jun. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature 333 (6172), 426–431.
Thyagarajan, B, Bloom, JD., 2014 Jul. The inherent mutational tolerance and antigenic evolvability of influenza hemagglutinin. Elife 3, e03300.
Lee, PS, Wilson, IA, 2015. Structural characterization of viral epitopes recognized by broadly cross-reactive antibodies. Curr. Top. Microbiol. Immunol. 386, 323–341.
Barrat-Charlaix, P, Huddleston, J, Bedford, T, et al., 2021 06 25. Limited predictability of amino acid substitutions in seasonal influenza viruses. Mol. Biol. Evol. 38 (7), 2767–2777.
Allen, JD, Ross, TM., 2018. H3N2 influenza viruses in humans: viral mechanisms, evolution, and evaluation. Hum. Vaccin. Immunother. 14 (8), 1840–1847.
Koel, BF, Burke, DF, Bestebroer, TM, et al., 2013 Nov. Substitutions near the receptor binding site determine major antigenic change during influenza virus evolution. Science 342 (6161), 976–979.
Ebranati, E, Pariani, E, Piralla, A, et al., 2015. Reconstruction of the evolutionary dynamics of A(H3N2) influenza viruses circulating in Italy from 2004 to 2012. PLoS One 10 (9), e0137099.
Russell, CA, Jones, TC, Barr, IG, et al., 2008 Apr 18. The global circulation of seasonal influenza A (H3N2) viruses. Science 320 (5874), 340–346.
Bahl, J, Nelson, MI, Chan, KH, et al., 2011 Nov 29. Temporally structured metapopulation dynamics and persistence of influenza A H3N2 virus in humans. Proc. Natl. Acad. Sci. U.S.A. 108 (48), 19359–19364.
Sullivan, SG, Carlson, S, Cheng, AC, et al., 2020. Where has all the influenza gone? The impact of COVID-19 on the circulation of influenza and other respiratory viruses, Australia, March to September 2020. Euro Surveill. 25 (47), 11.
Olsen, SJ, Azziz-Baumgartner, E, Budd, AP, et al., 2020 Sep 18. Decreased influenza activity during the COVID-19 pandemic - United States, Australia, Chile, and South Africa, 2020. MMWR Morb. Mortal. Wkly. Rep. 69 (37), 1305–1309.
Istituto Superiore di Sanità (ISS), 2022. Epicentro. Influnet. Available at. https://www.epicentro.iss.it/influenza/influnet. Last access: 29/11/.
Baker, RE, Park, SW, Yang, W, et al., 2020. The impact of COVID-19 nonpharmaceutical interventions on the future dynamics of endemic infections. Proc. Natl. Acad. Sci. U. S. A. 117 (48), 30547–30553, 12 01.
Adenaiye, OO, Lai, J, Bueno de Mesquita, PJ, et al., 2022 Aug 24. Infectious severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) in exhaled aerosols and efficacy of masks during early mild infection. Clin. Infect. Dis. 75 (1), e241–e248.
Leung, NHL, Chu, DKW, Shiu, EYC, et al., 2020. Respiratory virus shedding in exhaled breath and efficacy of face masks. Nat. Med. 26 (5), 676–680, 05.
Tang, JW, Bialasiewicz, S, Dwyer, DE, et al., 2021 Jul. Where have all the viruses gone? Disappearance of seasonal respiratory viruses during the COVID-19 pandemic. J. Med. Virol. 93 (7), 4099–4101.
Gomez, GB, Mahé, C, Chaves, SS., 2021. Uncertain effects of the pandemic on respiratory viruses. Science 372 (6546), 1043–1044, 06 04.
NUTS - Nomenclature of territorial units for statistics. Availabe at: https://ec.europa.eu/eurostat/web/nuts/background. Last accessed: 29/11/2022.
Galli, C, Orsi, A, Pariani, E, et al., 2020. In-depth phylogenetic analysis of the hemagglutinin gene of influenza A(H3N2) viruses circulating during the 2016-2017 season revealed egg-adaptive mutations of vaccine strains. Expert Rev. Vaccines 19 (1), 115–122, 01.
GISAID. Global initiative on sharing all influenza data. Available at https://www.gisaid.org/. Last accessed: 29/11/2022.
Hall, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 95–98.
Tamura, K, Stecher, G, Peterson, D, et al., 2013 Dec. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30 (12), 2725–2729.
Lindstrom, S, Sugita, S, Endo, A, et al., 1996. Evolutionary characterization of recent human H3N2 influenza A isolates from Japan and China: novel changes in the receptor binding domain. Arch. Virol. 141 (7), 1349–1355.
Wiley, DC, Skehel, JJ., 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annual Review of Biochemistry 56, p365–p394.
Yang, H, Carney, PJ, Chang, JC, et al., 2015 Mar. Structure and receptor binding preferences of recombinant human A(H3N2) virus hemagglutinins. Virology 477, 18–31.
Bonomo, ME, Deem, MW., 2018 Sep. Predicting Influenza H3N2 vaccine efficacy from evolution of the dominant epitope. Clin. Infect. Dis. 67 (7), 1129–1131.
Gupta, V, Earl, DJ, Deem, MW., 2006 May. Quantifying influenza vaccine efficacy and antigenic distance. Vaccine 24 (18), 3881–3888.
Bonomo, ME, Kim, RY, Deem, MW., 2019 May 27. Modular epitope binding predicts influenza quasispecies dominance and vaccine effectiveness: application to 2018/19 season. Vaccine 37 (24), 3154–3158.
Weaver, S, Shank, SD, Spielman, SJ, et al., 2018 Mar 01. Datamonkey 2.0: a modern web application for characterizing selective and other evolutionary processes. Mol. Biol. Evol. 35 (3), 773–777.
Dean AG, Sullivan KM, Soe MM. OpenEpi: open source epidemiologic statistics for Public Health, Versione. Available at: www.OpenEpi.com. Last access: 29/11/2022.
Midgley, CM, Haynes, AK, Baumgardner, JL, et al., 2017. Determining the seasonality of respiratory syncytial virus in the United States: the impact of increased molecular testing. J. Infect. Dis. 216 (3), 345–355, 08 01.
The European Centre for Disease Prevention and Control (ECDC), 2022. FluNews Europe. Available at: https://flunewseurope.org/. Last access: 29/11/.
Word Health Organization (WHO), 2022. Global influenza program. Influenza updates. Available at: https://www.who.int/teams/global-influenza-programme/surveillance-and-monitoring/influenza-updates/influenza-updates-archive. Last access: 29/11/.
Australian Influenza Surveillance Report and Activity Updates –2022. Available at: https://www1.health.gov.au/internet/main/publishing.nsf/Content/cda-ozflu-2022.htm. Last Accessed: 29/11/2022.
The European Centre for Disease Prevention and Control (ECDC). Seasonal influenza 2018–2019. Annual Epidemiological Report. Available at: https://www.ecdc.europa.eu/sites/default/files/documents/AER_for_2018_seasonal-influenza-corrected.pdf. Last Accessed: 29/11/2022.
The European Centre for Disease Prevention and Control (ECDC). Seasonal influenza 2017–2018. Annual Epidemiological Report. Available at: https://www.ecdc.europa.eu/sites/default/files/documents/AER_for_2018_seasonal-influenza-corrected.pdf. Last Accessed: 29/11/2022.
Pellegrinelli, L, Galli, C, Bubba, L, et al., 2022. Respiratory syncytial virus in pediatric influenza-like illness cases in Lombardy, Northern Italy, during seven consecutive winter seasons (from 2014-2015 to 2020-2021). Influenza Other. Respir. Viruses 16 (3), 481–491, 05.
The European Centre for Disease Prevention and Control (ECDC). Seasonal influenza. Annual Epidemiological Report for 2016. Available at: https://www.ecdc.europa.eu/sites/default/files/documents/AER_for_2016-influenza-seasonal.pdf. Last Accessed: 29/11/2022.
The European Centre for Disease Prevention and Control (ECDC), March 2022. Influenza virus characterisation - Summary Europe. Available at: https://www.ecdc.europa.eu

/sites/default/files/documents/Influenza-characterisation-report-march-2022.pdf. Last Accessed: 29/11/2022.
The European Centre for Disease Prevention and Control (ECDC). Influenza Virus Characterisation Reports, summary Europe. Available at: https://www.ecdc.europa.eu/en/seasonal-influenza/surveillance-and-disease-data/influenza-virus-characterisation. Last accessed: 29/11/2022.
Istituto Superiore di Sanità. ISS: Sorveglianza virologica dell'influenza (rete InfluNet). Available at: https://www.iss.it/sorveglianza-virologica-dell-influenza-influnet. Last access: 29/11/2022.
The Francis Crick Institute - Worldwide Influenza Centre lab. Annual and interim reports. Available at: https://www.crick.ac.uk/research/platforms-and-facilities/worldwide-influenza-centre/annual-and-interim-reports.Last access: 23/11/2022.
Chua, H, Feng, S, Lewnard, JA, et al., 2020 Jan. The use of test-negative controls to monitor vaccine effectiveness: a systematic review of methodology. Epidemiology 31 (1), 43–64.
Chung, JR, Kim, SS, Kondor, RJ, et al., 2022 Mar 11. Interim estimates of 2021-22 Seasonal influenza vaccine effectiveness - United States, February 2022. MMWR Morb. Mortal. Wkly. Rep. 71 (10), 365–370.
Emborg, HD, Vestergaard, LS, Botnen, AB, et al., 2022. A late sharp increase in influenza detections and low interim vaccine effectiveness against the circulating A(H3N2) strain, Denmark, 2021/22 influenza season up to 25 March 2022. Euro Surveill. 27 (15), 04.
Kim, S, Chuang, ES, Sabaiduc, S, et al., 2022. Influenza vaccine effectiveness against A (H3N2) during the delayed 2021/22 epidemic in Canada. Euro Surveill. 27 (38), 09.
Word Health Organization (WHO). Global influenza surveillance network. Manual for the laboratory diagnosis and virological surveillance of influenza. Available at: http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf. 2011. Last accessed: 29/11/2022.
Centers for Disease Control and Prevention (CDC). National Center for Immunization and Respiratory Diseases (NCIRD). Virology, surveillance, and diagnosis branch. Available at: https://www.cdc.gov/ncird/flu.html. Last accessed: 29/11/2022.
Centers for Disease Control and Prevention (CDC). Influenza SARS - CoV - 2 (Flu SC2) M ultiplex assay primers and probes. Available at: https://www.cdc.gov/coronavirus/2019-ncov/downloads/lab/multiplex-primers-probes-printer.pdf. Last accessed: 29/11/2022.
Word Health Organization. WHO information for the molecular detection of influenza viruses. Available at: https://cdn.who.int/media/docs/default-source/influenza/molecular-detention-of-influenza-viruses/protocols_influenza_virus_detection_feb_2021.pdf?sfvrsn=df7d268a_5. Last accessed: 29/11/2022.

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Surveillance

Epidemiological impact of human adenovirus as causative agent of respiratory infections: An Italian multicentre retrospective study, 2022–2023

Federica A.M. Giardina [a,1], Laura Pellegrinelli [b,1], Federica Novazzi [c,d,1], Elisa Vian [e], Valeria Biscaro [e], Cristina Russo [f], Stefania Ranno [f], Elisabetta Pagani [g], Elisa Masi [g], Claudia Tiberio [h], Martina Esposito [h], Sara Uceda Renteria [i], Annapaola Callegaro [i], Giulia Piccirilli [j], Tiziana Lazzarotto [j,k], Francesca Rovida [a,l], Cristina Galli [b], Eleonora Lalle [m], Fabrizio Maggi [m], Nicasio Mancini [c,d], Carla Acciarri [n], Stefano Menzo [n,o], Anna Maria Colacicco [p], Maria Scarasciulli [p], Antonio Piralla [l,*,l], Fausto Baldanti [a,l], Elena Pariani [b]

[a] Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy
[b] Department of Biomedical Sciences for Health, University of Milan, Milan, Italy
[c] Department of Medicine and Innovation Technology, University of Insubria (DIMIT), Varese, Italy
[d] Laboratory of Medical Microbiology and Virology University Hospital of Varese, Varese, Italy
[e] UOC Microbiology Treviso Hospital, Department of Specialist and Laboratory Medicine, AULSS 2 La, Marca, Italy
[f] Virology and Mycobacteria UOS, Microbiology and Diagnostic Immunology UOC, Bambino Gesù Children Hospital IRCCS, Roma, Italy
[g] Laboratory of Microbiology and Virology, Provincial Hospital of Bolzano (SABES-ASDAA), Lehrkrankenhaus der Paracelsus Medizinischen Privatuniversität, Bolzano, Italy
[h] UOC Microbiology and Virology, Cotugno Hospital AORN dei Colli, Naples, Italy
[i] Microbiology and Virology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy
[j] Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy
[k] Section of Microbiology, Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy
[l] Microbiology and Virology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
[m] Laboratory of Virology, National Institute for Infectious Diseases "Lazzaro Spallanzani" IRCCS, Rome, Italy
[n] Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy
[o] Virology Unit, Azienda Ospedaliero Universitaria delle Marche, Ancona, Italy
[p] Virology Laboratory - Microbiology and Virology Unit - University of Bari - Policlinic of Bari, Bari, Italy

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ABSTRACT

Human adenoviruses are the causative agents of 5–7% of viral respiratory infections, mainly caused by species B and C. They can infect all age groups, but children are usually at high risk of infections. Adenovirus epidemiology is well documented in East-Asian countries but little is known about adenovirus circulation in Europe in recent years. This multicentre retrospective study aimed to investigate the circulation and molecular epidemiology of hAdVs. This surveillance collected a total of 54463 respiratory specimens between January 1, 2022 and June 20, 2023 were tested for the presence of respiratory viruses. Our results showed that adenovirus was detected in 6.6

* Corresponding author. Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy.
E-mail addresses: federica.giardina01@universitadipavia.i (F.A.M. Giardina), laura.pellegrinelli@unimi.it (L. Pellegrinelli), federica.novazzi@asst-settelaghi.it (F. Novazzi), elisa.vian@aulss2.veneto.it (E. Vian), valeria.biscaro@aulss2.veneto.it (V. Biscaro), cristina.russo@opbg.net (C. Russo), stefania.ranno@opbg.net (S. Ranno), elisabetta.pagani@sabes.it (E. Pagani), elisa.masi@sabes.it (E. Masi), claudia.tiberio@ospedalideicolli.it (C. Tiberio), martina.esposito030@gmail.com (M. Esposito), sara.ucedarenteria@policlinico.mi.it (S. Uceda Renteria), annapaola.callegaro@policlinico.mi.it (A. Callegaro), giulia.piccirilli@aosp.bo.it (G. Piccirilli), tiziana.lazzarotto@unibo.it (T. Lazzarotto), f.rovida@smatteo.pv.it (F. Rovida), cristina.galli@unimi.it (C. Galli), eleonora.lalle@inmi.it (E. Lalle), fabrizio.maggi@inmi.it (F. Maggi), nicasio.mancini@uninsubria.it (N. Mancini), carla.acciarri@ospedaliriuniti.marche.it (C. Acciarri), s.menzo@staff.univpm.it (S. Menzo), annamaria.colacicco@uniba.it (A.M. Colacicco), mluisascarasciulli52@gmail.com (M. Scarasciulli), antonio.piralla@unipv.it (A. Piralla), f.baldanti@smatteo.pv.it (F. Baldanti), elena.pariani@unimi.it (E. Pariani).
[1] These authors contributed equally.

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% of all cases of acute respiratory infection included in the study and the median age of positive patients was 3 years, with male children in 1–2 years age group being the most affected. 43.5 % of adenovirus cases were co-infected with at least one other respiratory virus, and rhinovirus was co-detected in 54 % of cases. Genotyping of adenovirus allowed the identification of 6 different genotypes circulating in Italy, among which type B3 was the most frequently detected.

Abbreviations

AMCLI	Italian association of clinical microbiologists
EV	enterovirus
GLIViRe	working group on respiratory virus infections
hBoV	human bocavirus
hCoVs	human coronaviruses
hAdVs	Human adenoviruses
hRV	human rhinovirus
FluA	influenza A virus
FluB	influenza B virus
MPV	metapneumovirus
PIVs	parainfluenza viruses
RSV	respiratory syncytial virus

1. Introduction

Human adenoviruses (hAdVs) belong to the mammalian family *Adenoviridae* and are non-enveloped viruses. The hAdV genome is double-stranded DNA, approximately 34–36 kb in length, and encodes 40 different proteins, including the penton, the hexon and fiber proteins that make up the viral capsid [1]. To date, the *Adenoviridae* family comprises 7 species designed A to G and more than 110 genotypes [2]. HAdV can be transmitted directly or indirectly by aerosol or by the fecal-oral route. HAdVs are highly contagious pathogens causing several clinical syndromes, including upper and lower respiratory tract infections, conjunctivitis and acute gastroenteritis [3,4]. Moreover, some hAdV genotypes are associated with specific clinical pictures. For example, species B and C are mainly associated with respiratory infections [5]. Upper respiratory tract infections (URTI) are characterized by signs and symptoms such as fever, sore throat, cervical adenopathy, headache, myalgia, cough and chills [6]. Sometimes, usually in children or immunocompromised patients, hAdV infection can progress to more severe disease such as bronchiolitis or pneumonia, with severe and chronic lung damage [7,8]. No specific age limit has been observed for hAdV infections. However, hAdVs infections are particularly common in children under 5 years of age, accounting for at least 5–7% of the respiratory illnesses [9,10].

The epidemiology of hAdV-associated respiratory infections is well documented in East-Asian countries such as China or Taiwan [11–13], but little is known about their circulation in Europe: in fact, very few data on hAdV epidemiology or molecular characterization have been reported in the last decade [14–16]. Given the increasing interest in respiratory viruses and their role in causing severe respiratory syndromes, it is now necessary to improve our knowledge of the epidemiology of these viruses by evaluating the presence of a molecular signature that may correlate with the clinical picture.

This multicentre retrospective study involved twelve microbiology laboratories in Italy with the aim of investigating the circulation and molecular epidemiology of hAdVs in a18-month period between 2022 and 2023.

2. Methods

2.1. Study design

To evaluate the hAdV epidemiology, residual clinical specimens collected for diagnostic purposes from patients with respiratory infections between January 2022 and June 30, 2023 were used. Twelve clinical microbiology laboratories participated in this multicentre and retrospective study. The distribution of the laboratories participating in the study on the Italian territory is shown in Fig. 1. All these laboratories are part of the working group on respiratory virus infections (GLIViRe) of the Italian association of clinical microbiologists (AMCLI). All data included in this study were collected during routine clinical practice, and evaluated retrospectively and anonymously. All procedures performed were in accordance with the ethical standards and the Declaration of Helsinki and within the Italian law. Informed consent was waived because all data were de-identified.

2.2. Molecular detection of respiratory viruses

The presence of respiratory viruses in clinical specimens was performed with commercial assays used in the laboratories participating in the study: Allplex Respiratory Panel Assays (Seegene), BioFire® Respiratory 2.1 Panel (Biomerieux), QIAstat-Dx Respiratory SARS-CoV-2 Panel (Qiagen), Quanty HAdV (Clonit), HAdV ELITe MGB® kit (Elitech Group), HADV R-GENE® (Biomerieux), FTD Respiratory Panel 21 Assays (Siemens Healthineers). All assays were performed according to the manufacturer's instructions. Two laboratories used an *in-house* assay [17,18]. Alongside hAdV, clinical samples were screened for the presence of influenza viruses (FluA/FluB), parainfluenza viruses (PIVs), enterovirus (EV), metapneumovirus (MPV), seasonal coronaviruses (hCoV), human rhinovirus (hRV), respiratory syncytial virus (RSV), human bocavirus (hBoV) and SARS-CoV-2.

2.3. HAdV genotyping

HAdV DNA was amplified in a nested PCR targeting the *hexon* gene, according to Lu and Erdman 2006 [19]. Specifically, the first amplification was performed using AmpliTaqGold® with GeneAmp® (Life Technologies, NJ, USA) according to the manufacturer's instructions. The thermal profile was as follows: 95 °C for 10 min, then 50 cycles of 95 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min 30 s. The final step was 72 °C for 5 min. Nested amplification was performed with the same thermal profile, for a total of 40 cycles. The amplicons obtained have an expected size of approximately 800 nt. Amplicons were sequenced with the internal primers already used in the nested-PCR using different sequencing platforms (3500 xL Dx Genetic Analyzer, SeqStudio Genetic Analyser system and ABI PRISM® 3100 GeneticAnalyser, Applied Biosystem, NJ, USA). The resulting sequences were analyzed using Sequencher software, version 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA). BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to identify the hAdV genotype.

2.4. Statistical analysis

Comparisons between positivity rates in different age groups were calculated with χ 2 test. Statistical analyses were performed using GraphPad software version 8.3.0 (Prism).

3. Results

3.1. Study samples

A total of 54463 respiratory specimens collected between January 1, 2022 and June 20, 2023 were tested for the presence of respiratory viruses. Of these, 3583 (6.6 %) were hAdV-positive. Of the 3583 specimens that resulted positive to hAdV 2064 were nasal swabs (2064/3583, 57.7 %), 1449 nasopharyngeal aspirates (1449/3583, 40.4 %), and 70 bronchoalveolar lavages (70/3583, 1.9 %). The median age of the hAdV-positive patients was 3 years, (**25th percentile: 1 year, 75th percentile: 5 years**; range 6 days–98 years), including 1993 male and 1590 female subjects (55.6 % and 44.4 %, respectively). As shown in Fig. 2, hAdV-positive cases were reported in all age groups in both male and female subjects, with male individuals in the 1–2 years age group being the most affected. Overall, no statistically significant difference was observed between male and female subjects among all age groups ($p = 0.13$). However, a statistically significant difference was observed

Fig. 1. Geographical distribution of clinical microbiology laboratories participating in the study. The following microbiology laboratories have participated in the study: Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia/Microbiology and Virology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia; Department of Biomedical Sciences for Health, University of Milan; Department of Medicine and Innovation Technology, University of Insubria (DIMIT)/Laboratory of Medical Microbiology and Virology University Hospital of Varese; Virology Unit, Clinical Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan; Laboratory of Microbiology and Virology, Provincial Hospital of Bolzano; UOC Microbiology- Dept. Specialist and laboratory medicine, Treviso; Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna/Section of Microbiology, Department of Medical and Surgical Sciences, University of Bologna; Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona; Virology Unit, Azienda Ospedaliero Universitaria delle Marche, Ancona, Italy; Laboratory of Virology, National Institute for Infectious Diseases "Lazzaro Spallanzani" IRCCS, Rome; Department of Diagnostic and Laboratory Medicine, Unit of Microbiology and Diagnostic Immunology, Bambino Gesù Children Hospital IRCCS, Roma; Microbiology and Virology, Cotugno Hospital AORN dei Colli, Naples; Virology Laboratory - Microbiology And Virology Unit -University Of Bari - Policlinic Of Bari. For each center the total number of HAdV-positive samples and the total number of respiratory samples tested for the presence of HAdv is reported.

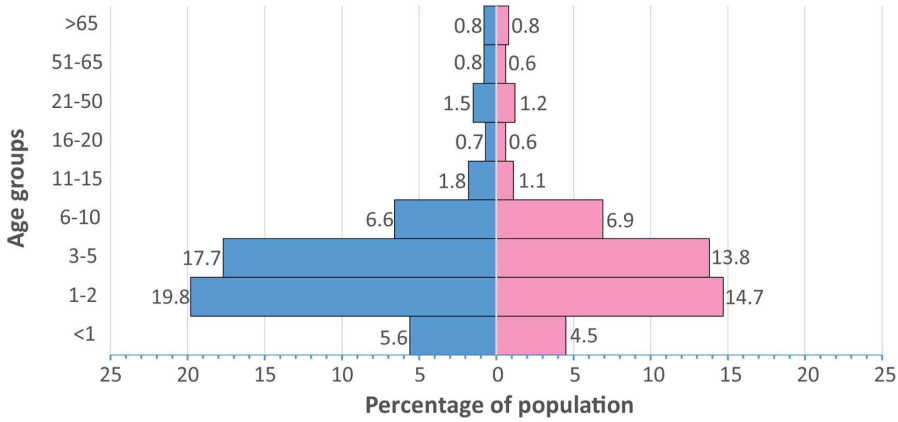

Fig. 2. Distribution of hAdV-positive cases by age group and by gender. Blue bars represent male subjects, pink bars represent female subjects. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

between the number of hAdV-positive male subjects (1541/1993, 77.3 %) as compared to the number of hAdV-positive female subjects under 5 years of age (1184/1590, 74.4 %, p = 0.02).

3.2. Temporal distribution of hAdV-positive cases

The majority of all hAdV cases (1648/3583, 46 % of the total) were observed between weeks 10–2023 (beginning of March) and 24–2023 (mid-June). Of note, since week 10–2023 the positivity rate was steadily higher than 10 %. (Fig. 3). Positivity rates varied throughout the study period, with the lower value observed in week 6–2022 (1.7 %, 8 positive specimens out of 471 specimens tested), while the higher value of the positivity rate was observed in week 22–2023 (15.8 %, 97 positive samples out of 614 samples tested) (Fig. 3).

3.3. Co-detection of other respiratory viruses

In 1561/3583 (43.5 %) hAdV-positive samples, at least one additional respiratory virus was detected. A single additional virus was detected in 1121/1561 cases (71.8 %), two other viruses in 348 (22.3 %), and three other viruses in 75 (4.8 %). Co-detection of 4 or 6 other respiratory viruses was reported in less than 1 % of cases. hRV was the most frequently co-detected virus (842/1561, 54 %) followed by RSV (206/1561, 13.2 %), while PIVs, MPV, EV and hBoV were detected in 12 %, 11.3 %, 9.7 % and 9 % of cases of co-detections (187, 177, 152 and 141 out of 1561 cases, respectively). Seasonal hCoVs and influenza A virus were co-detected in 8.6 % and 6.3 % of cases (134 and 99 out of 1561 cases, respectively). All other viruses detected, including influenza B virus, parechovirus and SARS-CoV-2 were detected in less than 6 % of cases.

3.4. HAdV genotyping

Genotyping results were available for 158 samples, collected between July 2022 and June 2023 out of 3583 (4.4 %) hAdV-positive cases. The genotypes detected were hAdV-B3 (98/158, 62 %), -C2 (30/158, 19 %), -C1 (19/158, 12 %), -C5 (7/158, 4.4 %), -B7 (3/158, 1.9 %) and -C6 (1/158, 0.7 %). From July 2022 to January 2023, hAdV-

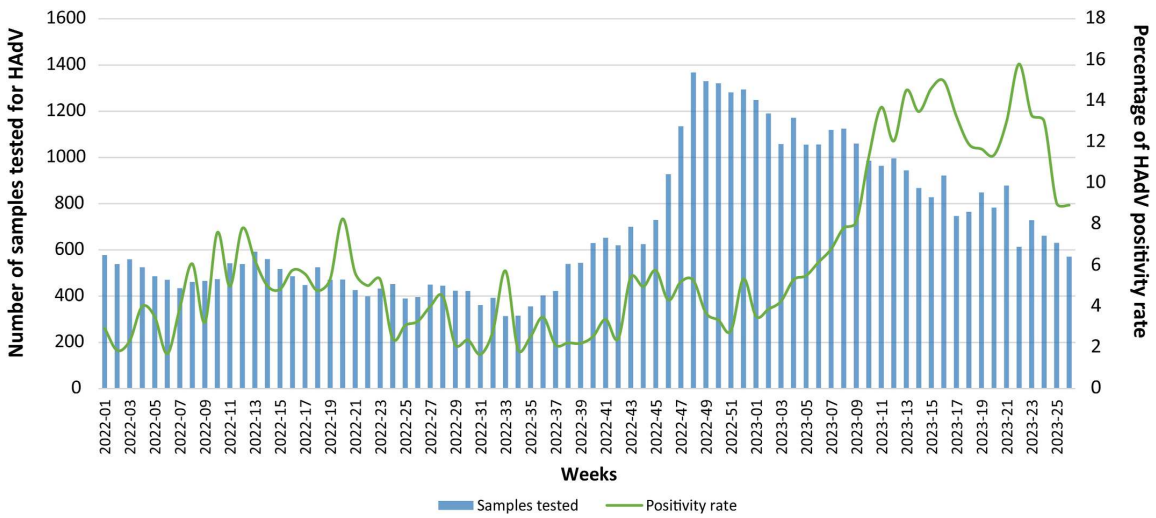

Fig. 3. Weekly distribution of the number of samples tested for hAdV and hAdV positivity rates by week throughout the study period (January 2022–June 2023).

C strains were the most frequently detected: 34/41 (82.9 %) strains sequenced belonged to hAdV-C1, -C2 or -C5, while 7/41 (17.1 %) belonged to hAdV-B species. From February 2023 to summer 2023, hAdV-B3 strains were the predominant circulating strains in Italy, with 92/117 (78.6 %) strains identified. The remaining hAdV strains, except for 2/117 (1.7 %) strains identified as hAdV-B7, 23/117 (19.6 %) strains belonged to hAdV-C species (Fig. 4).

4. Discussion

HAdV infection accounts for at least 5–7% of all viral respiratory infections, and children under 5 years of age are the most affected [4]. The present study investigated the epidemiology of hAdV respiratory infections in adult and pediatric populations. In a large retrospective study involving 12 laboratories distributed throughout Italy involving more than 50.000 samples, hAdV was detected in 6.6 % of specimens included in the study. Our results agree with those published by other groups [20,21], who reported an overall positivity rate of 6.6 % and 6.8 %, respectively. In contrast, the percentage of hAdV-positive cases in our study was higher than that reported by Radin et al. [22] and Pscheidt et al. [23], where hAdV accounted for approximately 2 % of all respiratory infections. Positivity rates varied throughout the study period, ranging from 1.7 % reported in February 2022 to 15.8 % reported in June 2023. This can be explained using non-pharmaceutical interventions (NPIs): during the COVID-19 pandemic, from 2020 to the fall season of 2022, the circulation of respiratory viruses decreased, mainly due to NPIs such as social distancing, smart working, school closure, hygiene measures, use of personal protective equipment such as facial masks to control SARS-CoV-2 circulation. Thus, the circulation of other respiratory viruses was also affected [24,25]. In the autumn of 2022, as health-restrictive measures were gradually reduced, the spread of respiratory viruses was restored to pre-pandemic levels with an increasing number of samples analyzed with a panel of respiratory viruses [26]. In our study population, the median age of hAdV-positive patients was 3 years, and, particularly, male children in the 1–2 years age group were most affected. These data are in line with other reports [14,23,27] These results also confirm that children under 5 years of age are more likely to be affected by hAdV than adults. There is a growing awareness of the importance of sex and gender in medicine and research. Females typically have stronger immune responses to self and foreign antigens than males, leading to sex differences in autoimmunity and infectious diseases [27]. In both animals and humans, males are generally more susceptible to bacterial infections than females. As hypothesised by Cheng et al. [28], the difference in infection rates between males and females could be explained by the fact that a gene that regulates the natural killer response is located on the X chromosome and is therefore expressed twice as much in females as in males. At the same time, gender differences have been reported in health-seeking behaviour, quality of health care and adherence to treatment recommendations. As in our studies most infections were reported in children <5 years, the difference in infection rate between males and females may depend on the recreational activities: males tend to play in larger groups than females and prefer rough-and-tumble games reducing personal distances and increasing the probability to get infection [29].

Viral co-infection may play a crucial role in the outcome of a respiratory syndrome: it has been reported that patients (mostly immunocompromised individuals or adults >65 years) with more than one respiratory virus, including rhinovirus and SARS-CoV-2, are more likely to report cough or dyspnoea or to be admitted to intensive care units [30,31]. However, other studies have not observed worsening of clinical syndromes due to viral co-detection, suggesting that disease severity is not correlated with the number of respiratory viruses detected [22,32,33]. In our study, the rate of co-infection accounted for at least 40 % of hAdV episodes. This value is higher than that reported in a study conducted in Vietnam in 2022, where the co-detection rate was at 20 % [13]. This difference in co-infection rates is probably due to the different length of the study period. While our study considered a period of 18 consecutive months, the study by Nguyen and colleagues only considered 1 month in 2022; moreover, as they reported, RSV was not included in the testing panel. Thus, some cases of co-detection may be missing. On the contrary, it is lower than the 81 % cases of viral co-detection reported in Jordan between 2010 and 2013 [34]. This may be explained by the nature of this study. It was indeed conducted in three years in hospitalized children under 2 years of age with acute respiratory disease, including those with underlying conditions. The population of our study, instead, included pediatric and adult patients, regardless of clinical syndromes. Similar to what was reported by Probst and colleagues, also in our study rhinovirus was the most commonly co-detected with hAdV (54 %), whereas in our study co-detection of influenza A virus was observed in 6 %. This may be explained by the seasonality of respiratory virus circulation: rhinovirus circulates throughout the year, with peaks in spring and autumn while other viruses such as influenza circulate during winter season. This study considered an 18-month period from January 2022 and June 2023,

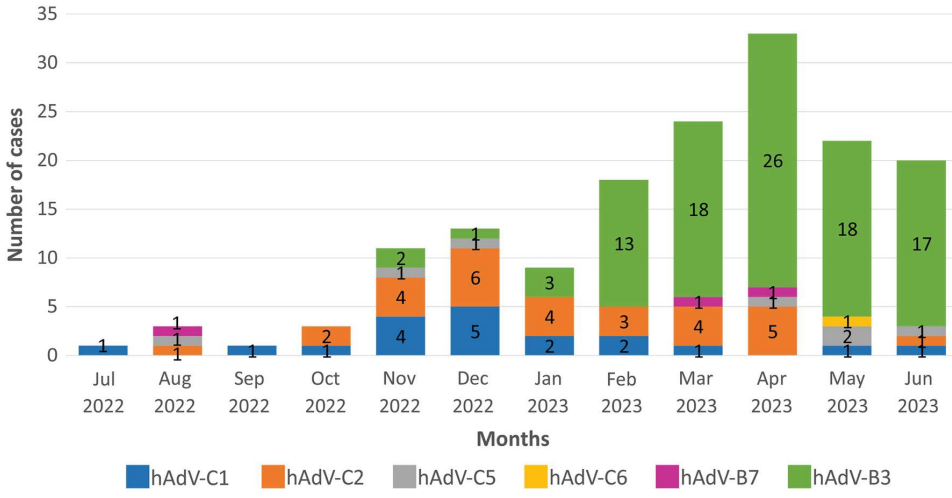

Fig. 4. Number of hAdV genotypes identified by month from July 2022 to June 2023.

including a period of reduced circulation of respiratory viruses due to NPIs.

Genotyping of the hAdV strains included in our study showed that hAdV-B, and, in particular hAdV-B3, was the most commonly detected (64 %), followed by hAdV-C strains (36 %). This finding is similar to studies reporting hAdV-B types as the most commonly detected [11,13, 35]. Some other studies instead have reported a different pattern of hAdV genotypes detected, with species C being the most commonly detected [34]. Nevertheless, our study confirms that hAdV species B and C are mainly associated with respiratory infections. Beyond the epidemiological purpose, genotyping of hAdV strains could also be useful as predictor of treatment effect as reported by Matthes-Martin et al., some antivirals showed higher *in vitro* efficacy for hAdV-C, with limited activity for species A, B and D [36].

Our study has several limitations: i) due to the nature of the study, focusing on epidemiological patterns rather than clinical outcomes, no clinical data of the patients included in the study were available, even if they would provide valuable insights into the impact of adenovirus infections. Thus we were not able to establish a possible correlation between hAdV genotypes and respiratory disease severity; ii) unfortunately, data on the total number of respiratory samples were not available, thus we could not calculate age group-specific positivity rates; iii)some of the commercial assays used in the different laboratories for hAdV detection are qualitative and it was not possible to investigate the potential correlation between viral loads and severity of disease and, iv) genotyping was performed only from July 2022 and, therefore, no data are available on hAdV types circulating before summer 2022. Besides, the number of genotyped strains is relatively small. Unfortunately, not all clinical laboratories can perform sequencing tests. Indeed, the genotyping data reported in the study were obtained from three laboratories all located in Lombardy (northern Italy). Regrettably, due to the retrospective nature of the study, it was not even possible to collect positive samples from each laboratory. Moreover, v) these results are based on local data from Italy and may not be applicable on an international scale, in particular with regard to the genetic analysis.

5. Conclusion

The results of this multicentre retrospective study show that hAdV accounts for nearly 7 % of all viral respiratory infections in the post-pandemic period, and that children under 5 years of age are at high risk of infection. Besides, genotyping revealed that hAdV-B3 was the most commonly detected genotype circulating in Italy in 2022–2023. The importance of hAdV as a causative agent of respiratory syndromes was emphasized, suggesting that further studies on circulating genotypes and their correlation with different clinical presentations are needed to fill in the knowledge on hAdV circulation in our country and in Europe.

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Contributors' statement

Federica A.M. Giardina, Laura Pellegrinelli, Federica Novazzi contributed to the study design; acquisition, analysis, and interpretation of the data; and drafting of the manuscript. Elisa Vian, Valeria Biscaro, Cristina Russo, Stefania Ranno, Elisabetta Pagani, Elisa Masi, Claudia Tiberio, Martina Esposito, Sara Uceda Renteria, Anna Paola Callegaro, Giulia Piccirilli, Tiziana Lazzarotto, Francesca Rovida, Cristina Galli, Eleonora Lalle, Fabrizio Maggi, Nicasio Mancini, Carla Acciarri, Stefano Menzo, Anna Maria Colacicco, Maria Scarasciulli contributed to the data collection and acquisition, formal analysis, interpretation of the data and investigation; Antonio Piralla had a major influence on the study design and contributed to the revision of the manuscript; Fausto Baldanti and Elena Pariani contributed to the study design, interpretation of the data, and revision of the manuscript. All authors contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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References

[1] Davison AJ, Benkő M, Harrach B. Genetic content and evolution of adenoviruses. J Gen Virol 2003;84(Pt 11):2895–908. https://doi.org/10.1099/vir.0.19497-0.
[2] Biserni GB, Scarpini S, Dondi A, Biagi C, Pierantoni L, Masetti R, et al. Potential diagnostic and prognostic biomarkers for adenovirus respiratory infection in children and young adults. Viruses 2021;13(9):1885. https://doi.org/10.3390/v13091885.
[3] Arnold A, MacMahon E. Adenovirus infections. Medicine 2021 Dec;49(12):790–3.
[4] Shieh WJ. Human adenovirus infections in pediatric population - an update on clinico-pathologic correlation. Biomed J 2022;45(1):38–49. https://doi.org/10.1016/j.bj.2021.08.009.
[5] Wen S, Lin Z, Zhang Y, Lv F, Li H, Zhang X, et al. The epidemiology, molecular, and clinical of human adenoviruses in children hospitalized with acute respiratory infections. Front Microbiol 2021;12:629971. https://doi.org/10.3389/fmicb.2021.629971.
[6] Dominguez O, Rojo P, de Las Heras S, Folgueira D, Contreras JR. Clinical presentation and characteristics of pharyngeal adenovirus infections. Pediatr Infect Dis J 2005;24(8):733–4. https://doi.org/10.1097/01.inf.0000172942.96436.2d.
[7] Narra R, Bono P, Zoccoli A, Orlandi A, Piconi S, Grasselli G, et al. Acute respiratory distress syndrome in adenovirus type 4 pneumonia: a case report. J Clin Virol 2016;81:78–81. https://doi.org/10.1016/j.jcv.2016.06.005.
[8] Lin TY, Tsai YS, Shen CF. Type 3 adenovirus respiratory infection complicated with catastrophic bronchiole damage and related barotrauma. Pediatr Neonatol 2022;63(3):319–21. https://doi.org/10.1016/j.pedneo.2021.11.011.
[9] Perez A, Lively JY, Curns A, Weinberg GA, Halasa NB, Staat MA, et al. Respiratory virus surveillance among children with acute respiratory illnesses - new vaccine surveillance network, United States, 2016-2021. MMWR Morb Mortal Wkly Rep 2022;71(40):1253–9. https://doi.org/10.15585/mmwr.mm7140a1.
[10] Teoh Z, Conrey S, McNeal M, Burrell A, Burke RM, Mattison C, et al. Burden of respiratory viruses in children less than 2 Years old in a community-based

longitudinal US birth cohort. Clin Infect Dis 2023;77(6):901–9. https://doi.org/10.1093/cid/ciad289.
[11] Chen Y, Lin T, Wang CB, Liang WL, Lian GW, Zanin M, et al. Human adenovirus (HAdV) infection in children with acute respiratory tract infections in Guangzhou, China, 2010-2021: a molecular epidemiology study. World J Pediatr 2022;18(8):545–52. https://doi.org/10.1007/s12519-022-00590-w.
[12] Xu D, Ji L, Chen L, Wu X. Molecular typing and epidemiology profiles of human adenovirus infection among children with severe acute respiratory infection in Huzhou, China. Lett Appl Microbiol 2022;75(5):1225–31. https://doi.org/10.1111/lam.13790.
[13] Nguyen DD, Phung LT, Thanh Tran HT, Ly HTT, Vo AHM, Dinh NP, et al. Molecular subtypes of Adenovirus-associated acute respiratory infection outbreak in children in Northern Vietnam and risk factors of more severe cases. PLoS Negl Trop Dis 2023;17(11):e0011311. https://doi.org/10.1371/journal.pntd.0011311.
[14] Esposito S, Zampiero A, Bianchini S, Mori A, Scala A, Tagliabue C, et al. Epidemiology and clinical characteristics of respiratory infections due to adenovirus in children living in milan, Italy, during 2013 and 2014. PLoS One 2016;11(4):e0152375. https://doi.org/10.1371/journal.pone.0152375.
[15] Parcell BJ, McIntyre PG, Yirrell DL, Fraser A, Quinn M, Templeton K, et al. Prison and community outbreak of severe respiratory infection due to adenovirus type 14p1 in Tayside, UK. J Public Health 2015;37(1):64–9. https://doi.org/10.1093/pubmed/fdu009.
[16] Cederwall S, Påhlman LI. Respiratory adenovirus infections in immunocompetent and immunocompromised adult patients. Epidemiol Infect 2020 Jan 3;147:e328. https://doi.org/10.1017/S0950268819002176.
[17] Piralla A, Pariani E, Rovida F, Campanini G, Muzzi A, Emmi V, et al. Segregation of virulent influenza A(H1N1) variants in the lower respiratory tract of critically ill patients during the 2010-2011 seasonal epidemic. PLoS One 2011;6(12):e28332. https://doi.org/10.1371/journal.pone.0028332.
[18] Jothikumar N, Cromeans TL, Hill VR, Lu X, Sobsey MD, Erdman DD. Quantitative real-time PCR assays for detection of human adenoviruses and identification of serotypes 40 and 41. Appl Environ Microbiol 2005;71:3131–6. https://doi.org/10.1128/AEM.71.6.3131-3136.2005.
[19] Lu X, Erdman DD. Molecular typing of human adenoviruses by PCR and sequencing of a partial region of the hexon gene. Arch Virol 2006;151(8):1587–602. https://doi.org/10.1007/s00705-005-0722-7.
[20] Zadheidar S, Yavarian J, Heydarifard Z, Nejati A, Sadeghi K, Ghavami N, et al. Molecular epidemiology of human adenoviruses in children with and without respiratory symptoms: preliminary findings from a case-control study. BMC Pediatr 2022;22(1):583. https://doi.org/10.1186/s12887-022-03625-3.
[21] Saha R, Majumdar A, Chaudhuri RD, Chatterjee A, Lo M, Dutta S, et al. Molecular epidemiology of circulating human adenoviruses among acute respiratory infection patients seeking healthcare facilities in West Bengal, India. Virology 2023;588:109912. https://doi.org/10.1016/j.virol.2023.109912.
[22] Radin JM, Hawksworth AW, Kammerer PE, Balansay M, Raman R, Lindsay SP, et al. Epidemiology of pathogen-specific respiratory infections among three US populations. PLoS One 2014;9(12):e114871. https://doi.org/10.1371/journal.pone.0114871.
[23] Pscheidt VM, Gregianini TS, Martins LG, Veiga ABGD. Epidemiology of human adenovirus associated with respiratory infection in southern Brazil. Rev Med Virol 2021;31(4):e2189. https://doi.org/10.1002/rmv.2189.
[24] Brañas P, Muñoz-Gallego I, Espartosa E, Moral N, Abellán G, Folgueira L. Dynamics of respiratory viruses other than SARS-CoV-2 during the COVID-19 pandemic in Madrid, Spain. Influenza Other Respir Viruses 2023;17(9):e13199. https://doi.org/10.1111/irv.13199.
[25] De Francesco MA, Pollara C, Gargiulo F, Giacomelli M, Caruso A. Circulation of respiratory viruses in hospitalized adults before and during the COVID-19 pandemic in brescia, Italy: a retrospective study. Int J Environ Res Publ Health 2021;18(18):9525. https://doi.org/10.3390/ijerph18189525.
[26] Chow EJ, Uyeki TM, Chu HY. The effects of the COVID-19 pandemic on community respiratory virus activity. Nat Rev Microbiol 2023;21(3):195–210. https://doi.org/10.1038/s41579-022-00807-9.
[27] Dias SP, Brouwer MC, van de Beek D. Sex and gender differences in bacterial infections. Infect Immun 2022 Oct 20;90(10):e0028322. https://doi.org/10.1128/iai.00283-22.
[28] Cheng MI, Li JH, Riggan L, Chen B, Tafti RY, Chin S, et al. The X-linked epigenetic regulator UTX controls NK cell-intrinsic sex differences. Nat Immunol 2023 May;24(5):780–91. https://doi.org/10.1038/s41590-023-01463-8.
[29] Barnadas C, Schmidt DJ, Fischer TK, Fonager J. Molecular epidemiology of human adenovirus infections in Denmark, 2011-2016. J Clin Virol 2018;104:16–22. https://doi.org/10.1016/j.jcv.2018.04.012.
[30] Le Glass E, Hoang VT, Boschi C, Ninove L, Zandotti C, Boutin A, et al. Incidence and outcome of coinfections with SARS-CoV-2 and rhinovirus. Viruses 2021;13(12):2528. https://doi.org/10.3390/v13122528.
[31] Trifonova I, Christova I, Madzharova I, Angelova S, Voleva S, Yordanova R, et al. Clinical significance and role of coinfections with respiratory pathogens among individuals with confirmed severe acute respiratory syndrome coronavirus-2 infection. Front Public Health 2022;10:959319. https://doi.org/10.3389/fpubh.2022.959319.
[32] Scotta MC, Chakr VC, de Moura A, Becker RG, de Souza AP, Jones MH, et al. Respiratory viral coinfection and disease severity in children: a systematic review and meta-analysis. J Clin Virol 2016;80:45–56. https://doi.org/10.1016/j.jcv.2016.04.019.
[33] Canela LNP, Magalhães-Barbosa MC, Raymundo CE, Carney S, Siqueira MM, Prata-Barbosa A, et al. Viral detection profile in children with severe acute respiratory infection. Braz J Infect Dis 2018;22(5):402–11. https://doi.org/10.1016/j.bjid.2018.09.001.
[34] Probst V, Rankin DA, Haddadin Z, Hamdan L, Rahman HK, Yanis A, et al. Adenovirus infection in hospitalized children with acute respiratory infection in Jordan. Pediatr Infect Dis J 2022;41(4):277–83. https://doi.org/10.1097/INF.0000000000003423.
[35] Wang L, Hu X, Huang Z, Zhang Y, Zhao X, Liu X, et al. Analysis of the typing of adenovirus and its clinical characteristics in children with acute respiratory tract infection. BMC Pediatr 2023;23(1):25. https://doi.org/10.1186/s12887-023-03840-6.
[36] Matthes-Martin S, Boztug H, Lion T. Diagnosis and treatment of adenovirus infection in immunocompromised patients. Expert Rev Anti Infect Ther 2013;11(10):1017–28. https://doi.org/10.1586/14787210.2013.836964.

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SARS-CoV-2 vaccine breakthrough infections with the alpha variant are asymptomatic or mildly symptomatic among health care workers

Francesca Rovida[1,2], Irene Cassaniti[1], Stefania Paolucci[1], Elena Percivalle[1], Antonella Sarasini[1], Antonio Piralla[1], Federica Giardina[1], Josè Camilla Sammartino[1], Alessandro Ferrari[1], Federica Bergami[1], Alba Muzzi[3], Viola Novelli[3], Alessandro Meloni[3,4], Sara Cutti[3], Anna Maria Grugnetti[5], Giuseppina Grugnetti[5], Claudia Rona[3], Marinella Daglio[3], Carlo Marena[3], Antonio Triarico[6], Daniele Lilleri[1✉] & Fausto Baldanti[1,2]

Vaccine breakthrough SARS-CoV-2 infection has been monitored in 3720 healthcare workers receiving 2 doses of BNT162b2. SARS-CoV-2 infection is detected in 33 subjects, with a 100-day cumulative incidence of 0.93%. Vaccine protection against acquisition of SARS-CoV-2 infection is 83% (95%CI: 58–93%) in the overall population and 93% (95%CI: 69-99%) in SARS-CoV-2-experienced subjects, when compared with a non-vaccinated control group from the same Institution, in which SARS-CoV-2 infection occurs in 20/346 subjects (100-day cumulative incidence: 5.78%). The infection is symptomatic in 16 (48%) vaccinated subjects *vs* 17 (85%) controls ($p = 0.01$). All analyzed patients, in whom the amount of viral RNA was sufficient for genome sequencing, results infected by the alpha variant. Antibody and T-cell responses are not reduced in subjects with breakthrough infection. Evidence of virus transmission, determined by contact tracing, is observed in two (6.1%) cases. This real-world data support the protective effect of BNT162b2 vaccine. A triple antigenic exposure, such as two-dose vaccine schedule in experienced subjects, may confer a higher protection.

[1] Molecular Virology Unit, Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy. [2] Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy. [3] Medical Direction, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy. [4] Department of Public Health, Experimental and Forensic Medicine, Section of Hygiene, University of Pavia, Pavia, Italy. [5] Health Professions Direction, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy. [6] Direzione Sanitaria, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy. ✉email: d.lilleri@smatteo.pv.it

Since the identification of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as the etiological agent of Coronavirus Disease 19 (COVID-19), several efforts have been made in order to prevent infection and disease. Moreover, recently, highly effective vaccines have been introduced[1–4].

The licensed vaccines showed high efficacy in protection from SARS-CoV-2 infection in clinical trials, ranging from 70 to 95%[1–4]. However, post-authorization real-life studies are an important complement to evaluate the vaccine effectiveness in different populations and in the face of non-controlled real-world challenges.

Initial nationwide data collection are confirming the effectiveness of the licensed vaccines, showing an effect size consistent with that reported in clinical trials [5–8].

However, clinical, virological, and immunological characteristics of breakthrough SARS-CoV-2 infections after vaccination have been poorly investigated, due to a lack of prospective systematical testing in vaccinated cohorts. Some studies conducted on healthcare workers reported a lower rate of symptomatic vs asymptomatic infections in vaccinated with respect to unvaccinated individuals[9–13], while data on the actual presence of infectious virus in SARS-CoV-2 RNA-positive samples recovered from vaccinated individuals are missing. Whether infected vaccinated subjects can transmit the infection, and to which extent, is a major concern for public health policy. Finally, whether post-vaccine infections are associated with a deficient immune response to vaccination has not been investigated yet.

Healthcare workers have a high risk of exposure to SARS-CoV-2, therefore representing a challenging cohort for the evaluation of vaccine effectiveness and breakthrough infections. In Italy, the vaccination campaign started on December 27th, 2020, prioritizing healthcare workers and fragile and elderly individuals [14].

The aim of the present study is to investigate prospectively the risk of SARS-CoV-2 infection in vaccinated healthcare workers in a single Italian Center (Fondazione IRCCS Policlinico San Matteo, Pavia). Data are compared with the group of healthcare workers of the same Institution that did not receive the vaccination during the study period.

The characteristics of breakthrough infections, the underlying immune response, and the risk of virus transmission to other individuals are investigated.

Results show that the BNT162b2 vaccine is effective in reducing the incidence of SARS-CoV-2 infection in healthcare workers, while breakthrough infections are poorly symptomatic and are infrequently transmitted.

Results

Incidence and clinical characteristics of SARS-CoV-2 infection in vaccinated and non-vaccinated healthcare workers. During the period January 18-April 19 2021, 3720 healthcare workers received the second dose of the BNT162b2 vaccine. Overall, before vaccination 507 subjects resulted from SARS-CoV-2-experienced and 2761 SARS-CoV-2-naïve, while SARS-CoV-2 serostatus was unknown for 426 subjects and dubious for 26 subjects (Fig. 1). After complete vaccination schedule, SARS-CoV2 infection was detected in 33 subjects (median time: 47, range 7-90, days after vaccination): 2 subjects among the 507 SARS-CoV-2-experienced, 24 among the 2761 SARS-CoV-2 naïve individuals, and 7 among the 452 individuals with unknown or dubious serostatus (Fig. 1).

Of the 33 infected subjects, 23 (70%) were nursing or health care assistants, 8 (24%) were physicians, 1 (3%) was a healthcare technician and 1 (3%) was a researcher. The infection was symptomatic in 16 (48%) and asymptomatic in 17 (52%) subjects (Table 1). The most common symptoms reported were rhinitis in 9 (27%), cough in 3 (9%), and arthralgia in 3 (9%) infected subjects; more details on symptoms are described in Table 1. No subjects required hospitalization. Virus isolation from nasal swab was attempted in 21 subjects (13 symptomatic and 8 asymptomatic subjects). Infectious virus was recovered in 7/13 (54%) symptomatic and 4/8 (50%) asymptomatic subjects. Lineage characterization was available in 23 subjects in whom the amount of viral RNA was sufficient for genome sequencing. All analyzed patients were infected by the B.1.1.7 variant, also recently renamed as an alpha variant. Evidence of virus transmission to family members or close contacts of the 33 infected subjects was observed in 2 (6.1%, 95% CI: 1.1–19.6%) cases, both of whom had a symptomatic infection.

The incidence of SARS-CoV-2 infection after vaccination was compared to that observed in the control group, including 346 healthcare workers of the same Institution that did not receive the vaccination during the study period (January-May 2021). The control group included 69 SARS-CoV-2 experienced and 134 SARS-CoV-2 naïve, while SARS-CoV-2 serostatus was unknown for 143 subjects (Fig. 1). During January–May 2021, SARS-CoV-2 infection was detected in 20 non-vaccinated subjects: 1 subject among the 69 SARS-CoV-2-experienced, 8 among the 134 SARS-CoV-2 naïve individuals, and 11 among the 143 subjects with unknown serostatus (Fig. 1). Of the 20 infected subjects, 12 (60%) were nursing or health care assistants, 4 (20%) were administrative workers, 2 (10%) were physicians, 1 (5%) was a healthcare technician and 1 (5%) was a maintenance worker. The infection was symptomatic in 17 (85%) and asymptomatic in 3 (15%) subjects, with a significantly higher occurrence of symptoms than in vaccinated subjects ($p = 0.010$, Table 1). The most common symptoms reported were fever in 13 (65%), anosmia in 12 (60%), ageusia in 10 (50%), and asthenia in 8 (40%) infected subjects (Table 1), which were significantly more frequent than in vaccinated subjects ($p \leq 0.004$). Dyspnea, which occurred in 4 (8%) non-vaccinated controls, was also significantly higher in this group ($p = 0.017$). Two subjects with pneumonia required hospitalization and one was transferred to the Intensive Care Unit due to worsening of symptoms.

The 100-day cumulative incidence of SARS-CoV-2 infection in the overall population of vaccinated healthcare workers was 0.93% vs. 5.78% ($p < 0.001$) in the non-vaccinated control group (Fig. 2a), with a hazard ratio (HR) of 0.17 (95%CI: 0.07–0.42%) and a protective effect in the prevention of infection of 83% (95% CI: 58–93%). Excluding the 452 subjects with unknown or dubious serostatus, and considering separately experienced and naïve subjects (Fig. 2b), the 100 day cumulative incidence was 0.42% in SARS-CoV-2-experienced and 0.90% in SARS-CoV-2 naïve subjects ($p = 0.272$). Among non-vaccinated controls (Fig. 2c), after exclusion of the 143 subjects with unknown serostatus the 100-day cumulative incidence was 1.45% in SARS-CoV-2-experienced and 5.97% in SARS-CoV-2 naïve subjects ($p = 0.139$). The HR for developing SARS-CoV-2 infection in vaccinated *vs* non-vaccinated naïve subjects was 0.15 (95% CI: 0.03–0.76), with a protective effect of 86% (95% CI: 24–98%). The HR for developing secondary SARS-CoV-2 infection in vaccinated *vs* non-vaccinated naïve subjects was 0.29 (95%CI: 0.01–8.74), with a protective effect of 71% (95% CI: > 0–99%). However, the number of non-vaccinated experienced subjects was too low ($n = 69$) to detect a potentially significant effect in this latter comparison. The HR for developing SARS-CoV-2 infection after vaccination in experienced subjects vs non-vaccinated naïve subjects was 0.07 (95% CI: 0.01–0.31), with a protective effect of 93% (95% CI: 69–99%).

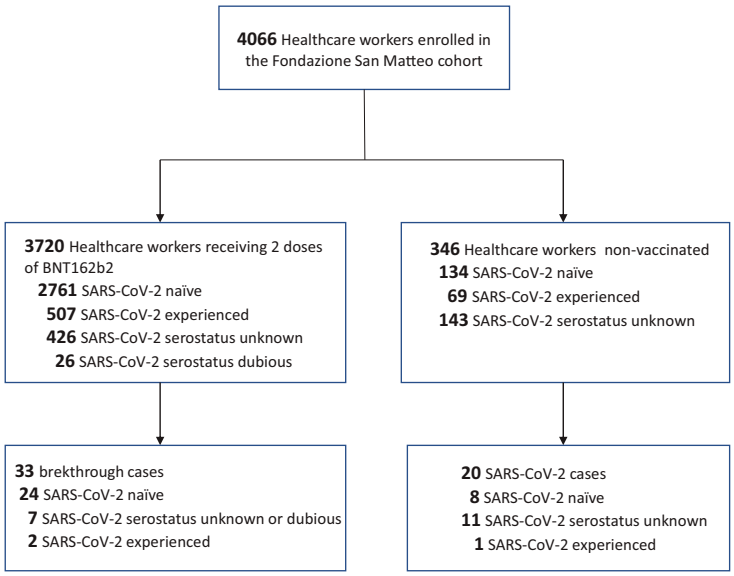

Fig. 1 Study profile. Data from 4066 healthcare workers of San Matteo hospital, Pavia, Italy were analysed: 3720 subjects received a complete schedule of BNT162b2 vaccine, while 346 subjects who did not receive vaccination during the study period were used as controls. According to serological or virological data subjects were considered SARS-CoV-2 experienced or naïve before initiation of the study, while SARS-CoV-2 serostatus was unknown or dubious for some individuals. SARS-CoV-2 infection was diagnosed during the study in 33 vaccinated subjects and 20 controls.

Table 1 Symptoms of SARS CoV in vaccinated and non-vaccinated subjects.

Symptoms	Vaccinated subjects ($n = 33$)	Non-vaccinated controls ($n = 20$)	*p*-value
any	16 (48%)	17 (85%)	0.010
fever (>37.5 °C)	2 (6%)	13 (65%)	<0.001
headache	2 (6%)	2 (10%)	0.627
asthenia	2 (6%)	8 (40%)	0.004
arthralgia	3 (9%)	5 (25%)	0.137
pharyngodynia	1 (3%)	2 (10%)	0.549
rhinitis	9 (27%)	2 (12%)	0.175
cough	3 (9%)	3 (15%)	0.661
pneumonia	0 (0%)	2 (10%)	0.138
dyspnea	0 (0%)	4 (20%)	0.017
anosmia	2 (6%)	12 (60%)	<0.001
ageusia	1 (3%)	10 (50%)	<0.001
nausea	1 (3%)	2 (10%)	0.549
diarrhea	1 (3%)	2 (10%)	0.549

Fisher's Exact test (two-sided) was used for statistical analysis.

Protective effect of immunity elicited by natural SARS-CoV-2 infection against secondary infections. To compare the protective effect of the immunity elicited by vaccination or natural infection, we analyzed the incidence of SARS-CoV-2 infection in SARS-CoV-2-experienced or naïve healthcare workers from the same Institution during the second pandemic wave, before the implementation of the vaccination campaign. In the period April 29-June 30 2020, 3810 healthcare workers were tested for previous SARS-CoV-2 infection according to serostatus determination: 336 subjects resulted in SARS-CoV-2 experienced and 3474 SARS-CoV-2 naïve. During the second pandemic wave, SARS-CoV-2 infection was detected in 9 SARS-CoV-2-experienced and 225 SARS-CoV-2 naïve subjects. The 3-months cumulative incidence of SARS-CoV-2 infection (Fig. 2d) was 2.68% in experienced vs. 6.48% in naïve subjects ($p = 0.006$), with a hazard ratio of 0.41 (95%CI: 0.26–0.61). The protective effect of the immunity elicited by natural infection was 59% (95% CI: 39–74%) Data on symptoms were available for 112 subjects: 1/4 (25%) naïve and 85/108 (79%) experienced subjects developed upper respiratory symptoms and no patient required hospitalization.

Immune response in vaccinated subjects with or without breakthrough SARS-CoV-2 infection. The antibody and T-cell response were determined in a subset of infected subjects within 48 h after diagnosis of infection and in a control subset of non-infected subjects who were SARS-CoV-2 naïve before vaccination. Anti-S1/S2 IgG antibodies were detected in all but one of 27 infected subjects at levels not significantly different from that observed in 143 non-infected controls (Fig. 3a). Neutralizing serum titer was determined in 24 infected subjects and compared to that observed in 128 controls (Fig. 3b). Median serum neutralizing titer was 1:320 (range 1:10-≥1:640), slightly lower (two-fold) than that observed in controls (median titer: ≥1:640; range: 1:20-≥1:640; $p = 0.045$). Finally, the T-cell response to the peptide pool of the Spike protein was not significantly different between 24 infected subjects and 132 controls (Fig. 3c).

Discussion

Results of this study suggest the protective effects of the BNT162b2 vaccine in the prevention of SARS-CoV-2 infection in healthcare workers. By comparing the incidence of vaccine breakthrough infections with the incidence of SARS-CoV-2 infection in healthcare workers that did not receive the vaccination during the study period, an 83% protection from infection was calculated. All vaccine breakthrough infections were asymptomatic or symptomatic mainly with few and mild symptoms as rhinitis. The frequency of symptomatic infections was lower in vaccinated than non-vaccinated subjects (48% vs 85%).

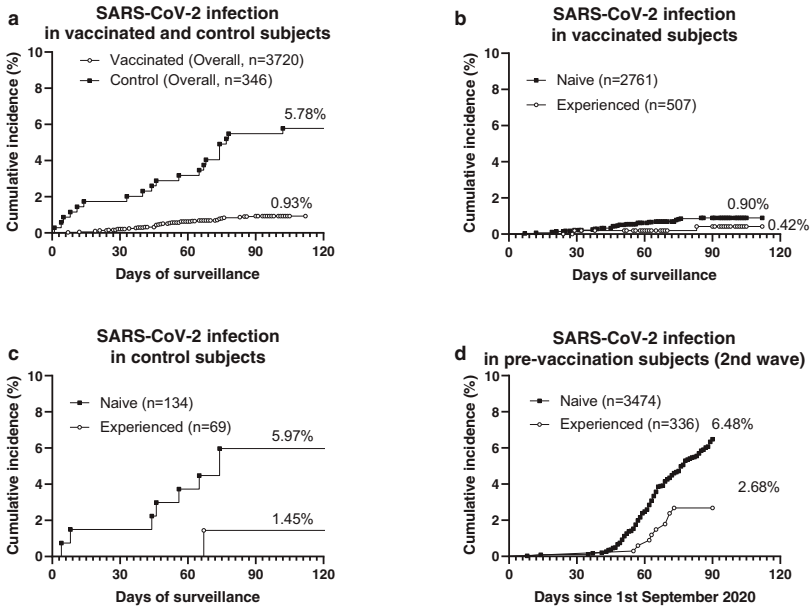

Fig. 2 SARS-CoV-2 infection in vaccinated and non-vaccinated healthcare workers of San Matteo hospital. a Cumulative incidence of SARS-CoV-2 infection in vaccinated subjects and non-vaccinated controls. **b** Cumulative incidence of SARS-CoV-2 infection in SARS-CoV-2-naïve and experienced vaccinated subjects. **c** Cumulative incidence of SARS-CoV-2 infection in SARS-CoV-2-naïve and experienced non-vaccinated control subjects. **d** Cumulative incidence of SARS-CoV-2 infection in SARS-CoV-2-naïve and experienced subjects before vaccine implementation during the second pandemic wave (period: September 1st-November 30th 2020).

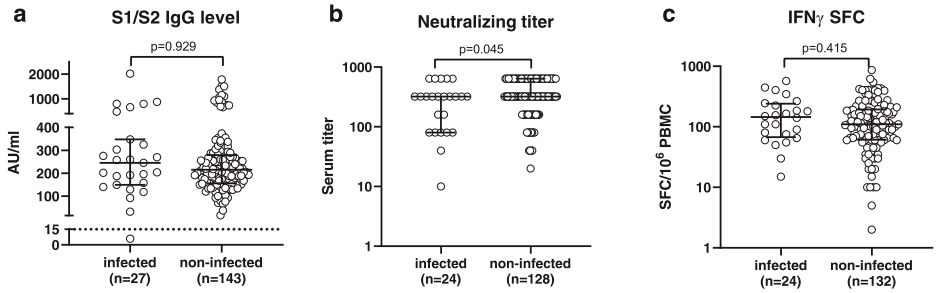

Fig. 3 Antibody and T-cell responses in vaccinated healthcare workers with or without SARS-CoV-2 breakthrough infections. a Anti-spike IgG level (AU: arbitrary units); **b** serum neutralizing titer; **c** IFNγ-producing spot forming cells (SFC)/10^6 peripheral blood mononuclear cells (PBMC). Immune response was determined within 48 h after diagnosis in infected subjects, and 21 days after complete vaccination schedule in non-infected subjects. Median values with interquartile ranges are shown. Mann–Whitney U-test (two-sided) was used for statistical analysis.

Live infectious virus was detected only in half of the cases of breakthrough infections and virus transmission to other individuals was documented in 6.1% of the cases. Finally, breakthrough infections were not associated with failure in developing antibody or T-cell response after vaccination.

The 83% effectiveness in the prevention of infection of BNT162b2 vaccine observed in our study is slightly lower than the 95% efficacy reported in the phase 2/3 clinical trial[1] but is consistent with those reported in a retrospective analysis conducted in Israel[9] and in the SIREN study conducted in England[10], both involving healthcare workers. The active surveillance and screening performed in our as well as in the other above-mentioned studies may have led to the identification of a number of subclinical infections that were not ascertained in the clinical trial. Nevertheless, vaccine effectiveness observed by this real-world analysis in healthcare workers remains high, and results are consistent among different countries.

Protection from secondary infections after primary SARS-CoV-2 infection appears effective and sustained for at least 10 months[15]. The protection from subsequent SARS-CoV-2 infection provided by the vaccine appears even higher than that induced by natural infection (83% vs. 59%, as observed in the retrospective analysis of our study). We analysed separately the incidence of breakthrough infections in individuals who were either SARS-CoV-2 naïve or experienced before vaccination. Although we observed a lower incidence of breakthrough infections in experienced than naïve subjects (0.42% vs. 0.90%) and a higher protective effectiveness (94% *vs* 83%), the population size was too low to verify whether SARS-CoV-2-experienced achieve significantly higher protection from infection after vaccination

than naïve subjects. In addition, we cannot exclude that previously infected individuals may have had different behaviors or underlying risk profiles. Nevertheless, immunogenicity studies showed that vaccination is able to boost the pre-existing immunity in experienced subjects, who develop higher antibody and T-cell levels than naïve subjects[16–18]. In addition, we observed that vaccination of experienced individuals elicited neutralizing antibody at levels that overcome the partial antibody escape of the B.1.351 variant[18]. It is tempting to speculate that three antigenic exposures, as in the case of SARS-CoV-2-experienced subjects receiving two vaccine doses, may confer a higher and broader protection.

Breakthrough infections were asymptomatic or symptomatic with few symptoms. To assess whether the detection of SARS-CoV-2 RNA in naso-pharyngeal samples of vaccinated subjects with breakthrough infection was associated with the presence of a live infectious virus, we attempted virus isolation on cell cultures in 21 of the 33 subjects, recovering infectious virus only in half of the cases. We could not compare the rates of infectious virus recovery in vaccinated *vs* unvaccinated SARS-CoV-2 RNA positive subjects, since we did not perform virus isolation in the control group. However, the low rate of detection of infectious virus is in line with a recent report showing decreased viral load in infected vaccinated subjects[19], and suggests a lower contagiousness, along with the lower severity, of the vaccine breakthrough infections. Thus, SARS-CoV-2 RNA detected in fully vaccinated is likely to be often a sign of an abortive infection limited and blocked on mucosal surfaces by the elicited immunity.

Most importantly, we documented a lack of transmission of the virus in the great majority of cases, since only 2/33 subjects transmitted the infection to a family member. The transmission was ascertained by contact tracing and analysis of naso-pharyngeal swabs of coworkers and family members. Although we cannot exclude that the actual rate of transmission could have been underestimated, this data suggests low contagiousness of SARS-CoV-2 infection in vaccinated subjects and the effectiveness of SARS-CoV-2 vaccination in blocking the infection spreading at the population level. However, this observation should be confirmed in controlled studies.

In order to verify the hypothesis that a poor individual response to the vaccine is the cause of breakthrough infections, we compared the antibody and T-cell responses of the infected individuals with a control group of vaccinated uninfected subjects. Since the immune response was evaluated in the peri-infection period (within 48 h after diagnosis), it is unlikely that the levels of antibody and T cells observed are the consequence of a boost due to the infection. No difference was observed for anti-Spike IgG and T-cells, while a slight reduction in the neutralizing serum titers was detected in the infected subjects. However, it was not possible to define a cutoff level of neutralizing titer able to identify poor responders that are at higher risk for vaccine breakthrough infections. We could exclude also that a viral variant associated to potential vaccine escape was causing the infection, since in all cases in which the RNA amount was sufficient for sequencing, the B.1.1.7 variant (alpha), which was accounting for the great majority of viral strains circulating in Italy during the study period, was detected. Before the end of the study, in Italy the first cases of delta variant have been reported, accounting for 0.02%[20]. No cases of delta variant were identified in vaccine breakthrough infections of our study.

The strength of this study resides in the prospective systematical collection of virological data (viral RNA detection and infectious virus recovery), clinical symptoms, immune response, and virus transmission to other individuals, providing an insight on the characteristics of vaccine breakthrough infection and initial observational evidence for their low contagiousness. The limitation resides in the small sample size of the control group for the evaluation of vaccine effectiveness, and the lack of control for the analysis of the contagiousness of breakthrough infections. Nevertheless, the observed vaccine effectiveness was similar to that observed in other real-world analysis on healthcare workers. Notwithstanding the non-random nature of the control group, we can assume that both vaccinated and control subjects were exposed to the same risk of SARS-CoV-2 infection, since all of them were healthcare workers of the same Institution. The decision to defer vaccination was taken by the individual subjects.

In conclusion, our analysis supports the effectiveness of the BNT162b2 vaccine in preventing SARS-CoV-2 infection, and suggest that breakthrough infections are poorly symptomatic and likely associated with a low contagiousness. Nevertheless, the duration of the vaccine protection should be evaluated in the next future.

Methods

Study subjects and design. The occurrence of SARS-CoV-2 infection was monitored prospectively in 3720 healthcare workers of Fondazione IRCCS Policlinico San Matteo, Pavia receiving 2 doses of the BNT162b2 vaccine. Subjects completed the vaccination schedule between January 18 and April 19, 2021 and data were collected until May 10, 2021.

Data on SARS-CoV-2 infection in 346 healthcare workers of the same Institution that did not receive the vaccination during the study period were used as control (data were collected in the period January 1–May 10, 2021).

Data on the incidence of SARS-CoV-2 infection during the second pandemic wave in 3810 healthcare workers from the same institution who had a serological definition of previous SARS-CoV-2 infection were used to compare protection from SARS-CoV-2 infection induced by the vaccine or by natural infection (data were collected in the period September 1st November 30, 2020). Subjects analysed are a subgroup of a cohort of healthcare workers partially described in [21].

Naso-pharyngeal swabs were collected and tested for SARS-CoV-2 RNA positivity in subjects with symptoms suggestive for SARS-CoV-2 infection or in case of contact with infected subjects as previously reported[22]. In vaccinated subjects who resulted in SARS-CoV-2 RNA-positive, blood samples for immune response analyses were collected 24–48 h after nasopharyngeal swab sampling. The transmission of the infection from SARS-CoV-2 RNA-positive vaccinated subjects was investigated by contact tracing and monitoring for SARS-CoV-2 RNA detection in nasal swabs in coworkers and family members of the infected subjects. Data on symptoms were collected during an interview by a physician and inserted into a specific database. Study procedures were approved by Fondazione IRCCS Policlinico San Matteo and Comitato Etico Area Pavia and all the subjects gave their written informed consent.

Virus isolation. Virus isolation was performed by inoculation of 200 µl naso-pharyngeal swab suspension medium, after decontamination with an antibiotics pool for 30 min at room temperature, on Vero E6 cells cultured in 24well plate, and detection of cytopathic effect after one-week culture.

Sequencing. Lineage characterization was available in 23 subjects in whom the amount of viral RNA was sufficient for genome sequencing. In detail, total RNAs were extracted from nasopharyngeal swabs by using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), followed by purification with Agencourt RNA Clean XP beads (Beckman Coulter, Inc., Brea, CA). Both the concentration and the quality of all RNA samples were measured and checked with the Nanodrop. Virus genomes were generated by using a multiplex approach, using version 1 of the CleanPlex SARS-CoV-2 Research and Surveillance Panel (Paragon Genomics, Hayward, USA), according to the manufacturer's protocol starting with 50 ng of total RNA. Briefly, the multiplex PCR was performed with two pooled primer mixtures and the cDNA reverse transcribed with random primers was used as a template. After ten rounds of amplification, the two PCR products were pooled and purified. Then the digestion reaction was performed to remove non-specific PCR products, followed by a second PCR reaction for barcoding with 24 rounds of amplification. Libraries were checked using High Sensitivity Labchip and quantified with the Qubit Fluorometric Quantitation system (Life Technologies, Carlsbad, CA, USA). An equimolar quantity of libraries was pooled, and the obtained run library mix was loaded at 1.5 pM into Illimuna MiSeq platform (Illumina, San Diego, CA, USA,) for sequencing[23]. NGS data were also analysed with an in-house pipeline and lineages were assigned from the alignment file using the Phylogenetic Assignment of Named Global Outbreak LINeages tool PANGOLIN v1.07 (https://github.com/hCoV-2019/pangolin).

SARS-CoV-2-specific antibody and T-cell determination. To detect subjects with SARS-CoV-2 infection after the first pandemic wave, serological analysis was performed in the period April 29-June 30 2020 using chemiluminescent assay (Liason SARS-CoV-2 S1/S2 IgG, Diasorin, Saluggia, Italy) for the quantitative measurement of SARS-CoV-2 anti-S1 and anti-S2 IgG antibody. Results higher than 15 AU/mL were considered positive and defined SARS-CoV-2 experienced subjects, whereas results below 12 AU/mL were considered negative and defined SARS-CoV-2 naïve subjects. Subjects with borderline results ranging from 12 and 15 AU/mL were not included in the analysis. A further serological screening was conducted after the second pandemic wave in the period December 15 2020-February 3 2021. Since this second screening overlapped with the initiation of the vaccination campaign, the electro-chemiluminescent assay Elecsys Anti-SARS-CoV-2 N (Roche Diagnostics Rotkreuz, Switzerland), which provides quantitative measures of mainly IgG (but also IgA and IgM) specific for SARS-CoV-2 Nucleocapsid protein (not present in the vaccine) was used. Results were given as units (U)/ml and are considered positive when ≥0.8 U/ml. Subjects with positive serological results after either or both screenings were considered SARS-CoV-2 experienced before vaccination.

Antibody and T-cell response to Spike protein was determined in vaccinated subjects after detection of SARS-CoV-2 infection and in a control group of SARS-CoV-2-naïve subjects 21 days after complete vaccination[18]. Anti-Spike IgG antibody was determined using Liason SARS-CoV-2 S1/S2 IgG (Diasorin). Neutralizing antibody serum titre was determined as following described[24]. Fifty µl of serum, from 1:10 to 1:640 in a serial fourfold dilution, were added in two wells of a flat bottom tissue culture microtiter plate (COSTAR, Corning Incorporated, NY 14831, USA). Then, the same volume of 100 TCID50 of SARS-CoV-2 strain was added and plates were incubated at 33 °C in 5% CO_2. All the dilutions were made in E-MEM with the addition of 1% penicillin, streptomycin, and glutammin and 5 γ/mL of trypsin. After 1 h incubation at 33 °C 5% CO_2, Vero E6 cells were added to each well. After 72 h of incubation at 33 °C 5% CO_2, plates were stained with Gram's crystal violet solution (Merck KGaA, 64271 Damstadt, Germany) plus 5% formaldehyde 40% m/v (Carlo ErbaSpA, Arese [MI], Italy) for 30 min. Microtiter plates were then washed under running water. Wells were scored to evaluate the degree of cytopathic effect (CPE) compared to the virus control. Blue staining of wells indicated the presence of neutralizing antibodies. Neutralizing titer was the maximum dilution with the reduction of 90% of CPE. Results higher or equal to 1:10 serum titer were considered positive.

Spike-specific T-cell response was determined with an IFNγ ELISpot assay after peripheral blood mononuclear cells (PBMC) stimulation with a peptide pool (15mers overlapping by 10 aminoacids) spanning the entire Spike protein[25]. In more details, PBMC were isolated from heparin-treated blood by standard density gradient centrifugation. The number of IFNγ-producing spot forming cells (SFC) was determined by ELISpot. Briefly, PBMC (2×10^5/100 µl culture medium per well) were stimulated in duplicate for 24 h in 96-well plates (coated with anti-IFN-γ monoclonal capture antibody) with peptide pools (15 mers, overlapping by 10 aminoacids, Pepscan, Lelystad The Netherlands) representative of the S at the final concentration of 0.25 µg/ml. Phytoheamagglutinin (PHA; 5 µg/mL) was used as a positive control, and medium alone as a negative control. After washing, plates were incubated for 90 min at 37 °C with biotinylated IFN-γ detection antibody. Then, streptavidin-alkaline phosphatase conjugate was added, and plates were incubated at 37 °C in a 5% CO_2 atmosphere for 1 h. Finally, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) was added for 20 min at room temperature. Wells were then washed several times under running water and air-dried overnight. Spots were counted using an automated AID ELISPOT reader system (AutoImmun Diagnostika GmbH, Strasburg, Germany). The mean number of spots from duplicate cultures were adjusted to 1×10^6 PBMC. The net spots per million PBMC was calculated by subtracting the number of spots in response to negative control from the number of spots in response to the S or N antigen. Responses ≥10 net spots/million PBMC were considered positive based on background results obtained with negative control (mean SFC + 2 SD).

Statistical analysis. The cumulative incidence of SARS-CoV-2 infection was calculated with the Kaplan-Meier method and the log-rank test was adopted for statistical comparison. The hazard ratio (HR) and 95% confidence interval (CI) for SARS-CoV-2 infection in the various groups of patients were calculated with the log-rank approach. The vaccine effectiveness in the prevention of SARS-CoV-2 infection was calculated as 100% x (1-HR), and the relevant 95% CI were calculated as 100% x (1–95% CI_{HR}), where CI_{HR} is the 95% CI of the HR. Similarly, the protective effectiveness of immunity elicited by SARS-CoV-2 infection against secondary infections was calculated as 100% × (1-HR), and the relevant 95% CI were calculated as 100% × (1–95% CI_{HR}). The frequency of symptoms in vaccinated and control subjects were compared with Fisher's exact test. The antibody and T-cell levels in vaccinated subjects with or without breakthrough infections were compared with the Mann-Whitney U-test.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The anonymized data relevant to SARS-CoV-2 infection during the study period in vaccinated and control subjects, along with serologic results indicating their previous exposure to SARS-CoV-2 infection, are available in the Dryad database under https://doi.org/10.5061/dryad.n2z34tmxk and in Supplementary Data 1. Raw data associated to Fig. 2a–c are present in the dataset. The 23 SARS-CoV-2 sequences obtained in this study are openly available on the GISAID portal and European Nucleotide Archive under the accession numbers EPI_ISL_3836237-EPI_ISL_3836259.

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References

1. Polack, F. P. et al. Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. *N. Engl. J. Med* **383**, 2603–2615 (2020).
2. Baden, L. R. et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. *N. Engl. J. Med* **382**, 1851–1852 (2021).
3. Sadoff, J. et al. Safety and efficacy of single-dose Ad26.COV2.S vaccine against Covid-19. *N. Engl. J. Med.* **384**, 2187–2201 (2021).
4. Voysey, M. et al. Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. *Lancet* **397**, 99–111 (2021).
5. Dagan, N. et al. BNT162b2 mRNA Covid-19 vaccine in a nationwide mass vaccination setting. *N. Engl. J. Med.* **384**, 1412–1423 (2021).
6. Vasileiou, E. et al. Interim findings from first-dose mass COVID-19 vaccination roll-out and COVID-19 hospital admissions in Scotland: a national prospective cohort study. *Lancet* **397**, 1646–1657 (2021).
7. Haas, E. J. et al. Impact and effectiveness of mRNA BNT162b2 vaccine against SARS-CoV-2 infections and COVID-19 cases, hospitalisations, and deaths following a nationwide vaccination campaign in Israel: an observational study using national surveillance data. *Lancet* **397**, 1819–1829 (2021).
8. Lopez Bernal, J. et al. Effectiveness of the Pfizer-BioNTech and Oxford-AstraZeneca vaccines on covid-19 related symptoms, hospital admissions, and mortality in older adults in England: test negative case-control study. *BMJ* **373**, n1088 (2021).
9. Angel, Y. et al. Association Between Vaccination With BNT162b2 and Incidence of Symptomatic and Asymptomatic SARS-CoV-2 Infections Among Health Care Workers. *JAMA*. **325**, 2457–2465. (2021).
10. Hall, V. J. et al. COVID-19 vaccine coverage in health-care workers in England and effectiveness of BNT162b2 mRNA vaccine against infection (SIREN): a prospective, multicentre, cohort study. *Lancet*. **397**, 1725–1735 (2021).
11. Amit, S. et al. Early rate reductions of SARS-CoV-2 infection and COVID-19 in BNT162b2 vaccine recipients. *Lancet* **397**, 875–877 (2021).
12. Thompson, M. G. et al. Interim estimates of vaccine effectiveness of BNT162b2 and mRNA-1273 COVID-19 vaccines in preventing SARS-CoV-2 infection among health care personnel, first responders, and other essential and frontline workers—eight U.S. locations, December 2020-March 2021. *Morb. Mortal. Wkly Rep.* **70**, 495–500 (2021).
13. Pritchard, E. et al. Impact of vaccination on new SARS-CoV-2 infections in the United Kingdom. *Nat. Med.* **27,** 1370–1378 (2021).
14. Legge 30 Dicembre 2020 n. 178 Piano Strategico Nazionale dei Vaccini per la prevenzione delle infezioni da SARS-CoV-2.
15. O. Murchu, E. et al. Quantifying the risk of SARS-CoV-2 reinfection over time. *Rev Med Virol.* **27**, e2260 (2021).
16. Manisty, C. et al. Antibody response to first BNT162b2 dose in previously SARS-CoV-2-infected individuals. *Lancet* **397**, 1057–1058 (2021).
17. Prendecki, M. et al. SARS-CoV-2 Effect of previous SARS-CoV-2 infection on humoral and T-cell responses to single-dose BNT162b2 vaccine. *Lancet* **397**, 1178–1181 (2021).
18. Cassaniti, I. et al. SARS-CoV-2 mRNA vaccine BNT162b2 elicited a robust humoral and cellular response against SARS-CoV-2 variants. *Res. Square* https://doi.org/10.21203/rs.3.rs-396284/v1 (2021).
19. Levine-Tiefenbrun, M. et al. Initial report of decreased SARS-CoV-2 viral load after inoculation with the BNT162b2 vaccine. *Nat. Med.* **27**, 790–792 (2021).
20. Istituto Superiore di Sanità. Prevalenza e distribuzione delle varianti del varianti del virus SARS-CoV-2 di interesse per la sanità pubblica in Italia. May 19th, 2021. Rome: ISS; 2021.
21. Rovida, F. et al. Incidence of SARS-CoV-2 infection in health care workers from Northern Italy based on antibody status: immune protection from secondary infection- A retrospective observational case-controlled study. *Int. J. Infect. Dis.* **109**, 199–202 (2021).
22. Giardina, F. et al. No evidence of SARS-CoV-2 circulation in the framework of influenza surveillance between October 2019 and February 2020 in Lombardy, Italy. *Travel Med. Infect. Dis.* **40**:102002 (2021).
23. Alteri, C. et al. Genomic epidemiology of SARS-CoV-2 reveals multiple lineages and early spread of SARS-CoV-2 infections in Lombardy, Italy. *Nat. Commun.* **12**, 434 (2021).

24. Percivalle, E. et al. Prevalence of SARS-CoV-2 specific neutralising antibodies in blood donors from the Lodi Red Zone in Lombardy, Italy, as at 06 April 2020. *Euro Surveill.* **25**, 2001031 (2020).
25. Cassaniti, I. et al. SARS-CoV-2 specific T-cell immunity in COVID-19 convalescent patients and unexposed controls measured by ex vivo ELISpot assay. *Clin. Microbiol. Infect.* **27**,1 029-1034 (2021).

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Author contributions

FR and DL analyzed and interpreted the data and drafted the manuscript; AM, VN, AM, AMG, GG, collected and managed the data; CS collected the data of the control group; CR, MD performed patients' follow-up; FeB and IC, performed experiments on T-cell response; JCS, AF, AS performed experiments on antibody response; EP performed virus isolation; SP, AP, FG sequenced viral genomes; CM, AT supervised participants enrollment; FaB supervised the study and revised the manuscript. All the authors critically reviewed the manuscripts.

Competing interests

The authors declare competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Daniele Lilleri.

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External quality assessment of HIV-1 DNA quantification assays used in the clinical setting in Italy

Ilaria Vicenti[1]✉, Filippo Dragoni[1], Alessia Giannini[1], Anna Casabianca[2], Francesca Lombardi[3], Laura Di Sante[4], Ombretta Turriziani[5], Sara Racca[6], Stefania Paolucci[7], Alessia Lai[8], Isabella Bon[9], Isabella Abbate[10], Gabriella Rozera[10], Simone Belmonti[11], Rossana Scutari[12], Claudia Alteri[13,14], Francesco Saladini[1], Maurizio Zazzi[1] & Italian HIV DNA Network*

Total cell-associated HIV-1 DNA is a surrogate marker of the HIV-1 reservoir, however, certified systems for its quantification are not available. The Italian HIV DNA Network was launched to validate HIV-1 DNA quantification methods in use at University and Hospital labs. A quality control panel including HIV-1 DNA standards, reconstructed blood samples (RBSs) and DNA from different HIV-1 subtypes was blindly tested by 12 participating labs by quantitative real-time PCR (n = 6), droplet digital PCR (n = 3) or both (n = 3). The median 95% hit rate was 4.6 (3.7–5.5) copies per test and linearity in the tested range was excellent (R^2 = 1.000 [1.000–1.000]). The median values obtained across labs were 3,370 (2,287–4,245), 445 (299–498), 59 (40–81) and 7 (6–11) HIV-1 DNA copies, for the 3,584, 448, 56 and 7-copy standards, respectively. With RBSs, measured values were within twofold with respect to the median in two thirds of cases. HIV-1 subtypes were missed (CRF01_AE by 3 labs) or underestimated by > 1 log (subtypes A, C, D, F by one lab; CRF01_AE by one lab; CRF02_AG by one lab). The overall performance was excellent with HIV-1 DNA standards, however detection of different HIV-1 subtypes must be improved.

Antiretroviral therapy achieves prolonged control of HIV-1 replication in the vast majority of patients[1]. However, eliminating HIV-1 infection remains an elusive goal due to indefinite persistence of the viral reservoir, characterized by latently infected cells carrying HIV-1 proviral DNA in their host genome[2]. Quantifying the HIV-1 reservoir in patients under successful treatment, as defined by undetectable HIV-1 RNA in plasma, is of great interest because a small sized reservoir would theoretically be suitable for different treatment strategies. On one hand, in patients with a limited HIV-1 reservoir it should be safer to reduce drug pressure with the aim of decreasing treatment toxicity and cost. On the other hand, such patients are likely to be the ideal candidates for pilot HIV-1 eradication studies through strategies targeting the latent HIV-1 reservoir[3]. Thus, reliable and practical markers are needed to analyze the viral reservoir[4].

Indeed, several systems have been proposed to quantify the viral reservoir[5]. Measuring virus outgrowth following stimulation of patient blood cells in vitro is considered the gold standard to quantify latent but replication competent virus[6]. However, there is no methodological consensus and the assays described differ in one or more features, including the patient cell population and the uninfected cells co-cultured, the approach

[1]Department of Medical Biotechnologies, University of Siena, Siena, Italy. [2]Department of Biomolecular Sciences, University of Urbino "Carlo Bo", Urbino, PU, Italy. [3]Fondazione Policlinico Universitario A. Gemelli IRCCS, UOC Malattie Infettive, Rome, Italy. [4]Virology Unit, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy. [5]Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy. [6]Laboratory of Microbiology and Virology, IRCCS San Raffaele Scientific Institute, Milan, Italy. [7]Molecular Virology Unit, Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy. [8]Department of Biomedical and Clinical Sciences Luigi Sacco, University of Milan, Milan, Italy. [9]UO Microbiologia, IRCSS Azienda Ospedaliero Universitaria di Bologna, Policlinico S. Orsola, Bologna, Italy. [10]Virology and Biosafety Laboratories Unit, National Institute for Infectious Diseases IRCCS L. Spallanzani, Rome, Italy. [11]Dipartimento di Sicurezza e Bioetica, Sezione Malattie Infettive, Università Cattolica del Sacro Cuore, Rome, Italy. [12]Department of Experimental Medicine, University of Rome "Tor Vergata", Rome, Italy. [13]Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy. [14]Multimodal Medicine Research Area, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy. *A list of authors and their affiliations appears at the end of the paper. ✉email: vicenti@unisi.it

used for reversing HIV-1 latency and the markers measured to quantify the induced virus[7–9]. In general, such methods are complex, time-consuming and difficult to standardize. In addition, they tend to underestimate the viral reservoir because not all the replication competent virus population can be effectively induced in vitro[10]. While viral outgrowth assays remain very valuable in investigating the nature, dynamics and pathogenesis of the HIV-1 reservoir, lower complexity methods are needed to integrate a measure of the latent HIV-1 reservoir into routine patient management.

Molecular assays such as the quantification of total cell-associated HIV-1 DNA can fulfill these requirements since they obviate the need for cell cultivation, biosafety level containment and high-level, specific expertise[11]. However, total HIV-1 DNA clearly overestimates the viral reservoir since it includes not only replication competent proviruses but also defective and more labile, unintegrated forms[10,12]. Nonetheless, extrachromosomal forms can contribute to HIV-1 pathogenesis[13] and total HIV-1 DNA load seems to correlate well with the frequency of cells containing replication-competent virus[14,15]. In addition, although discriminating between integrated and unintegrated HIV-1 DNA can add useful information in select studies, assays specifically targeting integrated HIV-1 DNA are complex to set up and require extensive replicate testing which makes the system not amenable to routine use[14,16]. Most importantly, the clinical role of total HIV-1 DNA is supported by sparse but relevant studies. A meta-analysis of six studies in untreated patients[17] indicated that total HIV-1 DNA is a stronger predictor of AIDS and of all-cause mortality compared to plasma HIV-1 RNA load. In addition, baseline total HIV-1 DNA load is predictive of the occurrence and severity of HIV-1 associated neurologic disorders[18] as well as of levels of T-cell activation[19]. More recently, the role of total HIV-1 DNA has been evaluated in treated patients with suppressed plasma HIV-1 RNA providing two important lines of evidence. First, total HIV-1 DNA is predictive of the time to plasma HIV-1 RNA rebound after treatment interruption, both in patients treated early during primary infection[20,21] and in patients treated late during chronic infection[22,23]. Second, higher total HIV-1 DNA levels are associated with an increased risk of virological failure following treatment de-escalation, as shown in the MONOI and MONET trials[24,25], comparing the outcomes of a switch to darunavir/ritonavir (DRV/r) monotherapy *vs*. combination therapy, and in the DOMONO trial evaluating the switch to DTG monotherapy[26].

The increasing interest for HIV-1 DNA quantification as a rough estimate of the viral reservoir has not yet been accompanied by the development of certified assays to measure this parameter. Few commercial assays have been developed but they have not been certified for in vitro diagnostic use. However, several homebrew HIV-1 DNA quantification protocols have been developed worldwide. Such assays typically undergo different kinds of internal validation but the results obtained from different methods can be hardly compared due to different genomic regions analyzed, different standards used and/or different principles and readouts, such as real time PCR (qPCR) or droplet digital PCR (ddPCR). The Italian HIV DNA Network was launched to investigate the features and performance of the HIV-1 DNA quantification methods in use at Italian University and Hospital labs, including homebrew systems and commercial kits not yet marked for in vitro diagnostic use. Here we report the results obtained from the first external quality assurance program involving 12 Italian labs receiving a comprehensive panel of HIV-1 DNA standards, reconstructed clinical samples and DNA extracts from different HIV-1 subtypes.

Methods

Participating centers. The quality control panel was assembled by the coordinating center and sent to the 12 labs participating to the Italian HIV DNA Network, including the coordinator, for blind testing, using the methodology routinely used at each center. The labs were originally asked to participate to the Network based either on the use of commercial assays or on publication of at least one peer-reviewed paper reporting quantification of HIV-1 DNA.

Quality control material for the external validation. Detailed instructions concerning the manipulation and processing of the quality assurance sample panel were provided to each lab. The number of replicates, dilutions and results required to validate the assay are indicated in Table 1.

To assess the accuracy, sensitivity, precision and linear range on reference standards, the pNL4-3 plasmid (code ARP2006), obtained from the Centre for AIDS Reagents (CFAR) was quantified with respect to an International Standard (code ARP956; CFAR) by qPCR[27] and ddPCR, using the same primers and probe as for qPCR in a reaction adapted for the QX200™ Droplet Digital™ PCR System (Bio-Rad), and by fluorometric quantification using the Qubit 4 fluorometer (ThermoFisher), based on the mean of the three measurements which differed from one another within 1.5-fold. The plasmid standard was then diluted in 10 ng/µl of human genomic DNA (code G3041; Promega), and frozen until shipment to each lab.

To assess inter-laboratory variability and intra-laboratory precision on clinical whole blood samples, reconstructed blood samples (RBS) were generated at the coordinator lab by diluting U1/HIV-1 lymphoblastoid cells (code ARP139; CFAR), carrying 2 copies of HIV-1 genome per cell, into HIV-1 negative human peripheral blood (code 297CTIPB.1.5; CTI Biotech), provided by the supplier in compliance with all relevant ethical guidelines. Briefly, U1 cells were counted and one million cells were added to 1 ml of HIV-1-negative blood and then serially diluted 1:10, 1:10, 1:3, 1:3. At the coordinating lab, aliquots of the above dilutions series were processed to extract DNA and quantify HIV-1 DNA to confirm that the HIV-1 DNA amount was in a range suitable for the purpose of the study. The same HIV-1 negative human peripheral blood used as diluent was included in the quality assurance panel to assess the specificity of the assays. Since these samples could not be considered as certified reference standards, the median of the HIV-1 DNA levels obtained at all the study labs was taken as a reference for the assessment of interlaboratory reproducibility, as proposed in a previous HIV-1 DNA quantification quality assurance study[28].

Parameter	Content	Replicates	Output
Sensitivity	Twofold dilution series containing nominal 1.75 to 112 copies of the reference standard	8 for each dilution (intra-run)	Frequency of positive results
Linear range	Tenfold dilution series containing nominal 7 to 70,000 copies of the reference standard	4 for each dilution (intra-run)	HIV-1 copies/test
Intra-run precision	Eightfold dilution series containing nominal 7 to 3,584 copies of the reference standard	4 for each dilution	HIV-1 copies/test
Inter-run precision	Eightfold dilution series containing nominal 7 to 3,584 copies of the reference standard	5 for each dilution	HIV-1 copies/test
Inter-laboratory reproducibility and intra-laboratory precision	5 reconstructed clinical samples estimated to contain 38,315/23,684, 2,189/1,942, 808/693, 160/162 and 0/0 copies per million cells at the coordinating lab by qPCR/ddPCR	3 DNA extractions for each sample, each quantified in duplicate in 3 separate runs (inter-run)	HIV-1 copies/million cells
Detection of different subtypes	7 DNA extracts from MOLT-4/CCR5 cells infected with different HIV-1 subtypes estimated to contain 664/1,068 (A), 469/948 (CRF01_AE), 102,494/233,579 (CRF02_AG), 31,305/36,765 (B), 97,343/121,256 (C), 70,599/83,835 (D), 214,738/297,904 (F) copies per 10^6 cells at the coordinating lab by qPCR/ddPCR	A duplicate for each sample in intra-run	HIV-1 copies/million cells

Table 1. Composition of the external quality assurance sample panel and output requested for the different parameters of performance.

To assess the ability to recognize the most representative subtypes, seven HIV-1 strains obtained by CFAR (codes ARP1089, ARP1112, 100595, ARP 169.6, ARP1121, 100215 and ARP1124, representing the A1, B, C, D, F, CRF01_AE and CRF02_AG variants, respectively) were used to infect the lymphoblastoid MOLT-4/CCR5 cell line expressing high levels of CCR5 receptor (code ARP5039; CFAR) and DNA was extracted at the coordinator lab. HIV-1 DNA was originally quantified in HIV-1 subtype extracts at the coordinating lab to ensure appropriateness of the material, however, similar to RBSs analysis, the median values derived from the study were used to estimate target underestimation or overestimation with the different subtypes.

Statistical analysis. Data were reported as mean ± SD or median (IQR) copies per test or copies per 10^6 cells, as appropriate for the distribution of data based on the Shapiro–Wilk test for normality. Comparisons between independent groups of data were done by the Student t-test or by Mann–Whitney U test. Analysis of paired data was done by the paired t test or by Wilcoxon signed rank sum test. Comparisons between frequencies were done by chi-square test. The trend between ordered independent variables and continuous dependent variables was analyzed by the Jonckheere-Terpstra test. Statistical analysis was performed with SPSS version 20.0.

Results

Assays used for total HIV-1 DNA quantification. The main features of the assays used at the 12 participating centers are shown in Table 2. Data sets provided are indicated by the lab number followed by _qPCR or _ddPCR, consistent with the method used. Four labs performed homebrew qPCR, 3 homebrew ddPCR, and 3 both methodologies. The remaining 2 labs tested one of two different commercial qPCR assays each, namely the Generic HIV DNA Cell kit (Biocentric) and the HIV-1 DNA Test PRO (Diatheva) (lab07_qPCR and lab11_qPCR, respectively).

Sensitivity and linear range on titrated standards. The sensitivity, defined as the smallest amount of HIV-1 DNA detectable in the 95% of cases (95% hit rate) was calculated on 8 replicates of a twofold dilution series of the reference standard containing 1.75 to 112 nominal HIV-1 DNA copies in 50 ng of human DNA. Results were delivered as frequency of qualitative positive reactions for each standard dilution. Based on probit analysis, the median analytical sensitivity of the assays was 4.6 (3.7–5.5) HIV-1 DNA copies per test at the 95% hit rate, including one outlier result (10.5 HIV-1 DNA copies per test) and without statistically significant difference between qPCR and ddPCR (4.7 [3.7–5.5] *vs.* 4.5 [4.0–5.5] HIV-1 DNA copies per test respectively; $P = 0.776$) (Fig. 1A). When analyzing 200 to 1,000 ng of total DNA as in a typical clinical application, this translates into a limit of detection of around 150 to 30 HIV-1 DNA copies per 10^6 cells, assuming as a reference that one million cells contain 6.66 µg of DNA and once confirmed that the same performance is maintained with clinical samples.

The linear dynamic range, as determined by testing a 5-point $\log_{10}$ dilution series of the standard (70,000 to 7 nominal copies per test), was expressed as the R^2 of the linear regression. The median R^2 value obtained was 1.000 (1.00–1.00), again without difference between qPCR and ddPCR (Fig. 1B). There was one outlier value ($R^2 = 0.92$).

Accuracy and precision on titrated standards. Accuracy was defined as the distance between measured and expected HIV-1 DNA copies and it was tested on 4 standard dilutions, each analyzed in quadruplicate. The median values obtained across labs were 3,370 (2,287–4,245), 445 (299–498), 59 (40–81) and 7 (6–11) HIV-1 DNA copies per test, for the 3,584, 448, 56 and 7-copy standards, respectively (Fig. 2). The ratio between nominal and measured values (fold difference) and the outlier values recorded by three labs are indicated in Supplementary Table 1. At individual sample level, fold-difference values were comparable across labs using

Lab	Extraction method	Method to determine the total DNA concentration		Method to determine the HIV-1 DNA concentration		PCR master mix	Instrument	References
		Method	Target	Method	Target			
lab01_qPCR	Abbott *m*Sample Preparation System DNA Kit on Abbott *m*2000*sp* instrument; Abbott	qPCR	hTERT	qPCR	Integrase	Abbott RealTime HIV-1 Amplification Reagent Kit; Abbott	7500 Fast Dx RTPCR Instrument; Applied Biosystems	[28,29]
lab02_qPCR	QIAamp DNA Blood Mini Kit; Qiagen	qPCR	Albumin	qPCR	pol	PrecisionPLUS qPCR Master Mix; PrimerDesign	RotorGene Q; Qiagen	[30]
lab03_ddPCR	High pure PCR template preparation kit; Roche	ddPCR	Albumin	ddPCR	LTR	ddPCR™ Supermix for Probes (No dUTP); Biorad	QX200 Droplet Digital PCR System; BioRad	[31]
lab04_ddPCR	AllPrep DNA/RNA Mini Kit; Qiagen	ddPCR	rpp30	ddPCR	gag	ddPCR™ Supermix for Probes (No dUTP); Biorad	QX200 Droplet Digital PCR System; BioRad	[32]
lab05_qPCR	Qiacube, Qiamp DNA mini kit; Qiagen	Spectrophotometry		qPCR	pol	iTaqUniversal probes supermix; Biorad	ABI 7900; Applied Biosystem	[33]
lab06_qPCR	High pure PCR template preparation kit; Roche	qPCR	Beta Globin	qPCR	LTR	GoTaq probe qPCR master Mix; Promega	Eco Real-Time PCR system; Illumina	[34]
lab06_ddPCR	High pure PCR template preparation kit; Roche	ddPCR	Albumin	ddPCR	LTR	ddPCR™ Supermix for Probes (No dUTP); Biorad	QX200 Droplet Digital PCR System; BioRad	[31]
lab07_qPCR	QIAamp DNA Blood Mini Kit; Qiagen	Spectrophotometry		qPCR	LTR	Generic HIV DNA Cell kit; Biocentric	QuantStudio 5 Dx Real-Time PCR System; Thermo Fisher	[35–37]Commercial kit
lab08_qPCR	QIA Symphony DNA Blood Mini Kit; Qiagen	qPCR	hTERT	qPCR	LTR	GoTaq Probe qPCR Reaction Mix; Promega	LightCycler 2.0; Roche	[34]
lab08_ddPCR	QIA Symphony DNA Blood Mini Kit; Qiagen	ddPCR	Albumin	ddPCR	LTR	ddPCR™ Supermix for Probes (No dUTP); Biorad	QX200 Droplet Digital PCR System; BioRad	[31,38]
lab09_ddPCR	High pure PCR template preparation kit; Roche	ddPCR	Albumin	ddPCR	LTR	ddPCR™ Supermix for Probes (No dUTP); Biorad	QX200 Droplet Digital PCR System; BioRad	[38]
lab10_qPCR	QIA Symphony DNA Blood Mini Kit; Qiagen	qPCR	Beta Globin	qPCR	LTR	homemade	RotorGene Q; Qiagen	[35]
lab11_qPCR	QIAamp DNA Blood Mini Kit; Qiagen	qPCR	hTERT	qPCR	LTR	HIV-1 DNA Test PRO; Diatheva	ABI 7500; Applied Biosystem	[39] Commercial Kit
lab12_qPCR	High pure viral nucleic acid kit; Roche	qPCR	Albumin	qPCR	LTR	PreMix ExTaq; Takara	LightCycler 96; Roche	[27]
lab12_ddPCR	High pure viral nucleic acid kit; Roche	ddPCR	Albumin	ddPCR	LTR	ddPCR™ Supermix for Probes (No dUTP); Biorad	QX200 Droplet Digital PCR System; BioRad	[27]

Table 2. Main features of the different methods used at each lab participating to the external quality assurance total HIV-1 DNA quantification program. *hTERT* human Telomerase Reverse Transcriptase.

ddPCR *vs.* qPCR (1.0 [0.7–1.2] *vs.* 0.7 [0.6–1.6], $P=0.456$; 1.1 [0.9–1.1] *vs.* 0.9 [0.6–1.7], $P=0.388$; 1.1 [1.0–1.5] *vs.* 0.8 [0.6–2.0], $P=0.224$; 1.1 [0.8–1.9] *vs.* 0.9 [0.8–2.3], $P=0.607$; for the 3,584, 448, 56 and 7-copy standards, respectively). When analyzing the whole fold-difference data set, there was a trend for ddPCR to yield higher values compared with qPCR (median fold-differences 1.1 [1.0–1.2] *vs.* 0.8 [0.6–2.0], $P=0.075$). However, ddPCR values were significantly closer to the nominal copy numbers compared with qPCR (median absolute deviation 0.04 [0.02–0.17] vs. 0.19 [0.08–0.43] log, $P=0.002$).

Precision was defined as the coefficient of variation (CV) of the HIV-1 DNA copies measured on 4 identical replicates of the standard at four different concentrations, both intra-run and inter-run (Supplementary Table 2). In the intra-run assessment, the median CV (considering both qPCR and ddPCR) was 5.5 [4.1–16.9], 9.9 [6.5–17.6], 20.9 [17.0–26.3] and 48.9 [33.4–71.5] for the 3,584, 448, 56 and 7-copy standards, respectively. CV values obtained by qPCR and ddPCR were comparable when considering the whole data set (18.8 [6.8–45.5] vs. 20.2 [5.7–32.0], respectively; $P=0.411$) and for any individual reference standard (data not shown). In the inter-run assessment, testing the same dilution series in five independent runs, the median CV was 16.2 [7.9–21.0], 17.1 [8.9–22.5], 24.7 [18.0–28.5] and 46.0 [35.3–58.5] for the 3,584, 448, 56 and 7-copy standards, respectively. A higher CV was scored for qPCR *vs.* ddPCR when considering the whole inter-run data set (23.9 [18.1–37.3] *vs.* 17.9 [10.1–24.5], $P=0.02$). This difference was more relevant at lower copy numbers (55.5 [38.5–71.7] *vs.* 37.4 [22.9–50.9], $P=0.088$; 25.9 [22.7–31.6] *vs.* 17.3 [13.1–23.2], $P=0.018$; 18.3 [12.4–22.9] *vs.* 9.8 [6.0–20.9], $P=0.181$; 20.1 [9.3–21.5] *vs.* 11.8 [6.8–17.4], $P=0.328$; at 7, 56, 448 and 3,584-copy numbers, respectively). Notably, both intra-run and inter-run CV values tended to increase with decreasing copy numbers ($P=0.042$).

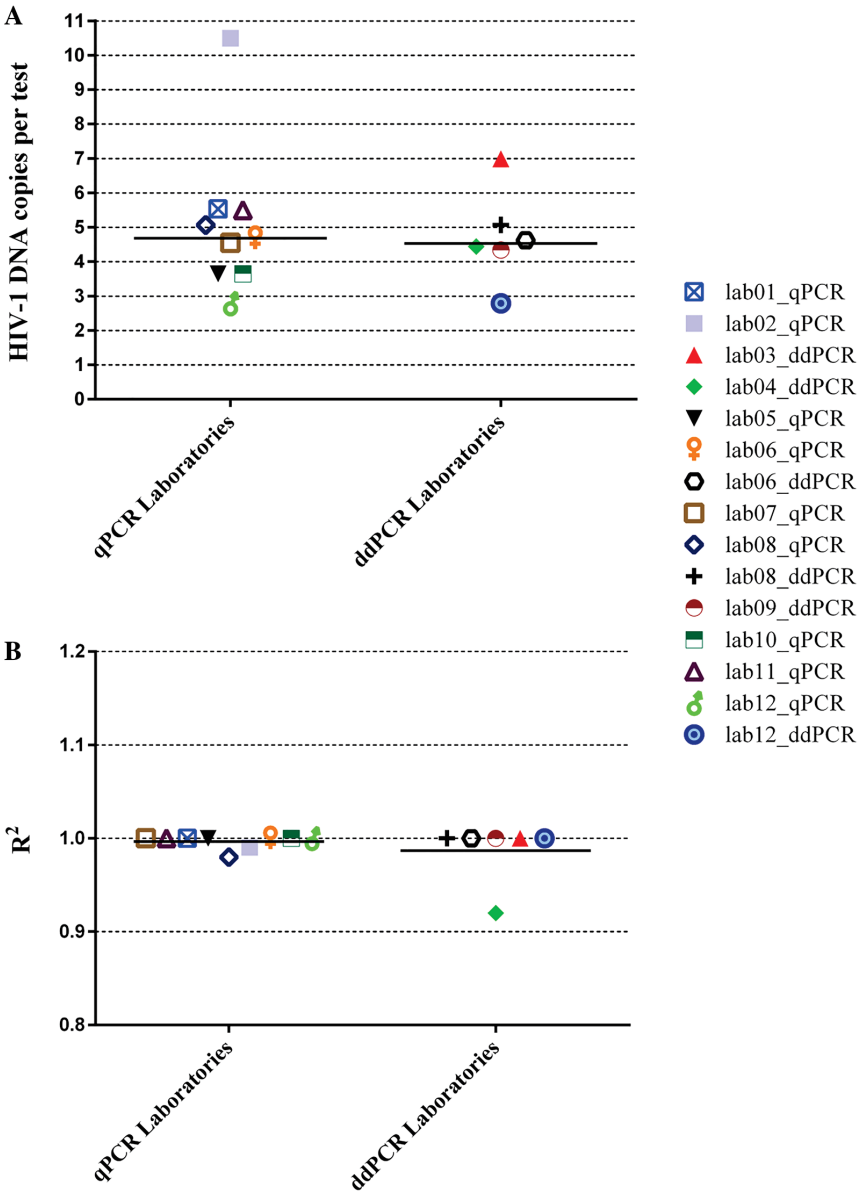

Figure 1. Distribution of the sensitivity, expressed as 95% hit rate (**A**), and linear range, expressed as R^2 value on a $\log_{10}$ dilution series (**B**), of the different HIV-1 DNA quantification assays. Bars indicate median values. Graphic elaboration was realized using the GraphPad Prism software version 6.0 (https://www.graphpad.com/scientific-software/prism/).

Inter-laboratory variability and intra-laboratory precision on reconstructed blood samples. Each lab extracted the five RBSs (Table 1) in 3 independent runs using its own extraction protocol, then each DNA extract was quantified in duplicate in 3 independent experiments. Due to blood clots, lab08_ddPCR did not process RBS-3 and RBS-4. The median values obtained for RBS-1, RBS-2, RBS-3 and RBS-4 considering all the results delivered by the participating labs were 19,469 (11,020–31,805), 1,903 (946–3,030), 684 (184–1,285) and 145 (65–273) HIV-1 DNA copies per 10^6 cells, respectively (Fig. 3). These values matched very closely those originally obtained at the coordinating lab (Table 1). When stratified by method, there was

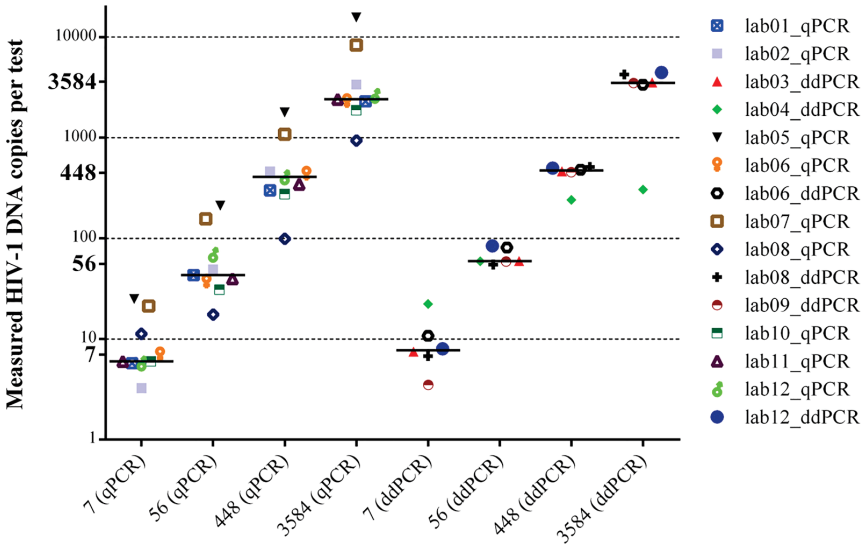

Figure 2. Expected and measured HIV-1 DNA copy values with the reference standard dilution series, stratified for qPCR and ddPCR. Bars indicate median values. Graphic elaboration was realized using the GraphPad Prism software version 6.0 (https://www.graphpad.com/scientific-software/prism/).

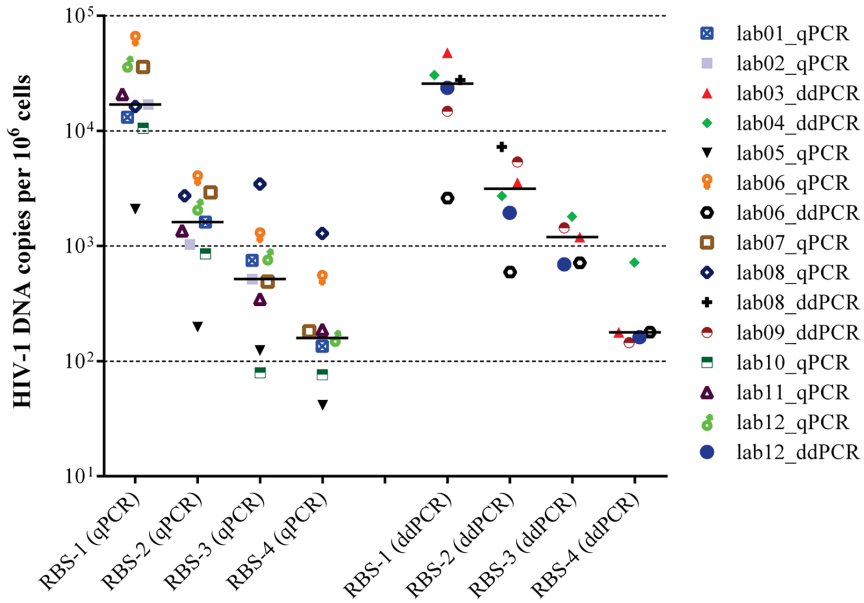

Figure 3. HIV-1 DNA (copies per 10^6 cells) measured by the participating laboratories in the different reconstructed blood samples (RBS), stratified for qPCR and ddPCR. Bars indicate median values. Graphic elaboration was realized using the GraphPad Prism software version 6.0 (https://www.graphpad.com/scientific-software/prism/).

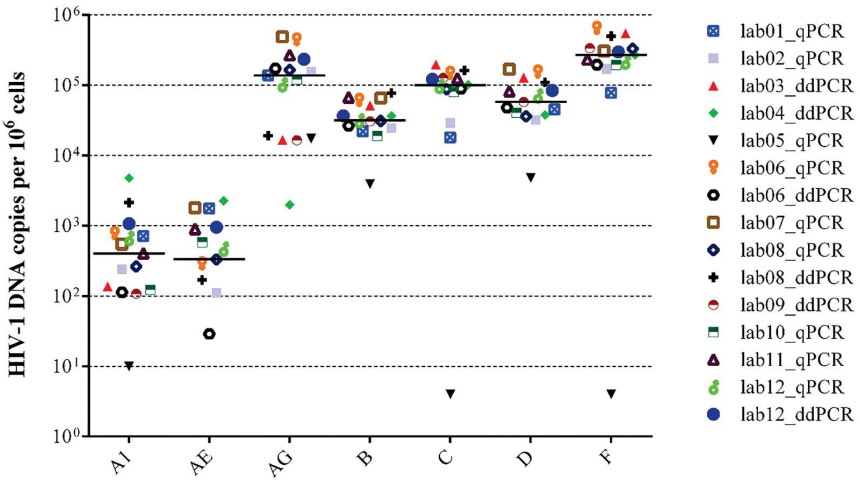

Figure 4. HIV-1 DNA (copies per 10^6 cells) measured by the participating laboratories in the DNA extracts from different HIV-1 subtypes. Bars indicate median values. Graphic elaboration was realized using the GraphPad Prism software version 6.0 (https://www.graphpad.com/scientific-software/prism/).

no statistically significant difference in ddPCR vs. qPCR values for any RBS (25,681 [11,755–34,906] vs. 16,977 [11,858–37,060], $P = 0.776$; 3,134 [1,605–5,843] vs. 1,610 [943–2,823], $P = 0.224$; 1,193 [703–1,622] vs. 516 [235–1,014], $P = 0.190$; 178 [154–449] vs. 159 [59–354], $P = 0.606$), although the median values were larger for ddPCR in all of the four samples.

When considering median values for each RBS as a reference, outlier values were detected for lab08_qPCR with RBS-3 and RBS-4, for lab06_qPCR with RBS-1 and RBS-4, for lab08_ddPCR with RBS-2 and for lab02_qPCR, lab04_ddPCR and lab05_qPCR with RBS-4. Thus, the lowest copy-number RBS (RBS-4) originated a larger number of outliers compared to the other RBSs ($P = 0.074$). Lab02_qPCR failed to detect HIV-1 DNA in RBS-4, this sample was excluded from the inter-laboratory variability analysis. The fold-difference between the measured and median values for each sample and lab are indicated in Supplementary Table 3. Overall, an absolute fold-difference > 2 was scored for one third of measurements, namely 4/15, 5/15, 5/14 and 4/13 for the four RBSs ordered by decreasing HIV-1 DNA titer, respectively, with no cases differing by more than 1 log with respect to the reference median value. When comparing fold-differences obtained by qPCR and ddPCR for all the individual RBSs, qPCR values were significantly closer to the median compared with ddPCR values (median 1.0 [0.6–1.6] *vs.* 1.4 [1.1–2.5], $P = 0.039$). This difference was prevalently driven by RBS-2 and RBS-3 (ratio between average ddPCR and qPCR values above twofold). Notably, the negative blood sample was scored as positive in 3 replicates (811.2 ± 615.3 HIV-1 copies per 10^6 cells) by lab04_ddPCR, in 9 replicates (110.0 ± 73.1 HIV-1 copies per 10^6 cells) by lab06_ddPCR and in 4 replicates (2.5 ± 1.0 HIV-1 copies per 10^6 cells) by lab07_qPCR. Overall, the rate of false positive reactions was significantly higher for ddPCR *vs.* qPCR (12/108 [11.1%] *vs.* 4/162 [2.5%], $P = 0.003$).

Precision on the same RBS panel was determined by measuring the CV both within and across extraction runs (Supplementary Table 4). The intra-extraction precision was calculated as the median CV of three extraction runs while the inter-extraction precision was calculated as the median CV of all the 18 replicates. In the intra-extraction assessment, the median CV was 20.7 (12.0–29.7), 24.8 (20.0–45.1), 35.8 (24.6–65.0) and 51.2 (31.4–67.4) for RBS-1, RBS-2, RBS-3 and RBS-4, respectively. In the inter-extraction assessment, the median CV for the same series was 25.0 (14.3–59.7), 33.7 (25.6–48.7), 50.3 (37.4–84.1) and 64.9 (36.1–82.4). Similar to what found with plasmid standards, there was a significant increase in CV values with decreasing HIV-1 DNA copy numbers, both in the intra- and inter-extraction assessment ($P = 0.042$). The median CVs observed in the inter-extraction assessment were significantly higher than those observed in the intra-extraction assessment ($P < 0.01$). No significant differences in CVs obtained by qPCR or ddPCR were observed for either the whole RBS panel or any individual RBS.

Ability to recognize different subtypes. To assess the ability to recognize and quantify the main HIV-1 subtypes, the coordinator lab sent to each lab seven tubes containing DNA extracted from a lymphoblastoid cell line infected with reference subtypes A1, CRF01_AE, CRF02_AG, B, C, D, F (Table 1). The median number of HIV-1 DNA copies per 10^6 cells obtained at all labs was: 404 (123–404) for subtype A, 333 (29–948) for CRF01_AE, 137,040 (17,316–233,579) for CRF02_AG, 31,305 (24,450–31,305) for subtype B, 99,560 (67,704–131,654) for subtype C, 57,975 (37,700–109,194) for subtype D and 269,932 (194,573–337,652) for subtype F (Fig. 4). Measured HIV-1 DNA was > 1 log lower than the median in 6 cases: CRF02_AG by lab04_ddPCR; subtypes A1, C, D, F by lab05_qPCR; CRF01_AE by lab06_ddPCR. In addition, CRF01_AE was not detected at all by

lab03_ddPCR, lab05_qPCR and lab09_ddPCR (Supplementary Table 5). By contrast, there was only one case where measured HIV-1 DNA was > 1 log higher than the median (lab04_ddPCR with subtype A1).

Alignment of available primer and probe sequences on the consensus of the different subtypes included in the panel revealed that, of the four labs underestimating or missing CRF01_AE, three used a forward primer affected by a 1-base deletion when hybridizing to the CRF01_AE and subtype F target sequence (Supplementary Fig. 1). Underestimation of CRF02_AG by lab04_qPCR coincided with the largest number of mismatches (6) affecting the forward primer among all the target subtypes considered, including one mismatch at position -3 bases with respect to the primer 3' end. Finally, lab05_qPCR primers and probes perfectly matched the consensus B sequence but were affected by 4–9 mismatches when aligned to the other subtypes.

Discussion

Despite conceptual limitations, total HIV-1 DNA is broadly considered as a measure of the viral reservoir and an attractive marker to monitor its changes following specific treatment strategies. Due to the lack of certified systems for in vitro diagnostic use, several homebrew assays have been developed, based on qPCR[27,29,40] or ddPCR[31]. However, the heterogeneity of methods may affect interpretation of data generated across different labs due to different sensitivity, accuracy and precision. Only two previously published papers[28,40] reported a multicenter quality control to evaluate the inter-laboratory reproducibility of total HIV-1 DNA quantification. In the French study[40], 4 peripheral blood mononuclear cell (PBMC) samples were tested by 10 labs all using the commercially available Biocentric system. In the Italian study[28], 7 labs tested 24 cellular samples carrying HIV-1 clade B and 40 HIV-1-negative PBMC samples spiked with different concentrations of plasmids containing the *gag* gene derived from different HIV-1 subtypes. All the labs participating to the latter study used hTERT as the housekeeping gene and one of two different primer sets to quantify HIV-1 DNA. The current study considerably expanded the scope of the quality control panel to assess the assays for their sensitivity, accuracy, linear range, precision and ability to recognize the different HIV-1 variants (Table 1). In addition, the variety of procedures used allowed an assessment of homebrew methods *vs.* commercial kits and of qPCR *vs.* ddPCR.

In total, 15 data sets were delivered by the 12 labs participating to the Italian HIV DNA Network. The overall performance with reference standards was good. Indeed, the linear range and sensitivity were excellent, with $R^2 = 1.000$ in 12/15 data sets and the 95% hit rate of 4.6 copies of target which translates into a sensitivity threshold of 150 to 30 copies of HIV-1 DNA per million cells when using 200 to 1,000 ng of total DNA in the reaction, provided PCR is run under optimal conditions without any inhibition. Also, the accuracy and precision measured on the titrated standards were high, with the difference from the expected value within twofold in 46/60 cases (15 centers testing 4 samples) and the CV < 50% in 44/60 and 47/60 cases in the intra- and inter-run assessment, respectively. However, an overall lower performance was obtained with RBSs. While the nominal number of copies was not certified for the RBSs, the difference from the median value, derived from the values obtained by all labs, was within twofold only in 36/58 cases. The CV was < 50% in 42/58 and 35/58 cases in the intra- and inter-extraction assessment, respectively. Although there may remain differences from clinical blood samples, the RBSs were prepared from a single source of HIV-1-negative human blood spiked with HIV-1-positive cells from infected cell lines, thus mirroring blood from infected patients. RBSs were then frozen to reproduce the most common material used in the clinic. The lower precision with RBSs compared to ready-to-use plasmid standards likely derived from additional sources of variability including HIV-1 DNA extraction and measurement. Noteworthy, precision appeared to decrease with lower numbers of target copies, as often occurring with individuals under prolonged successful therapy[37]. In addition, repeated RBS analysis revealed lower precision in inter-extraction compared with intra-extraction runs. These drawbacks may advise for same-run replicate analysis of samples obtained at different time points from the same patient in the clinical setting.

Comparative analysis of the 9 qPCR *vs.* the 6 ddPCR data sets revealed few significant differences. With titrated standards, ddPCR results matched more closely than qPCR the expected target copy numbers; in addition, qPCR had larger inter-run CV values with respect to ddPCR, particularly at low-copy numbers. With RBSs, ddPCR values tended to distribute above the median of the whole dataset and also generated a significantly higher rate of false positive reactions compared with qPCR when analyzing the negative control. Higher precision with respect to qPCR is indeed one of the expected benefits of ddPCR, particularly at low copy numbers[41]. The distribution of ddPCR generated values with RBSs may reflect the qPCR underestimation bias detected with plasmid standards. Indeed, median values obtained for the whole dataset were influenced more by qPCR values (9 datasets) than by ddPCR (6 datasets). On the other hand, the trend for ddPCR to generate some false positive signals might have derived from an incorrect setting of the threshold, a well-known key issue with ddPCR complicating low-level detection abilities and requiring training by expert users or system manufacturers[42,43]. Overall, these specific caveats highlighted by our study suggest using replicate testing with qPCR and advise for inclusion of multiple negative controls and adjustments in the computation of background noise with ddPCR.

Of the 9 qPCR data sets, only 2 were generated by commercial, research-use-only kits. There were no relevant differences between homebrew and commercial qPCR results. However, the Biocentric system yielded the only few cases of false positive qPCR results with RBSs and also overestimated HIV-1 DNA copies in the titrated standards (2.3, 2.4, 2.8, 3.0-fold for the 3,584, 448, 56 and 7-copy standards, respectively) but not in the RBSs. Nevertheless, the availability of standardized and ready to use reagents from a commercial source remains valuable, particularly for labs with limited experience, and ensures large-scale validation as well as updates of primers and probes which should minimize false negative reactions.

Significant issues were scored with the HIV-1 subtype panel. Substantial underestimation of HIV-1 DNA occurred at several labs, with CRF01_AE most affected. The primary reason for underestimation or failure to detect a specific subtype is suboptimal primers and probes, possibly chosen from outdated literature or published studies focusing on specific subtypes. The ability of published primers and probes to recognize different HIV-1

subtypes should be verified on a regular basis by checking for conservation of the target regions in the updated reference databases such as GenBank and the curated Los Alamos National Laboratory repository. For example, underestimation or even lack of detection of CRF01_AE by four participants to this study may have been driven by the occurrence of one extra nucleotide in the region targeted by the forward primer. By contrast, lab08_ddPCR using the same primer/probe combination correctly quantified CRF01_AE, implying that other factors such as mismatch tolerant experimental conditions may have a role. However, it must be noted that CRF01_AE was also the lowest concentration DNA extract provided for subtype analysis which may have impacted accuracy per se. Similarly, lab04_qPCR likely underestimated CRF02_AG due to six mismatches in the forward primer. Finally, underestimation of multiple subtypes by lab05_qPCR apparently derived from choosing primers and probe targeting HIV-1 subtype B without adequate consideration for target conservation across different subtypes.

This comprehensive external quality assurance study documented good performance parameters for non-certified quantitative HIV-1 DNA assays, however significant caveats were documented, including subtype specific issues and false positive reactions. Since the study involved only labs with HIV-1 DNA quantification documented in peer-reviewed papers, the same performance may be not guaranteed in other labs running the assay outside of these inclusion criteria. However, the above shortcomings need to be addressed at any individual lab willing to provide HIV-1 DNA measurements either in research studies or in the clinical setting, irrespective from the experience in the field.

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References

1. Phanuphak, N. & Gulick, R. M. HIV treatment and prevention 2019: current standards of care. *Curr. Opin. HIV AIDS.* **15**, 4–12 (2020).
2. Spivak, A. M. & Planelles, V. Novel latency reversal agents for HIV-1 cure. *Annu. Rev. Med.* **69**, 421–436 (2018).
3. Delagrèverie, H. M., Delaugerre, C., Lewin, S. R., Deeks, S. G. & Li, J. Z. Ongoing clinical trials of human immunodeficiency virus latency-reversing and immunomodulatory agents. *Open Forum Infect. Dis.* **3**, 10074 (2016).
4. Falcinelli, S. D., Ceriani, C., Margolis, D. M. & Archin, N. M. New frontiers in measuring and characterizing the HIV reservoir. *Front. Microbiol.* **10**, 11478 (2019).
5. Bruner, K. M., Hosmane, N. N. & Siliciano, R. F. Towards an HIV-1 cure: measuring the latent reservoir. *Trends Microbiol.* **23**, 192–203 (2015).
6. Siliciano, J. D. & Siliciano, R. F. Enhanced culture assay for detection and quantitation of latently infected, resting CD4+ T-cells carrying replication-competent virus in HIV-1-infected individuals. *Methods Mol. Biol.* **304**, 3–15 (2005).
7. Laird, G. M., Rosenbloom, D. I., Lai, J., Siliciano, R. F. & Siliciano, J. D. Measuring the frequency of latent HIV-1 in resting $CD4^+$ T cells using a limiting dilution coculture assay. *Methods Mol. Biol.* **1354**, 239–253 (2016).
8. Thomas, J., Ruggiero, A., Paxton, W. A. & Pollakis, G. Measuring the success of HIV-1 cure strategies. *Front. Cell Infect. Microbiol.* **10**, 1336 (2020).
9. Wang, Z., Simonetti, F. R., Siliciano, R. F. & Laird, G. M. Measuring replication competent HIV-1: advances and challenges in defining the latent reservoir. *Retrovirology.* **15**, 1002 (2018).
10. Ho, Y. C. *et al.* Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. *Cell* **155**, 540–551 (2013).
11. Rouzioux, C. & Avettand-Fenoël, V. Total HIV DNA: a global marker of HIV persistence. *Retrovirology.* **3**, 15–30 (2018).
12. Sharkey, M. E. *et al.* Persistence of episomal HIV-1 infection intermediates in patients on highly active anti-retroviral therapy. *Nat. Med.* **6**, 76–81 (2000).
13. Chan, C. N. *et al.* HIV-1 latency and virus production from unintegrated genomes following direct infection of resting CD4 T cells. *Retrovirology.* **13**, 1007 (2016).
14. Eriksson, S. *et al.* Comparative analysis of measures of viral reservoirs in HIV-1 eradication studies. *PLoS Pathog.* **9**, 537 (2013).
15. Kiselinova, M. *et al.* Integrated and total HIV-1 DNA predict ex vivo viral outgrowth. *PLoS Pathog.* **12**, 71 (2016).
16. De Spiegelaere, W. *et al.* Quantification of integrated HIV DNA by repetitive-sampling Alu-HIV PCR on the basis of poisson statistics. *Clin. Chem.* **60**, 886–895 (2014).
17. Tsiara, C. G. *et al.* Impact of HIV type 1 DNA levels on spontaneous disease progression: a meta-analysis. *AIDS Res. Hum. Retroviruses.* **28**, 366–373 (2012).
18. Valcour, V. G. *et al.* Southeast Asia Research Collaboration with the University of Hawaii 001 protocol team. HIV DNA and cognition in a Thai longitudinal HAART initiation cohort: the SEARCH 001 Cohort Study. *Neurology.* **72**, 992–8 (2009).
19. Weiss, L. *et al.* ANRS 116 SALTO Study Group. T-cell activation positively correlates with cell-associated HIV-DNA level in viremic patients with primary or chronic HIV-1 infection. *AIDS.* **28**, 1683–7 (2014).
20. Lafeuillade, A., Poggi, C., Hittinger, G., Counillon, E. & Emilie, D. Predictors of plasma human immunodeficiency virus type 1 RNA control after discontinuation of highly active antiretroviral therapy initiated at acute infection combined with structured treatment interruptions and immune-based therapies. *J. Infect. Dis.* **188**, 1426–1432 (2003).
21. Williams, J. P. *et al.* HIV-1 DNA predicts disease progression and post-treatment virological control. *Elife.* **3**, 1004 (2014).
22. Assoumou, L. *et al.* ANRS 116 SALTO study group. A low HIV-DNA level in peripheral blood mononuclear cells at antiretroviral treatment interruption predicts a higher probability of maintaining viral control. *AIDS.* **29**, 2003–7 (2015).
23. Piketty, C. *et al.* ANRS 116 SALTO Study Group. A high HIV DNA level in PBMCs at antiretroviral treatment interruption predicts a shorter time to treatment resumption, independently of the CD4 nadir. *J. Med. Virol.* **82**, 1819–28 (2010).
24. Geretti, A. M. *et al.* Dynamics of cellular HIV-1 DNA levels over 144 weeks of darunavir/ritonavir monotherapy versus triple therapy in the MONET trial. *HIV Clin. Trials.* **14**, 45–50 (2013).
25. Lambert-Niclot, S. *et al.* Factors associated with virological failure in HIV-1-infected patients receiving darunavir/ritonavir monotherapy. *J. Infect. Dis.* **204**, 1211–1216 (2011).
26. Wijting, I. *et al.* Predictors of virological failure in HIV-1-infected patients switching to dolutegravir maintenance monotherapy. *HIV Med.* **20**, 63–68 (2019).
27. Vicenti, I. *et al.* Development of an internally controlled quantitative PCR to measure total cell-associated HIV-1 DNA in blood. *Clin. Chem. Lab. Med.* **56**, 79 (2018).
28. De Rossi, A. *et al.* SIVIM (Italian Society of Medical Virology) Group. Quantitative HIV-1 proviral DNA detection: a multicentre analysis. *New Microbiol.* **33**, 293–302 (2010).

29. Di Sante, L. *et al.* Quantification of the HIV-1 total reservoir in the peripheral blood of naïve and treated patients by a standardised method derived from a commercial HIV-1 RNA quantification assay. *Clin. Chem. Lab. Med.* **59**, 609–617 (2020).
30. Stoszko, M. *et al.* Small molecule inhibitors of BAF; a promising family of compounds in HIV-1 latency reversal. *EBioMedicine* **3**, 108–121 (2015).
31. Alteri, C. *et al.* Quantification of HIV-DNA and residual viremia in patients starting ART by droplet digital PCR: Their dynamic decay and correlations with immunological parameters and virological success. *J. Clin. Virol.* **117**, 61–67 (2019).
32. Massanella, M., Gianella, S., Lada, S. M., Richman, D. D. & Strain, M. C. Quantification of total and 2-LTR (Long terminal repeat) HIV DNA, HIV RNA and herpesvirus DNA in PBMCs. *Bio Protoc.* **5**, 1492 (2015).
33. Chun, T. W. *et al.* Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* **387**, 183–188 (1997).
34. Viard, J. P. *et al.* Impact of 5 years of maximally successful highly active antiretroviral therapy on CD4 cell count and HIV-1 DNA level. *AIDS* **18**, 45–49 (2004).
35. Avettand-Fènoël, V. *et al.* French Pediatric Cohort Study ANRS-CO 01 Group. LTR real-time PCR for HIV-1 DNA quantitation in blood cells for early diagnosis in infants born to seropositive mothers treated in HAART area (ANRS CO 01). *J. Med. Virol.* **81**, 217–23 (2009).
36. Falasca, F. *et al.* Evaluation of HIV-DNA and inflammatory markers in HIV-infected individuals with different viral load patterns. *BMC Infect. Dis.* **17**, 763 (2017).
37. Bachmann, N. *et al.* Swiss HIV Cohort Study. Determinants of HIV-1 reservoir size and long-term dynamics during suppressive ART. *Nat. Commun.* **10**, 100047 (2019).
38. Jones, M. *et al.* Low copy target detection by Droplet Digital PCR through application of a novel open access bioinformatic pipeline, "definetherain". *J. Virol. Methods*. **202**, 46–53 (2014).
39. Bertoldi, A. *et al.* Development of C-TILDA: A modified TILDA method for reservoir quantification in long term treated patients infected with subtype C HIV-1. *J Virol Methods.* **276**, 113778 (2020).
40. Gantner, P. *et al.* ANRS-AC11 Quantification Working Group. Interlaboratory quality control of total HIV-1 DNA load measurement for multicenter reservoir studies. *J. Med. Virol.* **89**, 2047–2050 (2017).
41. Trypsteen, W., Kiselinova, M., Vandekerckhove, L. & De Spiegelaere, W. Diagnostic utility of droplet digital PCR for HIV reservoir quantification. *J Virus Erad.* **2**, 162–169 (2016).
42. Rutsaert, S., Bosman, K., Trypsteen, W., Nijhuis, M. & Vandekerckhove, L. Digital PCR as a tool to measure HIV persistence. *Retrovirology* **15**, 16 (2018).
43. Anderson, E. M. & Maldarelli, F. Quantification of HIV DNA using droplet digital PCR techniques. *Curr. Protoc. Microbiol.* **51**, 71 (2018).

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Author contributions

Conceptualisation and data management: I.V., F.D., M.Z. and F.S.; methodologies and experimental work: I.V., F.D., A.G., A.C., F.L., L.D.S., O.T., S.R., S.P., A.L., I.B., I.A., S.B., R.S., C.A. and all the members of the Italian HIV DNA Network Workgroup; original draft preparation: I.V. and M.Z.; funding: M.Z.; all authors have edited, reviewed and approved the final version of the manuscript.

Competing interests

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Additional information

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Correspondence and requests for materials should be addressed to I.V.

Italian HIV DNA Network

Chiara Orlandi[2], Mauro Magnani[2], Simona Di Giambenedetto[3], Roberta Longo[4], Stefano Menzo[4], Daniele Di Carlo[5], Laura Mazzuti[5], Anna Ardemagni[6], Massimo Clementi[6], Fausto Baldanti[7], Federica Giardina[7], Annalisa Bergna[8], Claudia Balotta[8], Alessia Bertoldi[9], Maria Rosaria Capobianchi[10], Francesca Ceccherini-Silberstein[12], Maria Antonello[13], Carlo Federico Perno[14] & Massimo Andreoni[15]

[15]Infectious Diseases Clinic, University Hospital "Tor Vergata", Rome, Italy.

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An Italian Multicenter Study on the Epidemiology of Respiratory Syncytial Virus During SARS-CoV-2 Pandemic in Hospitalized Children

Raffaella Nenna[1], Luigi Matera[1], Amelia Licari[2,3], Sara Manti[4], Gaia Di Bella[5], Alessandra Pierangeli[6], Anna Teresa Palamara[7,8], Luana Nosetti[5], Salvatore Leonardi[4], Gian Luigi Marseglia[2,3], Fabio Midulla[1] and ICHRIS Group*

[1] Department of Maternal Infantile and Urological Sciences, Sapienza University of Rome, Rome, Italy, [2] Pediatric Clinic, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy, [3] Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy, [4] Pediatric Respiratory and Cystic Fibrosis Unit, Department of Clinical and Experimental Medicine, San Marco Hospital, University of Catania, Catania, Italy, [5] Department of Pediatrics, Pediatric Sleep Disorders Center, F. Del Ponte Hospital, Insubria University, Varese, Italy, [6] Laboratory of Virology, Department of Molecular Medicine, Affiliated to Istituto Pasteur Italia, Sapienza University of Rome, Rome, Italy, [7] Laboratory Affiliated to Istituto Pasteur Italia-Fondazione Cenci Bolognetti, Department of Public Health and Infectious Diseases, Sapienza University of Rome, Rome, Italy, [8] Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy

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***Correspondence:**
Fabio Midulla
midulla@uniroma1.it

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Since the beginning of 2020, a remarkably low incidence of respiratory virus hospitalizations has been reported worldwide. We prospectively evaluated 587 children, aged <12 years, admitted for respiratory tract infections from 1 September 2021 to 15 March 2022 in four Italian pediatric hospitals to assess the burden of respiratory viruses during the COVID-19 pandemic in Italy. At admission, a Clinical Respiratory Score was assigned and nasopharyngeal or nasal washing samples were collected and tested for respiratory viruses. Total admissions increased from the second half of October 2021 to the first half of December 2021 with a peak in early November 2021. The respiratory syncytial virus (RSV) incidence curve coincided with the total hospitalizations curve, occurred earlier than in the pre-pandemic years, and showed an opposite trend with respect to the incidence rate of SARS-CoV-2. Our results demonstrated an early peak in pediatric hospitalizations for RSV. SARS-CoV-2 may exhibit a competitive pressure on other respiratory viruses, most notably RSV.

Keywords: respiratory infections, pediatrics, respiratory syncytial virus, SARS-CoV-2, COVID-19

INTRODUCTION

Respiratory viruses, and among them Respiratory Syncytial Virus (RSV), cause a large burden of respiratory diseases, accounting for most pediatric emergency visits and hospitalization worldwide, with high healthcare costs and significant morbidity (1). In the pre-Coronavirus Disease (COVID-19) era, RSV used to have a significant impact on children < 5 years, causing about 3.2 million hospital admissions globally (2) with annual winter epidemics peaking between December and February in the Northern Hemisphere (3). This trend is confirmed by other studies, including our previous published work (4, 5). Remarkably low incidence of respiratory viruses hospitalizations, with flat epidemiology, has been reported worldwide since early 2020, during the COVID-19

pandemic (5–8). Reduction in communicable disease was the lucky another side of the coin of coronavirus preventive measures, such as face masks use, hand washing, social distancing, and a ban on the mass gatherings (9).

A matter of concern was a possible resurgence of respiratory viruses diseases since spring-summer 2021, likely driven by relaxed community lockdown coupled with waning population immunity with a consequent increase in population susceptibility (8, 10, 11). Recently, data from the Southern Hemisphere reported a temporal shift in 2021 RSV seasonality that was not balanced by a more severe clinical presentation (10, 12). Despite the unusual onset of the RSV epidemic in the United States during summer, the relative timing of the RSV epidemic between states followed the usual spatial pattern (from East to North and West) (13). In Italy, normal epidemics showed a similar pattern (from North to South), following the changes in weather conditions, with peak RSV activity correlating with cold temperatures and higher relative humidity (14). Demonstrating the impact of SARS-CoV-2 periodic waves and the effects of COVID-19 preventive measures on the epidemiology of other respiratory viruses and anticipating epidemic timing for RSV can make policy decisions aimed at containing the morbidity and the spread of these viruses and at planning RSV passive and, eventually, active prophylaxis more effective.

For this study, we combined data sources from 4 major pediatric Hospitals, representative of the North, Center, and South of Italy, to examine trends in respiratory viruses hospitalization in autumn-winter 2021–2022 in Italy. We aimed at registering the resurgence of respiratory viruses diseases and describing the spatial variation in epidemic timing in Italy, during the COVID-19 pandemic. Moreover, we superimposed the incidence of COVID-19-related admissions, extracted from the national update of the Italian Superior Institute of Health to the incidence of RSV-related admissions to evaluate the relationship between viruses.

MATERIALS AND METHODS

We prospectively evaluated 587 children, aged < 12 years, consecutively admitted for respiratory tract infections from September 1, 2021, to March 15, 2022, at four Italian Pediatric University Hospitals: (1) The University of Insubria in Varese (North); (2) University of Pavia (North); (3) Sapienza University of Rome (Center) and (4) University of Catania (South). The Italian peninsula is divided into three different regions depending on the different latitudes, from continental Europe to the borders of Africa: the North, with a colder continental climate; the Center, with a more temperate climate; the South, with a warmer climate. This is reflected in the spread of viruses, which are particularly affected by climatic conditions. Thus, including in this study four centers from different Italian regions (Varese and Pavia for the North, Rome for the Center, and Catania for the South), we were able to evaluate not only the general trend of the RSV epidemic but also its spread throughout the Italian peninsula. The total number of hospitalized children due to respiratory tract infections was enrolled in the participating hospitals, even those with comorbidities.

This prospective observational study was approved by the Ethics Committees of the recruiting centers. Informed consent was waived, as the analysis was performed on de-identified data. Demographic and clinical data were collected from patients' clinical charts. A Clinical Respiratory Score (CRS) was assigned at admission. The score included the child's color, respiratory rate, presence of wheeze, use of accessory muscles, mental status, and oxygen saturation, and each variable ranges from 0 to 2. Thus, CRS total score ranges from 0 to 12 and defines into three categories: Mild (<3), Moderate (4–7), and Severe (8–12) (15).

From all children, we collected a nasopharyngeal (NP) washing (NPW) (in infants up to 1 year of age) or from NP swabs (NPS) (for older children). The children underwent NPW or NPS within 24 h of hospitalization. To test RSV and other respiratory viruses, the four centers used different PCR-based molecular methods, either commercially available kits or homemade real-time PCRs. In the latter tests, a sample was considered positive to a viral target when its Ct value was < 40. In particular, in Varese, a qualitative multiplex real-time RT- PCR intended for testing RSV, hRV, and human influenza type A and B (FluA and FluB), was performed. In Pavia, detection of RSV RNA was performed with a specific one-step real-time RT-PCR assay targeting RSV-A and -B together; hRV, FluA and FluB, human metapneumovirus (hMPV), human parainfluenza virus (hPiV) type 1–4 and human adenoviruses (hAdV) were tested using a panel of laboratory-developed real-time RT-PCR, previously validated and tested (16). In Rome, purified RNA from respiratory samples was retrotranscribed using random-primers; cDNA was tested by real-time PCRs (17) to detect and subtype RSV and by home-made, qualitative PCRs for FluA and FluB, human coronavirus h (CoV) OC43, 229E, NL-63 and HUK1, hAdV, hRV, hPiV type 1–3, human bocavirus (hBoV) and hMPV (18). In Catania, the rapid molecular test for testing RSV, FluA, and FluB (Xpert® Xpress, Cepheid) or the R-GENE® qualitative multiplex real-time RT-PCR (Biomerieux) were used.

Data on the weekly incidence of COVID-19-related admissions per 1,000,000 inhabitants, were extracted from the national update of the Italian Superior Institute of Health, published on March 16, 2022 (19).

We analyzed the data using SPSS version 27 (IBM Corp., New York, United States). Continuous variables were described as means ± standard deviations and categorical variables as frequencies and percentages. Comparisons between continuous variables were assessed using the analysis of the Variance test and categorical variables using the Chi-Square test. A p-value < 0.05 was considered statistically significant.

RESULTS

We consecutively enrolled 587 children admitted for respiratory tract infection from the North to the South of Italy: 105 (17.7%) in Varese, 130 (22.1%) in Pavia, 218 (37.1%) in Rome, and 134 (22.8%) in Catania. Children had a median age of 0.6 years (IQ:0.18–2.2) and 309/587 (52.6%) were males.

Concerning the CRS, the mild forms of respiratory diseases were predominant (54%), while in 39.7% of patients they were moderate, and in 6.3% they were severe. **Table 1** shows the

TABLE 1 | The main characteristics of the population studied in the participant centers.

	Center				Total
	Varese	**Pavia**	**Rome**	**Catania**	
N. of case	105	130	218	134	587
Age < 5 years, *n* (%)	105 (100)	124 (96.1)	211 (96.8)	108 (81.2)	548 (93.7)
Male sex, *n* (%)	48 (45.7)	71 (54.6)	131 (60.1)	59 (44.0)	309 (52.6)
Family history for asthma, n (%)[a]	26 (24.8)	18 (15.8)	33 (20.0)	35 (26.3)	112 (21.7)
Virus[b]					
RSV (+), *n* (%)	76 (72.4)	54 (49.1)	114 (54.3)	43 (32.1)	287 (51.3)
hRV (+), *n* (%)	1 (1.0)	19 (17.3)	16 (7.6)	6 (4.5)	42 (7.5)
Other viruses (+), *n* (%)	2 (1.9)	20 (18.2)	3 (1.4)	16 (11.9)	41 (7.3)
Severe cases, *n* (%)[c]	14 (13.3)	9 (6.9)	5 (2.3)	9 (6.7)	37 (6.3)
Epidemic peak (week number)	42–43	46–47	46–47	48–49	46–47
Latitudine	45°49′N	45°12′N	41°54′N	37°30′N	

[a] *Parental asthma.*
[b] *Viruses are: RSV, Respiratory Syncytial Virus; hRV, human Rhinovirus.*
Other viruses = Adenoviruses, Bocavirus, Metapneumovirus.
[c] *Severe cases are defined as having CRS = 8.*

main characteristics of the population studied according to the participant centers (**Table 1**).

Dividing our observational period into weeks' timeframes, the total admissions for respiratory diseases increased from the second half of October 2021 to the first half of December 2021 with a peak at the beginning of November 2021 (**Figure 1**).

Considering viral etiology, RSV ($n = 306$, 52.1%) was the most frequent identified virus in the four participant centers (Varese: $n = 76$, 72.4%; Pavia: $n = 65$, 50%; Roma: $n = 122$, 55.9%; Catania: $n = 43$, 32%). RSV incidence curve coincided with the total hospitalizations curve.

When we analyzed the single centers' data, we found that the peak in admissions occurred earlier in the North of Italy, in Varese, and, subsequently, it spread southwards, through Pavia, Rome, and Catania, according to latitude and different climatic conditions ($p < 0.01$) (**Table 1** and **Figure 1**). In all centers, the RSV epidemic peak occurred earlier than in pre-pandemic years, during which the earliest RSV-associated hospitalizations occurred in mid-December (13).

When we compared RSV admissions incidence to the weekly hospitalization rate for COVID-19, we found that they had an opposite trend ($p < 0.001$). RSV circulation had a surge in early autumn and peaked in November 2021, while the low activity of SARS-CoV-2 was registered in children; on the contrary, RSV incidence was drastically reduced when the novel Omicron (B.1.1.529) variant of SARS-CoV-2 started to circulate in children (**Figure 2**).

Finally, considering CRS, we found that the mild forms were prevalent: mild 317 (54%), moderate 233 (39.7%), and severe 37 (6.3%).

DISCUSSION

In this study, we reported an unseasonal RSV circulation that lasted from early autumn to early winter 2021 and was apparently blunted by the arrival of the novel SARS-CoV-2 variant. That delay did not affect the timing pattern of respiratory viruses spreading from North to South in Italy. To the best of our knowledge, we are the first to report an abrupt drop in this early RSV epidemic season and to hypothesize a competition between RSV and SARS CoV-2 in children.

Several factors may have contributed to the unseasonal RSV epidemiology, as well as that of other respiratory viruses. An explanation could come from restrictive measures adopted to slow the SARS-CoV-2 pandemic that dramatically reduced childhood respiratory infections, particularly RSV, except for rhinovirus (5). As expected, a resurgence of respiratory viruses was registered, when lockdown measures were relaxed. Moreover, a possible decline in the population immunity due to the disappearance of respiratory viruses during winter 2020–2021 with a possible increase in population susceptibility may have contributed to the rapid increase in cases in early autumn. Therefore, the re-emergence of respiratory viruses was not-unexpected during the COVID-19 pandemic. However, the abrupt decrease of cases by late December and the ending of RSV season by early January, concurrently with a sudden increase of SARS-CoV-2 pediatric cases, was unexpected (20). To understand this phenomenon, we examined the epidemiological curve of SARS-CoV-2 cases in Italy and noted that the surge of pandemic cases in fall was delayed with respect to other European countries with the exception of Spain, probably due to climatic conditions. Our data shows that the sharp decrease in RSV cases paralleled the SARS-CoV-2 surge during December 2021. One of the possible explanations is that a viral interference phenomenon may explain the sudden RSV disappearance observed in Italy. It has been long hypothesized and then proved in different experimental contexts (21, 22) that a respiratory viral infection could prevent the super-infection of other respiratory pathogens due to the activation of the innate immunity that confers to respiratory mucosal cells the ability to counteract a second virus replication, mainly through the interferon response (23). Other data supporting viral interference come from epidemiological studies; it has been well documented that the circulation of

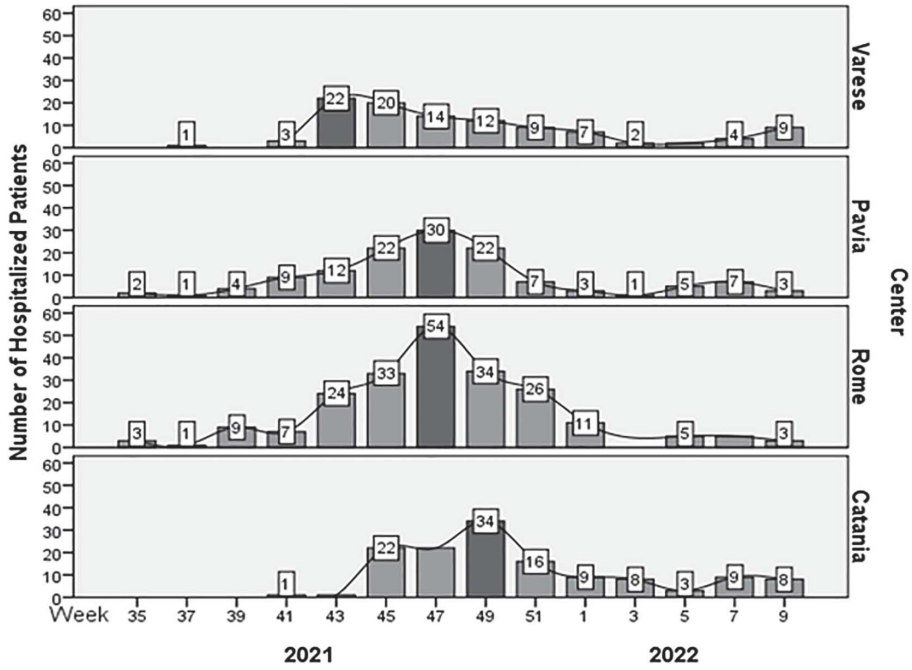

FIGURE 1 | Distribution of hospitalized patients for respiratory diseases from September 1, 2021 to March 15, 2022. The epidemic peaks are highlighted in dark gray.

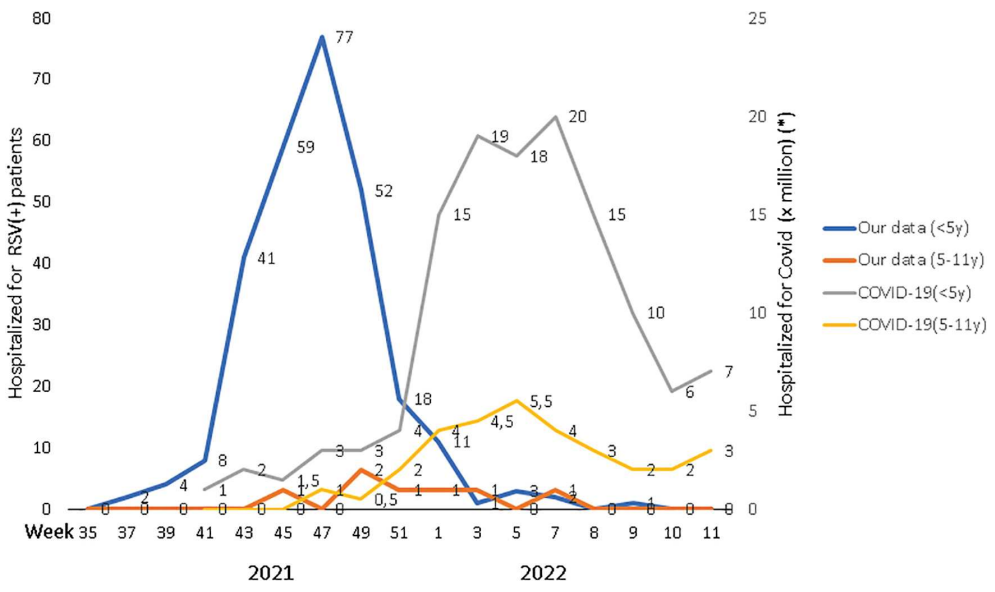

FIGURE 2 | Pediatric admissions for RSV + respiratory diseases by age vs. COVID-19 + admissions during the epidemic timeframe 2021–2022. COVID-19 admissions were collected from the national update of ISS (18).

Influenza Virus H1N12009 during the first pandemic winter has been delayed by the Rhinoviruses (hRVs) cases in September-October in several countries and other large studies followed (23, 24). Similarly, during the 2009 influenza pandemic, an average delay of 0.58 months in the onset of RSV season was reported (25). Moreover, hRVs circulation in autumn can also influence RSV epidemic seasons (26, 27). Recently, it has been demonstrated that hRV triggers an interferon response that blocks SARS-CoV-2 replication (28). Influenza Virus, but not RSV, reduced SARS-CoV-2 replication and in turn, SARS-CoV-2 interfered with RSV-A replication in Nasal Epithelial Cells *in vitro* (29). Accordingly, it is possible to hypothesize that RSV circulation this year was halted by the ongoing spread of the SARS-CoV-2 Omicron variant surge that was particularly contagious and abundant in unvaccinated children (30). Further studies are needed to investigate this possibility.

Finally, our results showed that, regarding CRS, the mild forms were predominant. This finding let us speculate that there is no evidence for a possible overlap of the immune response against RSV and SARS-CoV-2 and their mutual enhancement, leading to a worse clinical picture.

CONCLUSION

In conclusion, our multicenter study demonstrated an early and intense peak in RSV-associated pediatric admissions. Considering that SARS-CoV-2 is becoming endemic, its circulation will affect that of other respiratory viruses, and vice-versa. Reliably predicting the onset of the RSV epidemic, has become a major challenge for those involved in preventing respiratory infections. Clinicians need to be prepared for the advent of respiratory viruses to timely use healthcare sources and effectively plan RSV passive and, eventually, active prophylaxis.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ICHRIS (ITALIAN CHILDREN RESPIRATORY INFECTIONS SURVEILLANCE) GROUP

Massimo Agosti, Guido Antonelli, Fausto Baldanti, Flaminia Bonci, Maria Giulia Conti, Greta Di Mattia, Guglielmo Ferrari, Antonella Frassanito, Ginevra Gargiulo, Federica Giardina, Manuela Lo Bianco, Fabrizio Maggi, Paola Magri, Enrica Mancino, Matteo Naso, Federica Novazzi, Giuseppe Oliveto, Giuseppe Fabio Parisi, Maria Papale, Paola Papoff, Laura Petrarca, Antonio Piralla, Santiago Presti, and Gaia Vanzù.

AUTHOR CONTRIBUTIONS

RN, LM, and FM: conceptualization, project administration and supervision. RN, AL, SM, GD, LM, AP, ATP, LN, SL, GM, FM, and ICHRIS Group: data curation and writing—review and editing. LM and RN: formal analysis and writing—original draft. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Nair H, Simões EA, Rudan I, Gessner BD, Azziz-Baumgartner E, Zhang JSF, et al. Global and regional burden of hospital admissions for severe acute lower respiratory infections in young children in 2010: a systematic analysis. *Lancet.* (2013) 381:1380–90.
2. Shi T, McAllister DA, O'Brien KL, Simoes EAF, Madhi SA, Gessner BD, et al. Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: a systematic review and modelling study. *Lancet.* (2017) 390:946–58.
3. Cangiano G, Nenna R, Frassanito A, Evangelisti M, Nicolai A, Scagnolari C, et al. Bronchiolitis: analysis of 10 consecutive epidemic seasons. *Pediatr Pulmonol.* (2016) 51:1330–5. doi: 10.1002/ppul.23476
4. Obando-Pacheco P, Justicia-Grande AJ, Rivero-Calle I, Rodríguez-Tenreiro C, Sly P, Ramilo O, et al. Respiratory syncytial virus seasonality: a global overview. *J Infect Dis.* (2018) 217:1356–64. doi: 10.1093/infdis/jiy056
5. Nenna R, Matera L, Pierangeli A, Oliveto G, Viscido A, Petrarca L, et al. First COVID-19 lockdown resulted in most respiratory viruses disappearing among hospitalised children, with the exception of rhinoviruses. *Acta Paediatr.* (2022) 111:1399–403. doi: 10.1111/apa.16326
6. Agha R, Avner JR. Delayed seasonal RSV surge observed during the COVID-19 pandemic. *Pediatrics.* (2021) 148:e2021052089. doi: 10.1542/peds.2021-052089
7. Guitart C, Bobillo-Perez S, Alejandre C, Armero G, Launes C, Cambra FJ, et al. Bronchiolitis, epidemiological changes during the SARS-CoV-2 pandemic. *BMC Infect Dis.* (2022) 22:84. doi: 10.1186/s12879-022-07041-x
8. Van Summeren J, Meijer A, Aspelund G, Casalegno JS, Erna G, Hoang U, et al. Low levels of respiratory syncytial virus activity in Europe during the 2020/21 season: what can we expect in the coming summer and autumn/winter? *Euro Surveill.* (2021) 26:2100639. doi: 10.2807/1560-7917.ES.2021.26.29.2100639
9. Matera L, Nenna R, Rizzo V, Ardenti Morini F, Banderali G, Calvani M, et al. SARS-CoV-2 pandemic impact on pediatric emergency rooms: a multicenter study. *Int J Environ Res Public Health.* (2020) 17:8753. doi: 10.3390/ijerph17238753
10. Hatter L, Eathorne A, Hills T, Bruce P, Beasley R. Respiratory syncytial virus: paying the immunity debt with interest. *Lancet Child Adolesc Health.* (2021) 5:e44–5. doi: 10.1016/S2352-4642(21)00333-3
11. Di Mattia G, Nenna R, Mancino E, Rizzo V, Pierangeli A, Villani A, et al. During the COVID-19 pandemic where has respiratory syncytial virus gone? *Pediatr Pulmonol.* (2021) 56:3106–9. doi: 10.1002/ppul.25582
12. Saravanos GL, Hu N, Homaira N, Muscatello DJ, Jaffe A, Bartlett AW, et al. RSV epidemiology in Australia before and during COVID-19. *Pediatrics.* (2022) 149:e2021053537. doi: 10.1542/peds.2021-053537
13. Zheng Z, Warren JL, Artin I, Pitzer VE, Weinberger DM. Relative timing of respiratory syncytial virus epidemics in summer 2021 across the United States was similar to a typical winter season. *Influenza Other Respir Viruses.* (2022) 16:617–20. doi: 10.1111/irv.12965
14. Nenna R, Evangelisti M, Frassanito A, Scagnolari C, Pierangeli A, Antonelli G, et al. Respiratory syncytial virus bronchiolitis, weather conditions and air pollution in an Italian urban area: an observational study. *Environ Res.* (2017) 158:188–93. doi: 10.1016/j.envres.2017.06.014
15. Nayani K, Naeem R, Munir O, Naseer N, Feroze A, Brown N, et al. The clinical respiratory score predicts paediatric critical care disposition in children with

respiratory distress presenting to the emergency department. *BMC Pediatr.* (2018) 18:339. doi: 10.1186/s12887-018-1317-2

16. Piralla A, Lunghi G, Percivalle E, Viganò C, Nasta T, Pugni L, et al. FilmArray® respiratory panel performance in respiratory samples from neonatal care units. *Diagn Microbiol Infect Dis.* (2014) 79:183–6. doi: 10.1016/j.diagmicrobio.2014.02.010
17. Scagnolari C, Midulla F, Selvaggi C, Monteleone K, Bonci E, Papoff P, et al. Evaluation of viral load in infants hospitalized with bronchiolitis caused by respiratory syncytial virus. *Med Microbiol Immunol.* (2012) 201:311–7. doi: 10.1007/s00430-012-0233-6
18. Pierangeli A, Gentile M, Di Marco P, Pagnotti P, Scagnolari C, Trombetti S, et al. Detection and typing by molecular techniques of respiratory viruses in children hospitalized for acute respiratory infection in Rome, Italy. *J Med Virol.* (2007) 79:463–8. doi: 10.1002/jmv.20832
19. Istituto Superiore di Sanità Epicentro. *Epidemia COVID-19. Report Esteso ISS*. (2020). Available online: https://www.epicentro.iss.it/coronavirus/sars-cov-2-dashboard (accessed February 16, 2022).
20. Methi F, Størdal K, Telle K, Larsen VB, Magnusson K. Hospital admissions for respiratory tract infections in children aged 0-5 years for 2017/2023. *Front Pediatr.* (2022) 9:822985. doi: 10.3389/fped.2021.822985
21. Haller O, Kochs G, Weber F. The interferon response circuit: induction and suppression by pathogenic viruses. *Virology.* (2006) 344:119–30. doi: 10.1016/j.virol.2005.09.024
22. Waterlow NR, Flasche S, Minter A, Eggo RM. Competition between RSV and influenza: limits of modelling inference from surveillance data. *Epidemics.* (2021) 35:100460. doi: 10.1016/j.epidem.2021.100460
23. Wu A, Mihaylova VT, Landry ML, Foxman EF. Interference between rhinovirus and influenza A virus: a clinical data analysis and experimental infection study. *Lancet Microbe.* (2020) 1:e254–62. doi: 10.1016/S2666-5247(20)30114-2
24. Casalegno JS, Ottmann M, Duchamp MB, Escuret V, Billaud G, Frobert E, et al. Rhinoviruses delayed the circulation of the pandemic influenza A (H1N1) 2009 virus in France. *Clin Microbiol Infect.* (2010) 16:326–9. doi: 10.1111/j.1469-0691.2010.03167.x
25. Li Y, Wang X, Msosa T, de Wit F, Murdock J, Nair H. The impact of the 2009 influenza pandemic on the seasonality of human respiratory syncytial virus: a systematic analysis. *Influenza Other Respir Viruses.* (2021) 15:804–12. doi: 10.1111/irv.12884
26. Achten NB, Wu P, Bont L, Blanken MO, Gebretsadik T, Chappell JD, et al. Interference between respiratory syncytial virus and human rhinovirus infection in infancy. *J Infect Dis.* (2017) 215:1102–6. doi: 10.1093/infdis/jix031
27. Karppinen S, Toivonen L, Schuez-Havupalo L, Waris M, Peltola V. Interference between respiratory syncytial virus and rhinovirus in respiratory tract infections in children. *Clin Microbiol Infect.* (2016) 22:208.e1. doi: 10.1016/j.cmi.2015.10.002
28. Dee K, Goldfarb DM, Haney J, Amat JAR, Herder V, Stewart M, et al. Human rhinovirus infection blocks severe acute respiratory syndrome coronavirus 2 replication within the respiratory epithelium: implications for COVID-19 epidemiology. *J Infect Dis.* (2021) 224:31–8. doi: 10.1093/infdis/jiab147
29. Fage C, Hénaut M, Carbonneau J, Piret J, Boivin G. Influenza A(H1N1)pdm09 virus but not respiratory syncytial virus interferes with SARS-CoV-2 replication during sequential infections in human nasal epithelial cells. *Viruses.* (2022) 14:395. doi: 10.3390/v14020395
30. Wang L, Berger NA, Kaelber DC, Davis PB, Volkow ND, Xu R. Incidence rates and clinical outcomes of SARS-CoV-2 infection with the omicron and delta variants in children younger than 5 years in the US. *JAMA Pediatr.* (2022) e220945. doi: 10.1001/jamapediatrics.2022.0945 [Epub ahead of print].

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Evaluation of the Neutralizing Antibodies Response against 14 SARS-CoV-2 Variants in BNT162b2 Vaccinated Naïve and COVID-19 Positive Healthcare Workers from a Northern Italian Hospital

Josè Camilla Sammartino [1], Irene Cassaniti [1,*], Alessandro Ferrari [1], Federica Giardina [1], Guglielmo Ferrari [1], Federica Zavaglio [1], Stefania Paolucci [1], Daniele Lilleri [1], Antonio Piralla [1], Fausto Baldanti [1,2] and Elena Percivalle [1]

[1] Molecular Virology Unit, Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy; jose.sammartino@iusspavia.it (J.C.S.); alessandro.ferrari04@universitadipavia.it (A.F.); federica.giardina01@universitadipavia.it (F.G.); guglielmo.ferrari01@universitadipavia.it (G.F.); f.zavaglio@smatteo.pv.it (F.Z.); s.paolucci@smatteo.pv.it (S.P.); d.lilleri@smatteo.pv.it (D.L.); a.piralla@smatteo.pv.it (A.P.); fausto.baldanti@unipv.it (F.B.); e.percivalle@smatteo.pv.it (E.P.)

[2] Department of Clinical, Surgical, Diagnostics and Pediatric Sciences, University of Pavia, 27100 Pavia, Italy

* Correspondence: i.cassaniti@smatteo.pv.it

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Abstract: SARS-CoV-2 still represents a global health burden, causing more than six million deaths worldwide. Moreover, the emergence of new variants has posed new issues in terms of vaccine efficacy and immunogenicity. In this study, we aimed to evaluate the neutralizing antibody response against SARS-CoV-2 variants in different cohorts of vaccinated and unvaccinated subjects. Four-fold diluted sera from SARS-CoV-2 naïve and recovered subjects vaccinated with two or three doses of the BNT162b2 vaccine were challenged against 14 SARS-CoV-2 variants, and the SARS-CoV-2 neutralizing antibody titer was measured. Results were compared with those obtained from unvaccinated COVID-19 recovered patients. Overall, a better SARS-CoV-2 NT Abs response was observed in recovered vaccinated subjects after three doses of the vaccine when compared to unvaccinated patients and vaccinated subjects with only two doses. Additionally, the lowest level of response was observed against the Omicron variant. In conclusion, third doses of BNT162b2 vaccine seems to elicit a sustained response against the large majority of variants.

Keywords: SARS-CoV-2; immune response; RNA vaccine; variants of concern

1. Introduction

Since the beginning of the Coronavirus infectious disease 2019 (COVID-19) pandemic in March 2020 [1] caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), there have been 508,827,830confirmed cases and 6,227,291deaths worldwide [2]. SARS-CoV-2 is a novel human coronavirus first reported in China in December 2019 which spread worldwide with over 500 million confirmed cases that led the WHO to declare a state of pandemic in March 2020 [2]. The rapid spread of the disease has prompted intense research activity to identify potential treatments, including investigations on existing drugs and the parallel de novo development of innovative treatments. In January 2020, the Chinese Center for Disease Control and Prevention released the genetic sequence of the first SARS-CoV-2 isolated in Wuhan, China, which led to the development of the BNT162b2 vaccine [3]. BNT162b2 is a novel RNA-based vaccine encoding the full-length SARS-CoV-2 Spike protein, with a 95% protection rate against COVID-19, approved for emergency use by the WHO in December 2020, only 11 months after its development started [4]. The rapidity of approval reflects the seriousness of the current situation [5]. The availability of a vaccine is particularly important in inducing neutralizing humoral and cellular immunity and,

more importantly, reducing COVID-19 infections, hospitalizations, and deaths in clinical trials. Further, in a hospital context, a vaccine is important for healthcare workers, not only for their own safety while working on the front line, but also to protect the patients from the spread of the disease [6]. Moreover, the development of mutations in the Spike sequence, which codes for the principal antigenic target of the new vaccines, leads to the insurgence of new variants. This raises concern over the neutralizing activity of vaccine-induced antibody responses, and the ability of antibodies triggered by previous infection to protect against re-infection [7], thus evaluating the BNT162b2 immunogenicity is fundamental. The Omicron variant, first identified in Botswana and South Africa, and which started spreading in November 2021, consists of over 30 mutations within the Spike protein and represents the last variant that can trigger the effect of the vaccines. In this context, we aimed to assess the humoral immune response elicited by the BNT162b2 vaccination in naïve and previously COVID-19 positive healthcare workers from the Fondazione IRCCS Policlinico San Matteo of Pavia, after two and three doses of vaccine, in comparison with a historical cohort of COVID-19 convalescent unvaccinated plasma donors. We determined the neutralizing titers of sera from all subjects of the study against the original virus (Wuhan, A), the Italian reference strain (D614G, B.1) and an additional 12 different variants of SARS-CoV-2, including the main variants of concern (VOC) with particular focus on the Omicron variant.

2. Materials and Methods

2.1. Study Population

All subjects included in the study were presented with an informed written consent prior to the sampling of the sera. An overview of the test population is reported in Table 1.

Table 1. Overview of the study populations. For the experienced-HCW and the Omicron-HCW populations all the available subjects were included, while the groups convalescent-PD and naïve-HCW were randomly selected from the pool of plasma donors and healthcare workers of Policlinico San Matteo, respectively.

	n	Drop-Outs	COVID-19	Times Assayed	Vaccination
Convalescent-PD	30	none	yes	Pre-vaccination	no
Naive-HCW	30	8	no	1 month after 2nd dose; 6 months after 2nd dose;	full course
Experienced-HCW	16	6	yes	1 month after 3rd dose	full course
Omicron-HCW	15	none	yes	1 month after COVID-19 positivity post full course vaccination	full course

2.1.1. COVID-19 Convalescent Plasma Donors (PD) Cohort Characteristics

The cohort is composed of 30 subjects who did not receive BTN162b2 vaccination and had a previously reported history of COVID-19 positivity. In the group, there are 80% male and 20% female subjects with a median age of 67 years (range 35–84). All presented mild common symptoms, and none needed to be hospitalized. The sera were collected during the first wave of COVID-19 infections between May and July 2020, at 26.4 days on average after the onset of symptoms (median days 17; range 12–103).

2.1.2. Naïve Healthcare Workers (Naïve HCW) Cohort Characteristics

The cohort is composed of 30 subjects who did not have a reported history of COVID-19 and tested negative for antibodies against the SARS-CoV-2 Nucleoprotein. The group consists of 20% male and 80% female subjects with a median age of 52 years (range 30–66). Eight subjects were excluded from the follow-ups.

2.1.3. COVID-19 Exposed Healthcare Workers (Exposed-HCW) Cohort Characteristics

The cohort is composed of 16 subjects who did receive at least two doses of the BTN162b2 vaccination at enrollment and had a previously reported history of COVID-19 positivity. In the group, there are 13% male and 87% female subjects with a median age of 42 years (range 25–61). In all, 14 subjects presented mild common symptoms, while 2 had none. No subject of the group needed hospitalization. During follow-ups, two subjects did not receive the third dose of vaccine and four did not participate in the follow-up analysis, thus being excluded.

2.1.4. Omicron Healthcare Workers (Omicron-HCW) Cohort Characteristics

The cohort is composed of 15 subjects who received three doses of the BTN162b2 vaccination but got infected with the Omicron variant during an Omicron outbreak in one of the Policlinico San Matteo departments. There are a 54% male and 46% female subjects with a median age of 28 (range 26–34). In all, 12 subjects had mild symptoms, with only one reporting dyspnea; none needed to be hospitalized. The symptoms lasted a mean of 7.7 days (median: 6; range 2–20). The first positivity was reported on average after 54.8 days (median: 71; range 2–75) after the 3rd dose and lasted for about 14.7 days (median: 15; range 7–19). One subject had reported history of COVID-19 (infected with the Delta variant) at 7 months from the 2nd dose. The sera were sampled on average 25.6 days after the first positive molecular test (median: 27; range 18–30).

2.2. Virus Isolation

All variants were isolated from 200 uL of nasopharyngeal swabs seeded on confluent VERO E6 cells (VERO C1008 (Vero 76, cloneE6, Vero E6); ATCC® CRL-1586™) in a 24-well flat-bottom tissue-culture microtiter plate (COSTAR, Corning Incorporated, Corning, NY, USA) decontaminated and incubated at 33 °C with 5% CO_2 for 1 h. After inoculum removal, fresh MEM eagle (EMEM, Lonza Group Ltd., Basel, Switzerland) supplemented with 1% *v/v* Penicillin, Streptomycin, Glutamine (Euroclone SpA) and 0.1% *v/v* Trypsin was added before incubation, in the same conditions, until cytopathic effect development. All the samples inoculated were observed under an inverted microscope, 10× magnification, every other day, until cytopathic effect (CPE) development was observed. SARS-CoV-2 CPE on VERO E6 cells is characterized by cell enlargement and syncytia formation. After the first isolation, all the variants were propagated in a VERO E6 25 cm^2 cell culture flask (Corning Incorporated, Corning, NY, USA) to increase virus titer and to prepare virus stock for microneutralization testing.

2.3. Whole Genome Sequencing

All variants were confirmed through complete genome sequencing [8] in order to confirm the presence of variant-defining mutations, and sequences were submitted to the Global Initiative on Sharing Avian Influenza Data (GISAID).

2.4. Virus Titration and Microneutralization Test

The titer of each variant's stock was measured at the 50% tissue culture infectious dose (TCID50) in six replicas in a 96-well flat-bottom tissue-culture microtiter plate. Briefly, logarithmic dilutions of previously stocked virus in presence of 3×10^4 VERO E6 cells were incubated for 72 h at 33 °C in 5% CO_2. The cells were observed under a microscope for cytopathic effect development and stained with Gram's crystal violet solution (Merck KGaA, Darmstadt, Germany) plus 5% *v/v* formaldehyde 40% m/v (Carlo Erba SpA, Arese, Italy). The value of TCID50 mL^{-1} was calculated with the Reed–Muench method [9]. After virus titration, 50 µL of 100 TCID50 was incubated with 50 µL of serial dilutions (1:10 to 1:640) of the subject's sera in duplicate in a 96-well flat-bottom tissue-culture microtiter plate. After 1 h incubation at 33 °C in 5% CO_2, 3×10^4 VERO E6 cells were added to each well. After 72 h incubation, wells were stained with Gram's crystal violet solution as

previously reported. The neutralizing titer demonstrated the maximum dilution with the reduction of 90% of cytopathic effect. A positive titer was equal to or greater than 1:10 [7,8].

2.5. Statistical Analysis

Comparison between groups was performed using two-way analysis of variance (ANOVA) with post hoc Dunnett's correction. GraphPad Prism 8.3.0 (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses. A two-sided p value < 0.05 is considered statistically significant.

3. Results

3.1. Viral Isolation and Characterization

SARS-CoV-2 variants were isolated from nasopharyngeal swabs, and whole genome sequencing was performed (Table 2). All the variants were titrated and the growth curves for the Wuhan, D614G, Delta (B.1.617.2), and Omicron (B.1.1529; BA.1) strains were determined (Figure 1). We observed that the Wuhan strain grew faster, reaching the growth peak at 48 h and slowly decreasing afterwards. On the other hand, D614G and Delta both peaked at 72 h, followed by the Omicron variant that grew slowly. In detail, The Wuhan and Delta variant titers were 1.0 ± 0.2 log lower in comparison to the D614G values at each time point but had the same trend. The overall trend of the Omicron growth curve mimics the other variants but is slightly translated to the right, growing at a lower level than the other VOC. In particular, Omicron had a titer 2.5 log lower at 24 h, and a 1.4 log lower at 48 h in comparison to D614G values. At 72 h, there was a slight recovery in the growth speed, with a titer of only a 1.2 log lower in comparison to D614G but was almost identical to the Wuhan and Delta values at the same time points. At 96 h, the Omicron variant showed a drop, with titer values log 2 that were lower than those of D614G.

Table 2. Overview of the SARS-CoV-2 variants used in the study, reported with their corresponding mutations, the date of isolation, the lineage following Pangolin [10] and, where applicable, the WHO nomenclature [11].

Strain Name	WHO Nomenclature	Lineage (Pangolin)	Spike Mutations	Spike Deletions	GISIAD
hCoV-19/Italy/LAZ-INMI1-isl/2020	-	A	N679S	-	EPI_ISL_410545
hCoV-19/Italy/LOM-INMI-10734/2020	-	B.1	S247R, D614G	-	EPI_ISL_568579
hCoV-19/Italy/LOM-Pavia-10833/2020	Alpha	B.1.1.7	N501Y, A570D, D614G, P681H, R685H, T716I, S982A, D1118H	69–70, 144	EPI_ISL_7043618
hCoV-19/Italy/LOM-Pavia-10858/2021	Gamma	P1	L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I, V1176F	-	EPI_ISL_7043637
hCoV-19/Italy/LOM-Pavia-10860/2021	Beta	B.1.351	S171L, D80A, D215G, L242H, K417N, E484K, N501Y, D614G, A701V	243–245	EPI_ISL_7043650
hCoV-19/Italy/LOM-Pavia-10870/2021	-	B.1.258.17	L189F, N439K, D614G, V772I	69–70	EPI_ISL_7043668

Table 2. *Cont.*

Strain Name	WHO Nomenclature	Lineage (Pangolin)	Spike Mutations	Spike Deletions	GISIAD
hCoV-19/Italy/LOM-Pavia-10881/2021		C.36.3(1)	S12F, W152R, R346S, L452R, T547I, D614G, Q677H, A899S	69–70	EPI_ISL_7043684
hCoV-19/Italy/LOM-Pavia-10882/2021	Eta	B.1.525	Q52R, A67V, E484K, D614G, Q677H, F888L	69–70, 144	EPI_ISL_7043697
hCoV-19/Italy/LOM-Pavia-10916/2021	Delta	B.1.617.2	T19R, G142D, E156G, A222V, L452R, T478K, D614G, P681R, R682W, D950N, E990A	157–158	EPI_ISL_7043718
hCoV-19/Italy/LOM-Pavia-10919/2021	Mu	B.1.621.1	T95I, Y144T, R346K, N501Y, D614G, P681H, D950N	-	EPI_ISL_7462685
hCoV-19/Italy/LOM-Pavia-10921/2021	-	C.36.3(2)	S12F, W152R, R346S, L452R, D614G, A899S	69–70, 675–679	EPI_ISL_7043733
hCoV-19/Italy/LOM-Pavia-10924/2021	Lambda	C.37	G75V, T76I, R246N, L452Q, D614G, T859N	247–253, 675–679	EPI_ISL_7043746
hCoV-19/Italy/LOM-Pavia-10940/2021	DeltaPlus	AY.4.2.3	T19R, T95I, G142D, Y145H, R158G, A222V, L452R, T478K, D614G, P681R, D950N	156–157	Submitted
hCoV-19/Italy/LOM-Pavia-10943/2021	Omicron	B.1.1.529 (BA.1)	A67V, T95I, Y145D, L212I, G339D, S371L, S373P, S375F, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981	69–70, 142–144, 211	Submitted

3.2. Assessing Antibody Response through Microneutralization Test

At 1 month post-2nd dose, SARS-CoV-2 NT-Abs in naïve healthcare workers (HCW) and exposed-HCW were compared to results obtained in unvaccinated COVID-19 convalescent plasma donors (PD) (Figure 2).

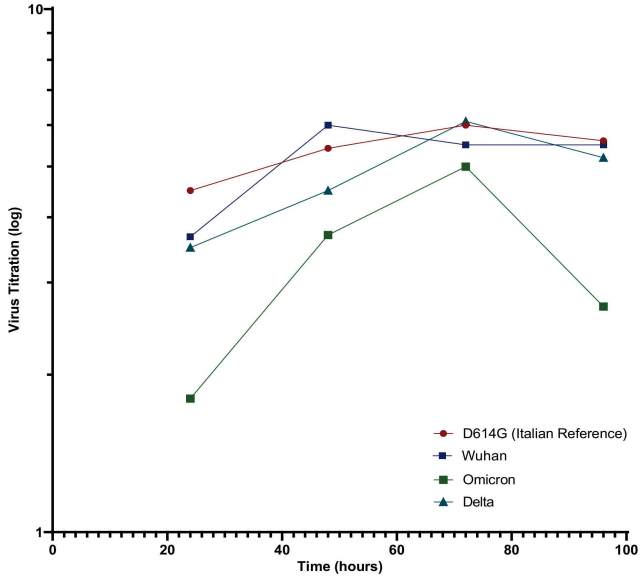

Figure 1. SARS-CoV-2 variants titration curves.

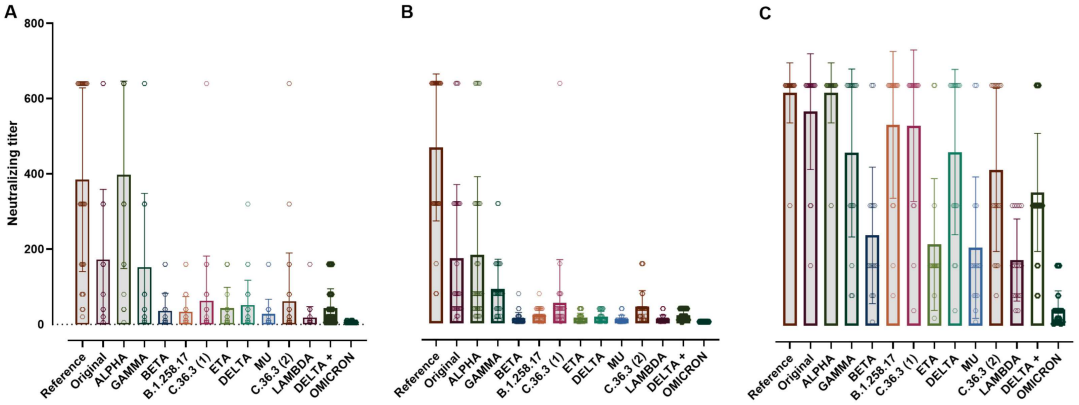

Figure 2. Scatter dot plot of the neutralizing titer for convalescent-PD (**A**), naïve-HCW (**B**) and exposed-HCW (**C**) following 2 doses of BTN162b2. In the graph, the bars stop at the titer mean for each variant. The convalescent-PD group has a high response only for the reference strain while the naïve-HCW has a high response to both the reference and alpha variants, but overall mimics the convalescent-PD trend. The experienced-HCW cohort achieved the highest response to all the different SARS-CoV-2 variants between the groups. High titers were associated with a larger variability (higher SD). Reference: D614G strain; Original: Wuhan.

The highest response levels were observed in vaccinated COVID-19 exposed-HCW (Figure 2C), SARS-CoV-2 NT-Ab titers were lower in COVID-19 convalescent-PD (Figure 2A) and naïve-HCW (Figure 2B). The convalescent-PD group had the higher response to Alpha and D614G strains, followed by Wuhan and Gamma strains, while the results obtained with other variants scored between 1:20 and 1:80, except for Lambda and Omicron variants, which were lower. The naïve-HCW had the highest response to D614G, followed by Alpha and Wuhan, while the response to the other variants was lower, with Delta, Eta, Beta, Lambda, Mu, and Omicron values below 1:20. The exposed-HCW group had the strongest response in comparison to the other groups, with NT-Ab against D614G and Alpha scoring the highest

followed by Wuhan, B.1.258.17. C.36.3 (1), Delta and Gamma, with values ranging 1:460 to 1:620, while for C.36.3 (2), Delta+ (1:400 and 1:350 respectively), Beta, Eta, Mu, Lambda, and Omicron (in range 1:40 to 1:250), there was a significant reduction in the NT-Ab response in comparison to the response to D614G (Figure 2). Detailed data on the NT-Ab response can be found in the Supplementary Table S1.

The kinetics of the neutralizing antibody titer (NT-Ab) in the naïve-HCW and exposed-HCW cohorts following two doses (1- and 6-month follow-up) and three doses of BTN162b2 vaccination is reported in Figure 3.

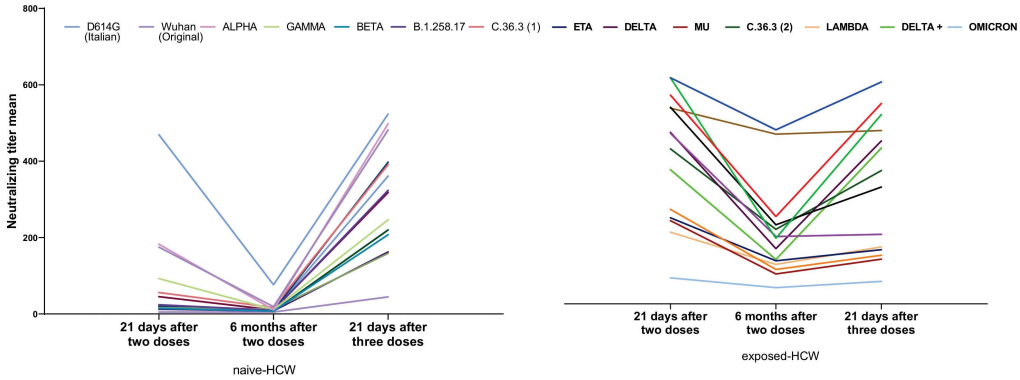

Figure 3. Data show the progression of the neutralizing antibody titer (NT-Ab) in the naïve HCW (**left**) and exposed-HCW cohorts (**right**). In both groups, there was a reduction in the NT-Ab at 6 months post-2nd dose, but the titer was recovered at 1 month after the 3rd dose.

As expected, at 6 months post-2nd dose, the overall NT-Abs response decreased, with a 65% mean reduction for the naïve HCW and 55% for the exposed-HCW, which, however, maintained higher values. The NT-Ab against the D614G variant had the highest reduction at 6 months and the least recovery after the 3rd dose in the naïve-HCW cohort, which had the opposite trend in the exposed-HCW. Alpha had the highest reduction at 6 months in both groups, while Delta and Delta+ had the highest recovery at 1 month post-3rd dose. After three doses, the D614G variant had the overall higher response in all cohorts, while Omicron had the lowest (Figure 3).

Data obtained from a group of triple dosed vaccinated HCW with subsequent exposure to the Omicron variant (Omicron-HCW) were compared to data obtained by the other groups of the study to investigate how the NT-Ab response changes in relation to the infection with a different, more recent variant of SARS-CoV-2 especially as the infection occurred after the full course of vaccinations was administered (Figure 4).

The highest difference was observed between the convalescent-PD and Omicron-HCW groups. On average, the convalescent-PD NT-Ab values were 8-fold lower than the Omicron-HCW with the highest differences observed for the Lambda (20-fold), B.1.258.17 (15-fold), Beta, Delta, and C.36.3 (1) (9-fold each) variants. Differently, the naïve-HCW and exposed-HCW had similar responses against the variants of SARS-CoV-2, thus scoring similarly to the Omicron-HCW, with average NT-Ab values respectively 1.4 and 1.7 times lower than the Omicron-HCW. The smallest significative differences between groups were observed in response to the D614G and the Alpha variants, while there was no significative difference in the response to the Omicron variant between the groups of the study (Figure 4).

In particular, the convalescent-PD cohort responded with titers higher than 1:300 only to the D614G and the Alpha variants, while both naïve-HCW and exposed-HCW had titers higher than 1:300 for 8 of the 14 variants tested after 3rd dose administration. Overall, the Omicron-HCW had the best response, with a NT-Ab titer higher than 1:300 for 12 of the 14 variants tested. Interestingly, the lower level of SARS-CoV-2 NT-Abs response was

observed against the Omicron variant in all of the study cohorts with median titers ranging from negative to 1:60 (Table 3; Table S1).

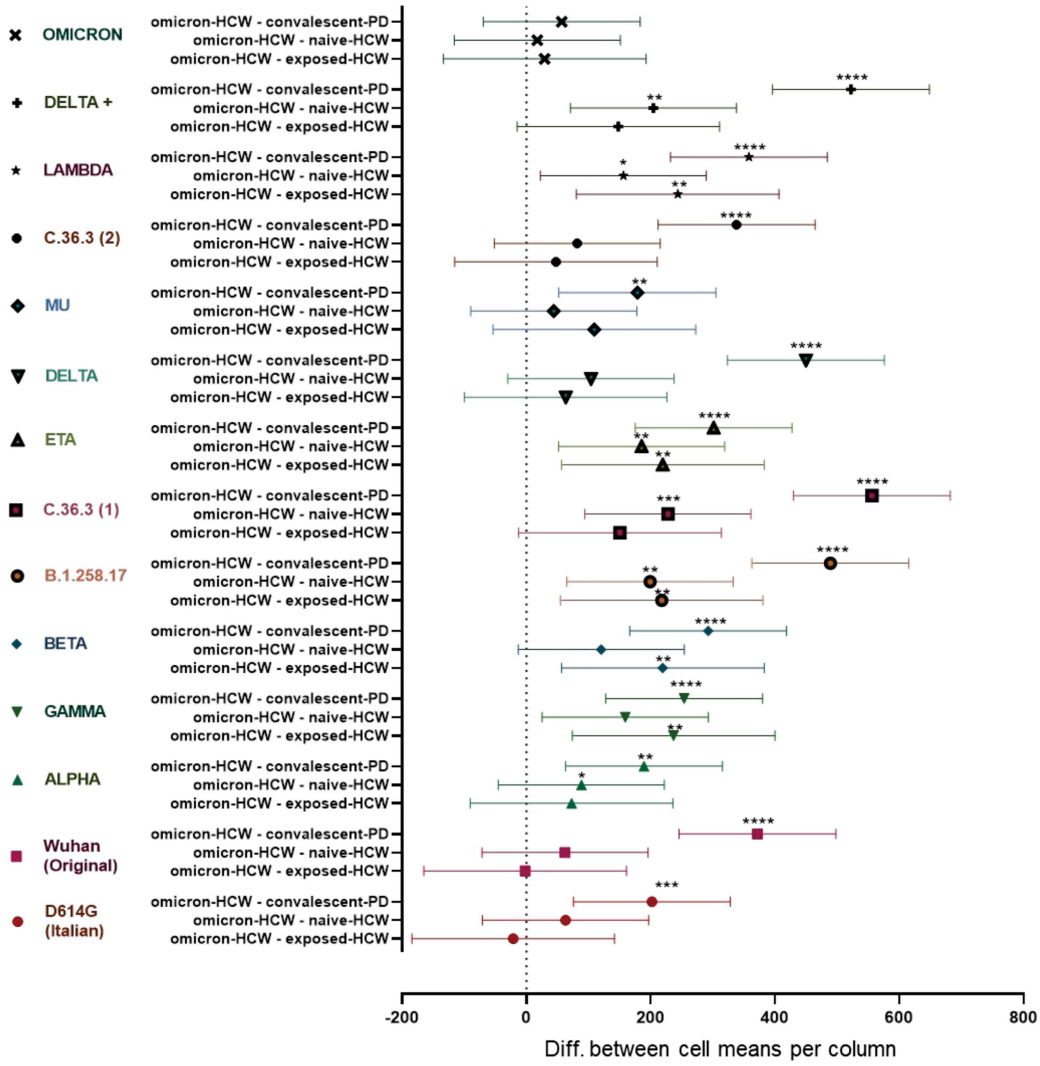

Figure 4. Data show the difference in means of the neutralizing titer (NT-Ab) against 14 different SARS-CoV-2 variants in naïve-HCW and exposed-HCW after 3 doses of BTN162b2 and in unvaccinated convalescent-PD, in comparison to the fully vaccinated Omicron-HCW NT-Ab values. The more distant the values are from the origin line (0), the higher the difference between the paired populations. **** $p < 0.0001$; *** $p = 0.0001$; ** $p = 0.001$; * $p < 0.05$. (Omicron-HCW—convalescent-PD: mean difference between Omicron-HCW and convalescent-PD groups; Omicron-HCW—naïve-HCW: mean difference between Omicron-HCW and naïve-HCW groups; Omicron-HCW—experienced-HCW: mean difference between Omicron-HCW and experienced-HCW groups).

Table 3. Neutralizing titers of the different population of the study against the 14 SARS-CoV-2 variants tested. The results are expressed as means and standard deviations.

	Convalescent-PD		**Naive-HCW**		**Exposed-HCW**		**Omicron-HCW**	
Variant	Mean	SD	Mean	SD	Mean	SD	Mean	SD
D614G	384.7	244.1	523.6	157.6	608	101.2	586.7	144
Wuhan	172.3	186.3	481.8	205.3	546	210.4	544	168.9
Alpha	397.5	249.2	498.2	200.5	514	218.7	586.7	144
Gamma	151.7	196.4	246.4	230.7	168.5	94.81	405.4	240.8
Beta	35.67	46.75	207.5	220.5	108.5	58.79	328	215
B.1.258.17	33.5	40.81	323.6	215.6	305	242.5	522.7	176
C.36.3 (1)	62.67	119.1	390.9	226.3	468	235.6	618.7	82.62
Eta	42.83	55.83	158.6	164	124.5	59.84	344	229.2
Delta	51.5	65.8	397.3	222.6	438	272.7	501.3	180.1
Mu	27.83	38.9	162.3	201.9	97	58.51	206.4	189.4
C.36.3 (2)	61.83	128.2	318.2	225.4	352.5	222.9	400	215.9
Lambda	18	29.14	220	217.3	132.5	58.56	376	235.1
Delta +	43.17	51.55	360.9	245.2	417.5	260.8	565.3	158.5
Omicron	5.5	1.526	44.77	50.01	33	22.01	62.4	61.9

4. Discussion

Vaccination with new mRNA vaccines can stimulate a substantial humoral response with the production of neutralizing antibodies highly specific for the defined antigens. On the other hand, thousands of COVID-19 variants were detected all over the world and few of them are still considered a concern [12]. Coronaviruses, including SARS-CoV-2, develop many mutations that are often not detrimental for their biological behavior and their structures [13]. It is conceivable that the variants would not undermine vaccine effectiveness in preventing severe COVID-19 but can have a role in reducing the vaccine immunogenicity. In particular, the rapid emergence of the new Omicron variant at the end of 2021, with more than 30 mutations at the Spike sequence, in the background of high Beta immunity, implies that the virus may have evolved to escape neutralization in Beta-specific serum raising new issues in terms of vaccine efficacy and the use of monoclonal antibodies in clinical practice. In agreement with our results, it has been observed that in two dosed vaccinated subjects SARS-CoV-2 NT-Ab levels were reduced against Omicron but improved when the third dose of vaccine was administered [14,15]. Interestingly, we observed as other authors did, a reduced replication capacity on permissive VERO E6 cells of the Omicron variant in comparison to the reference strain and Delta variant. Differences in the morphology of infected cells between Omicron and Delta were also observed, suggesting that Omicron is less fusogenic than Delta [16]. These data are confirmed also by the clinical features of infection characterized by an attenuated pathogenicity.

Based on our results, two doses of BNT162b2 vaccine are able to elicit an antibody response in naïve subjects against variants harboring the sequence used for vaccine construction, while in previously infected subjects the response is broader, covering different SARS-CoV-2 variants, as previously reported [17]. An overall reduction in SARS-CoV-2 NT-Abs against each variant tested was observed at six months after vaccination, as expected. However, after the third dose, the NT-Ab response was recovered.

As the convalescent-PD cohort was sampled before the start of the vaccination campaign, when there were few circulating variants, the presence of antibodies against different newer strains shows how natural infection provides wider coverage maintaining a reasonable effectiveness also against the new Omicron variant. So far, a humoral and a potent cell-mediated response, which are involved in protection against severe disease in COVID-19 patients, was observed for at least 15 months after the onset of symptoms, suggesting a long-term response elicited by natural infection [18]. Nonetheless, vaccinated subjects infected with the newer Omicron variant show the highest response against all previously characterized strains of SARS-CoV-2, which is in accordance with previously published

data [19]. However, Omicron can escape NT-Abs, causing milder symptoms, which can be attributed to its reduced fitness in the lower airways [20] and reduced replication in comparison to the other variants. Moreover, as stated by other authors, the antigenic profile of the Omicron receptor binding domain (RBD) is different from previous VOC, giving a reduced antigenicity in its new receptor binding sites (RBS) [21]. This evolutionary trend of decreasing antigenicity was also described for the old circulating coronavirus hCoV229E [21], which could confirm this hypothesis for the Omicron variant as well.

5. Conclusions

Altogether, these data show how important it is to vaccinate naïve subjects, but also that vaccination is helpful for previously infected subjects as it boosts their immune system and helps to keep the NT-Ab high in time. Moreover, as demonstrated by the high level of response in the Omicron-HCW cohort, boosting the immune system with different, newer variants could be beneficial in terms of antibody coverage. These findings are strengthened by the use of wild type isolated viruses better mimicking what could happen in a realistic setting. Therefore, it could be reasonable to suggest boosting vaccinated subjects with vaccines derived from circulating variants to implement immunity stimulation and ensure a broader coverage of immunity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vaccines10050703/s1, Table S1: Descriptive statistics of the data analyzed in the study.

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References

1. World Health Organization. Available online: https://www.who.int/emergencies/diseases/novel-coronavirus-2019 (accessed on 16 February 2022).
2. Jee, Y. WHO International Health Regulations Emergency Committee for the COVID-19 outbreak. *Epidemiol. Health* **2020**, *42*, e2020013. [CrossRef] [PubMed]
3. World Health Organization. Available online: https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19---15-march-2022 (accessed on 19 March 2022).
4. Polack, F.P.; Thomas, S.J.; Kitchin, N.; Absalon, J.; Gurtman, A.; Lockhart, S.; Perez, J.L.; Pérez Marc, G.; Moreira, E.D.; Zerbini, C.; et al. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N. Engl. J. Med.* **2020**, *383*, 2603–2615. [CrossRef] [PubMed]
5. Lamb, Y.N. BNT162b2 mRNA COVID-19 Vaccine: First Approval. *Drugs* **2021**, *81*, 495–501. [CrossRef] [PubMed]
6. Maltezou, H.C.; Theodoridou, K.; Ledda, C.; Rapisarda, V.; Theodoridou, M. Vaccination of healthcare workers: Is mandatory vaccination needed? *Expert Rev. Vaccines* **2019**, *18*, 5–13. [CrossRef] [PubMed]

7. Pulliam, J.R.C.; van Schalkwyk, C.; Govender, N.; von Gottberg, A.; Cohen, C.; Groome, M.J.; Dushoff, J.; Mlisana, K.; Moultrie, H. Increased risk of SARS-CoV-2 reinfection associated with emergence of Omicron in South Africa. *Science* **2022**. Epub ahead of print. [CrossRef] [PubMed]
8. Alteri, C.; Cento, V.; Piralla, A.; Costabile, V.; Tallarita, M.; Colagrossi, L.; Renica, S.; Giardina, F.; Novazzi, F.; Gaiarsa, S.; et al. Genomic epidemiology of SARS-CoV-2 reveals multiple lineages and early spread of SARS-CoV-2 infections in Lom-bardy, Italy. *Nat. Commun.* **2021**, *12*, 434. [CrossRef] [PubMed]
9. Reed, L.J.; Muench, H. A simple method of estimating fifty percent endpoints. *Am. J. Hygiene* **1938**, *27*, 493–497.
10. Lineage List. Available online: https://cov-lineages.org/lineage_list.html (accessed on 18 February 2022).
11. World Health Organization. Tracking SARS-CoV-2 Variants. Available online: https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/ (accessed on 18 February 2022).
12. European Centre for Disease Prevention and Control. SARS-CoV-2 Variants of Concern as of 10 February 2022. Available online: https://www.ecdc.europa.eu/en/covid-19/variants-concern (accessed on 18 February 2022).
13. Tao, K.; Tzou, P.L.; Nouhin, J.; Gupta, R.K.; de Oliveira, T.; Kosakovsky Pond, S.L.; Fera, D.; Shafer, R.W. The biological and clinical significance of emerging SARS-CoV-2 variants. *Nat. Rev. Genet.* **2021**, *22*, 757–773. [CrossRef] [PubMed]
14. Dejnirattisai, W.; Huo, J.; Zhou, D.; Zahradník, J.; Supasa, P.; Liu, C.; Duyvesteyn, H.M.E.; Ginn, H.M.; Mentzer, A.J.; Tuekprakhon, A.; et al. SARS-CoV-2 Omicron-B.1.1.529 leads to widespread escape from neutralizing antibody responses. *Cell* **2022**, *185*, 467–484.e15. [CrossRef] [PubMed]
15. Garcia-Beltran, W.F.; St Denis, K.J.; Hoelzemer, A.; Lam, E.C.; Nitido, A.D.; Sheehan, M.L.; Berrios, C.; Ofoman, O.; Chang, C.C.; Hauser, B.M.; et al. mRNA-based COVID-19 vaccine boosters induce neutralizing immunity against SARS-CoV-2 Omicron variant. *Cell* **2022**, *185*, 457–466.e4. [CrossRef] [PubMed]
16. Suzuki, R.; Yamasoba, D.; Kimura, I.; Wang, L.; Kishimoto, M.; Ito, J.; Morioka, Y.; Nao, N.; Nasser, H.; Uriu, K.; et al. Attenuated fusogenicity and pathogenicity of SARS-CoV-2 Omicron variant. *Nature* **2022**, *603*, 700–705. [CrossRef] [PubMed]
17. Cassaniti, I.; Bergami, F.; Percivalle, E.; Gabanti, E.; Sammartino, J.C.; Ferrari, A.; Adzasehoun, K.M.G.; Zavaglio, F.; Zelini, P.; Comolli, G.; et al. Humoral and cell-mediated response against SARS-CoV-2 variants elicited by mRNA vaccine BNT162b2 in healthcare workers: A longitudinal observational study. *Clin. Microbiol. Infect.* **2022**, *28*, 301.e1–301.e8. [CrossRef] [PubMed]
18. Marcotte, H.; Piralla, A.; Zuo, F.; Du, L.; Cassaniti, I.; Wan, H.; Kumagai-Braesh, M.; Andréll, J.; Percivalle, E.; Sammartino, J.C.; et al. Immunity to SARS-CoV-2 up to 15 months after infection. *iScience* **2022**, *25*, 103743. [CrossRef] [PubMed]
19. Zeng, C.; Evans, J.P.; Qu, P.; Faraone, J.; Zheng, Y.M.; Carlin, C.; Bednash, J.S.; Zhou, T.; Lozanski, G.; Mallampalli, R.; et al. Neutralization and Stability of SARS-CoV-2 Omicron Variant. *bioRxiv* **2021**. Preprint.
20. Servellita, V.; Syed, A.M.; Morris, M.K.; Brazer, N.; Saldhi, P.; Garcia-Knight, M.; Sreekumar, B.; Khalid, M.M.; Ciling, A.; Chen, P.Y.; et al. Neutralizing immunity in vaccine breakthrough infections from the SARS-CoV-2 Omicron and Delta variants. *Cell* **2022**, *185*, 1539–1548. [CrossRef]
21. Tubiana, J.; Xiang, Y.; Fan, L.; Wolfson, H.J.; Chen, K.; Schneidman-Duhovny, D.; Shi, Y. Reduced antigenicity of Omicron lowers host serologic response. *bioRxiv* **2022**. Preprint.

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Detection of SARS-CoV-2 in Cancellous Bone of Patients with COVID-19 Disease Undergoing Orthopedic Surgery: Laboratory Findings and Clinical Applications

Edoardo Guazzoni [1,*], Alberto Castelli [1], Alberto Polizzi [1], Giacomo Galanzino [1], Antonio Piralla [2], Federica Giardina [2], Fausto Baldanti [2,3], Eugenio Jannelli [4], Laura Caliogna [1], Gianluigi Pasta [1], Mario Mosconi [1] and Federico Alberto Grassi [1]

[1] Department of Orthopaedics and Traumatology, IRCCS Fondazione Policlinico San Matteo, University of Pavia, 27100 Pavia, Italy
[2] Microbiology and Virology Department, IRCCS Fondazione Policlinico San Matteo, 27100 Pavia, Italy
[3] Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, 27100 Pavia, Italy
[4] Multidisciplinary Department of Medico-Surgical and Dentistry Specialties, Luigi Vanvitelli, University of Campania, 80138 Naples, Italy
* Correspondence: e.guazzoni@gmail.com

Abstract: An emerging issue for orthopedic surgeons is how to manage patients with active or previous COVID-19 disease, avoiding any major risks for the surgeons and the O.R. personnel. This monocentric prospective observational study aims to assess the prevalence of SARS-CoV-2 viral RT-PCR RNA in cancellous bone samples in patients with active or previous COVID-19 disease. We collected data about 30 consecutive patients from our institution from January 2021 to March 2021 with active or previous COVID-19 disease. The presence of SARS-CoV-2 in the samples was determined using two different PCR-based assays. Eighteen of the thirty patients included in the study had a positive nasopharyngeal swab at the time of surgery. Twelve patients had a negative nasopharyngeal swab with a mean days since negativization of 138 ± 104 days, ranging from 23 to 331 days. Mean days of positivity to the nasal swab were 17 ± 17. Twenty-nine out of thirty (96.7%) samples were negative for the presence of SARS-CoV-2 RNA. In one sample, low SARS-CoV-2 load (Cycle threshold (Ct) 36.6.) was detected but not confirmed using an additional confirmatory assay. The conducted study demonstrates the absence of the viral genome within the analyzed cancellous bone. We think that the use of personal protection equipment (PPE) to only protect from aerosol produced during surgery, both in active and recovered patients, is not strictly necessary. We think that the use of PPE should not be employed by surgeons and the O.R. personnel to protect themselves from aerosols produced from the respiratory tract. Moreover, we think that our results could represent a valid basis for further studies related to the possibility of bone donation in patients that suffered and recovered from COVID-19.

Keywords: COVID-19; bone; orthopedic surgery; operative room; risk of infection; tissue bank

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1. Introduction

On 11 March 2020, the World Health Organization (WHO) declared the SARS-CoV-2 disease as a global pandemic. Little is known about how SARS-CoV-2 infection can negatively affect the musculoskeletal system [1]. Otherwise, recent studies have shown that patients with moderate and severe coronavirus disease (COVID-19) have a variable involvement of the musculoskeletal system [2].

SARS-CoV-2 belongs to the coronavirus family and represents a new viral strain never identified in humans before. This virus is transmitted by air through the emission of droplets. Infection is favored by close contact among people, particularly in closed environments such as hospitals; therefore, nosocomial infections from SARS-CoV-2 are

widely reported in the literature [3,4]. SARS-CoV-2 is thought to predominantly infect type-II pneumocytes that line the respiratory epithelium, which express ACE2 and TMPRSS2. Disser et al. have shown that in the musculoskeletal system many cells express TMPRSS2 (pericytes, muscle stem cells, macrophages, adaptive immune cells (B, T, or natural killer cells), and myonuclei (muscle fibers), though only pericytes and smooth muscle cells express ACE2 [2].

However, it is important to emphasize that air transport may not be the only way of transmission. In particular, contagion by direct or indirect contact with mucous membranes of the eyes, mouth, and nose has been described [5]. SARS-CoV-2 is responsible for respiratory and flu-like symptoms. In more severe cases, the infection may cause pneumonia, acute respiratory distress syndrome, and onset of acidosis with kidney damage. Death may occur due to the progression of these conditions. Furthermore, recent studies have shown an increased thrombotic risk in patients with COVID-19 disease [6].

The COVID-19 outbreak resulted in a severe reduction in operating capacity all around the world and cessation of routine elective surgery to reduce the pressure on the intensive care unit (ICU) [3,5].

Due to the COVID-19 pandemic, resources have been allocated to ICUs and COVID-19 wards and many "non-urgent" orthopedic surgeries have been postponed or canceled to preserve medical resources. The "Non urgent orthopaedic surgery" was defined as planned non-trauma-related surgery [7–9].

An emerging issue for orthopedic surgeons is how to manage patients with active COVID-19 disease or patients who have recovered from it and need urgent trauma surgery for fracture management. Another compelling issue is the return towards full trauma operating capacity and elective orthopedic services, avoiding any major risks for the patients and the healthcare worker team, once the peak of COVID-19 pandemic begins to fall [6].

This topic will be a major issue for any orthopedic surgeons and health workers who have to perform surgical procedures on patients with active COVID-19 or recovered patients.

Does any long persistence of SARS-CoV-2 occur in cancellous bone? Should all the operative room (O.R.) team treating healed patients wear protection devices as is done for those infected by COVID-19? How long should we wait before treating a healed patient without preventive measures concerning to O.R. organization and personal protective equipment (PPE)?

The presence or absence of the virus in cancellous bone could help us answer questions about the management of bone donation in COVID-19-recovered patients.

All these questions need to be answered and this paper aims to assess the prevalence of SARS-CoV-2 RNA in cancellous bone samples in patients hospitalized with active or previous COVID-19 disease.

2. Materials and Methods

2.1. Study Aims

2.1.1. Primary Endpoint

This monocentric prospective observational study aims to assess the prevalence of SARS-CoV-2 RNA in cancellous bone samples in patients with active or previous COVID-19 disease, who have been hospitalized in our hospital Policlinico San Matteo di Pavia (Italy) and have undergone orthopedic surgery. These findings will improve our knowledge on infection from SARS-CoV-2 and its tropism for human tissues.

2.1.2. Secondary Endpoints

We intend to assess the presence level of SARS-CoV-2 RNA in cancellous bone and its association with: (i) disease status (healed or active); (ii) the time since healing in healed COVID-19 patients; and (iii) the viral load and the time since recovery in recovered COVID-19 patients.

2.2. Study Design

This is a cross-sectional observational study with both retrospective and prospective collection of data from stored biological samples and clinical charts.

We collected data about 30 consecutive patients from our institution from January 2021 to March 2021. These patients were either affected by acute COVID-19 disease or achieved recovery (negative nasal swab) and required urgent orthopedic surgery. We enrolled them in this clinical trial after informed consent was acquired.

Inclusion criteria were as follows: (i) patients who underwent urgent orthopedic surgery for fractures or other musculoskeletal injury requiring surgery; (ii) patients affected by acute COVID-19 disease or patients who achieved healing (negative nasal swab); (iii) informed consent signed.

Exclusion criteria were as follows: (i) patients younger than 18 years old; (ii) inability to understand the information about the study and to provide informed consent; (iii) incomplete information/data about the previous COVID-19 disease.

Patients' demographics and surgical data were collected from the databases which included: age, sex, medical history, BMI, date of admission, date of surgery, procedure and injury type, and COVID-19-related symptoms. The American society of anesthesiologists classification (ASA) score for preoperative risk was recorded on the theatre database and used to assign physical status.

COVID-19 infection was diagnosed in respiratory samples by using two different PCR-based assays in the preoperative period. Specific, real-time reverse transcriptase-polymerase chain reaction (RT-PCR) targeting RNA-dependent RNA polymerase (RdRp) and E genes were used to detect the presence of SARS-CoV-2, according to WHO guidelines and Corman et al. protocols [10,11].

Spongy bones from SARS-CoV-2-positive patients were collected in a 3 mL universal transport medium (UTM™, Copan Italia, Brescia, Italy) and stored at −80 °C until analyzed. Extraction of SARS-CoV-2 RNA was performed on inactivated UTM using the Quick-Viral RNA kit (Zymo Research, Irvine, CA, USA) per the manufacturer's protocol. Viral RNA was eluted with 50 µL of DNase/RNase-free water. The presence of SARS-CoV-2 in the samples was determined using the same assays used for diagnosis [10,11]. A confirmatory assay Cepheid GeneXpert® Xpert® SARS-CoV-2 assay (Cepheid, Sunnyvale, CA, USA) was used to prove positivity obtained previously.

2.3. Statistical Aspects

All analyses will be used using Stata 16 (StataCorp, College Station, TX, USA). All tests will be 2-sided. A p-value < 0.05 will be statistically significant.

2.3.1. Sample Size

The sample size calculation is based on the desired precision of the prevalence. We plan to be able to enroll about 40 patients in the next 3 months. With this sample size, we will obtain a precision of the estimate of the prevalence at most of 16% (calculated as half the 95% confidence interval (95% CI) in the less-favorable scenario from a mathematical point of view, in the absence of any prior information, of a prevalence of 50%).

2.3.2. Descriptive Statistics

Continuous data will be described with the mean and standard deviation or the median and quartiles, depending on the distribution; categorical variables will be described as counts and percent.

2.3.3. Analysis of the Primary Endpoint

The prevalence will be computed as the ratio of the positive patients to the total enrolled patients, together with its 95% binomial exact confidence interval.

2.3.4. Analysis of the Secondary Endpoints

1. The prevalence for each type of sample will be computed as described above. The comparison will be performed with Fisher's exact test; the mean difference and 95% CI will be computed.
2. The SARS-CoV-2 load expressed as cycle threshold will be compared between groups with the Mann–Whitney U test.
3. The association of SARS-CoV-2 load and time since healing will be assessed with the Spearman R and its 95% CI.

3. Results

Our population consists of: 30 patients, 19 females and 11 males; the mean age was 77 ± 15 years old with a range from 30 to 93 years old; the mean BMI was 25 ± 5.

We divided the orthopedics diagnosis into four main groups: the first one was femoral fracture, the second one was tibial fracture, the third one was humeral fracture and the last one was all the other. Half of the patients (15) were diagnosed with femoral fracture (group 1), three (3) were diagnosed with tibia fracture (group 2), two (2) were diagnosed with humeral fracture (group 3) and ten (10) with other diagnosis (group 4) (Table 1).

Table 1. Diagnosis of fracture.

Diagnosis	N° of Patients (30)
Femoral fracture (group 1)	15
Tibia fracture (group 2)	3
Humeral fracture (group 3)	2
Other diagnosis (group 4)	10

We divided patients into three main groups based on different types of surgical operation. The first group included patients undergoing open reduction and internal fixation (ORIF) or close reduction and internal fixation (CRIF). The second group included patients who underwent hip hemiarthroplasty surgery and the last group involved patients undergoing all the other invasive surgery (Table 2). Sixteen (16) patients underwent ORIF or CRIF surgery, six (6) underwent hip hemiarthroplasty, and the remaining eight (8) patients underwent other types of surgery.

Table 2. Type of surgical operation.

Surgical Operation	N° of Patients (30)
ORIF or CRIF	16
Hip hemiarthroplasty	6
All the other	8

Fourteen patients had an ASA score of 3, thirteen had an ASA score of 2, and the remaining three had an ASA score of 1 (Table 3).

Table 3. ASA risk score.

ASA Score	N° of Patients (30)
ASA 1	3
ASA 2	13
ASA 3	14

Mean days of hospitalization was 6.7 ± 10 ranging from 0 to 39. Eighteen of the thirty patients included in the study had a positive nasopharyngeal swab at the time of surgery. Twelve patients had a negative nasopharyngeal swab at the time of surgery with a mean

days since negativization of 138 ± 104 days ranging from 23 to 331 days. Mean days of positivity to the nasal swab were 17 ± 17. As we checked COVID-19-positive patients every 3 days by means of nasal swab, all the patients considered positive in the O.R. had a positive nasal swab after less than 72 h before surgery. Half of the patients (15) had radiological signs of COVID-19 at the chest X-ray, executed right after the positive nasal swab, and nine (9) of them had bilateral signs (Table 4).

Table 4. Signs of COVID-19 infection in chest X-rays.

X-rays Signs of COVID-19 Infection	N° Patients (30)	Bilateral Signs
Yes	15	9
No	15	

We collected a number of COVID-19-related signs and symptoms, including fever, cough, fatigue, sore throat, dyspnea, spO_2, chest pain, nasal congestion, headache, dizziness, diarrhea, and abdominal pain. All the patients had one or more of these symptoms during their COVID-19 infection. None of them had COVID-19-specific therapy or were vaccinated.

Twenty-nine out of thirty (96.7%) samples were negative for the presence of SARS-CoV-2 RNA. In one sample, low SARS-CoV-2 load (Cycle threshold (Ct) 36.6) was detected but not confirmed using an additional confirmatory assay.

4. Discussion

The new coronavirus pandemic severely affected health care systems worldwide. All surgical specialties had to adapt to different innovations, and changes have been introduced with regard to surgical and outpatient activities.

The necessity of placing more attention and resources on the clinical management of COVID-19 patients has caused these changes. The introduction of some new features was necessary to limit the transmission of the virus and thus the infection in health care personnel [12]. In fact, many health care workers treating COVID-19 patients became infected and subsequently died.

It is known that the virus is present in the blood. The current attitude is to exclude blood donors in cases of COVID-19 positivity. Despite this, transmission of infection has never been reported from donor to recipient either in transfusion of blood products or cellular therapies [13].

According to Xia et al., the presence of the virus in tears and conjunctival secretions has only been demonstrated in SARS-CoV-2-infected patients with conjunctivitis, whereas in SARS-CoV-2 patients without conjunctivitis, the presence of the virus was not demonstrated on RT-PCR analysis. Therefore, it has been proved that tears and conjunctival secretions are not a route of virus transmission from patients SARS-CoV-2 who are not suffering from conjunctivitis [14].

Grassi et al. searched for viral RNA within synovial fluid and bone tissue samples taken post-mortem in patients who died of SARS-CoV-2 respiratory complications. Again, the results were negative [15]. To the best of our knowledge, this study is the first one searching for viral nucleic acid in vivo within bone tissue samples.

It is well-known how the virus is transmitted directly. Less known are the capabilities of indirect transmission.

During orthopedic surgery, many power instruments, such as bone saws, drills and reamers are used, and they are known to be producers of blood aerosols. The theory has been therefore postulated that virus transmission to health care workers may occur through these aerosols. Consequently, some authors have defined a series of preventive measures to be taken in case of COVID-19-positive patients undergoing orthopedic surgery [16]. For example, patients requiring skeletal traction, a hand drill can be used, as well as cutting bone with an osteotome, rather than an oscillating saw blade. This could help reduce

aerosol production. Another case is hip replacement surgery, where it is preferable to use spoons to prepare the femur, keep the femoral canal dry with gauze and use saline solution in a syringe, instead of pulse lavage to prevent splash of particles of blood [16].

Personal protection equipment (PPE), such as FFP2 masks, face shields and goggles, are commonly used during surgery of COVID-19-active patients to protect the surgeon. The same equipment is used during anesthesiologic procedures and for O.R. cleaning [17]. Orthopedic surgeons and O.R. personnel showed concern about the lack of comparative efficacy, scientific evidence, compliance, shortage of materials, and the side effects of PPE usage. It is known that PPE can negatively affect surgical performance due to the limitation of vision and communication, discomfort, and fatigue. For this reason, we think that PPE should be used only in cases of necessity [18].

The objective of our study was to understand whether or not there was viral RNA shedding with aerosol, posing a risk to health care providers during orthopedic surgical procedures on positive or previously infected patients.

After informed consent was signed, we obtained cortical spongiosa bone tissue samples from the patients during surgery for standard RT-PCR assay. Almost all samples were negative, and the only positivity was not reconfirmed with an additional assay.

Due to its specificity and sensitivity, RT-PCR is a simple, convenient, and efficient technique to search for viral nucleic acid in the samples. Moreover, it is the gold standard for diagnosis. On the other hand, Lin et al. identified in their review several false positives and negatives due to contamination and damage of samples [19].

These results are obtained either among COVID-19-positive patients during surgery or healed COVID-19 patients. We did not find any association between the disease status (healed or active) and the prevalence of positive RT-PCR. We did not find any association either between the time since healing and the prevalence of SARS-CoV-2 positivity or level of SARS-CoV-2 load. In any case, we did not detect the presence of the virus in the bone samples, indicating that it is not a common way of virus transmission.

Considering these findings, we think that the use of PPE to only protect from aerosols produced during surgery is not strictly necessary in both active and healed patients. This does not mean that the use of PPE should not be employed by surgeon and the O.R. personnel to protect themselves from aerosols produced from the respiratory tract.

Another challenge created by the COVID-19 pandemic is the management of the tissue bank, primarily due to the possibility of human tissue contamination or the risk of disease transmission following transplantation. Therefore, tissue banks all over the world established strict preventive measures, or in some cases stopped collecting samples. On the other hand, at the moment, no documented reports have proved any COVID-19 infection following tissue transplantation [20,21]. We think that our results could represent a valid basis for further studies related to the possibility of bone donation in patients who suffered and healed from COVID-19.

The main limitation of the study is the small size of the sample due to the availability of resources. In order to improve the study reliability, it should be implemented with further cases in future studies.

5. Conclusions

Our study demonstrates the absence of the viral genome within the analyzed cancellous bone. This leads us to assume that SARS-CoV-2 has no direct bone affinity. We think that the aerosol generated during orthopedic surgery, in patients with active COVID-19 or healed, does not represent a risk of infection for the surgeons and the O.R. personnel. Therefore, PPE should not be necessary for this part of the surgery only.

Despite this, normal activities in the operating room with COVID-19-active patients, such as intubation, moving the patient to the operating table, etc., can promote the dispersion of viral particles into the environment. Consequently, this may cause the infection of health care personnel. It is therefore imperative to take preventive measures in the operat-

ing room when performing surgery on a COVID-19-active patient, especially through the use of PPE, in order to prevent virus transmission.

The small number of patients is the main limitation of the study, and for this reason further studies are needed to better understand the exact natural history of the disease.

We think that our results could represent an important basis for further studies related to this topic.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of IRCCS Fondazione Policlinico San Matteo (protocol code 2020084310 and date of approval 23 September 2020) for studies involving humans.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Agrawal, S.; Goel, A.D.; Gupta, N. Emerging prophylaxis strategies against COVID-19. *Monaldi Arch. Chest Dis.* **2020**, *90*. [CrossRef] [PubMed]
2. Disser, N.P.; De Micheli, A.J.; Schonk, M.M.; Konnaris, M.A.; Piacentini, A.N.; Edon, D.L.; Toresdahl, B.G.; Rodeo, S.A.; Casey, E.K.; Mendias, C.L. Musculoskeletal Consequences of COVID-19. *J. Bone Jt. Surg. Am.* **2020**, *102*, 1197–1204. [CrossRef] [PubMed]
3. Peng, J.; Ren, N.; Wang, M.; Zhang, G. Practical experiences and suggestions for the 'eagle-eyed observer': A novel promising role for controlling nosocomial infection in the COVID-19 outbreak. *J. Hosp. Infect.* **2020**, *105*, 106–107. [CrossRef] [PubMed]
4. Jannelli, E.; Castelli, A.; Calderoni, F.; Annunziata, S.; Maccario, G.; Ivone, A.; Pasta, G.; Mosconi, M. Fractures in Patients With COVID-19 Infection: Early Prognosis and Management. A Case Series of 20 Patients in a Single Institution in Lombardy, Northern Italy. *J. Orthop. Trauma* **2020**, *34*, 10. [CrossRef] [PubMed]
5. Carlos, W.G.; Dela Cruz, C.S.; Cao, B.; Pasnick, S.; Jamil, S. Novel Wuhan (2019-nCoV) Coronavirus. *Am. J. Respir. Crit. Care Med.* **2020**, *201*, P7–P8. [CrossRef] [PubMed]
6. Tang, N.; Bai, H.; Chen, X.; Gong, J.; Li, D.; Sun, Z. Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. *J. Thromb. Haemost.* **2020**, *18*, 1094–1099. [CrossRef] [PubMed]
7. Ciatti, C.; Maniscalco, P.; Quattrini, F.; Gattoni, S.; Magro, A.; Capelli, P.; Banchini, F.; Fiazza, C.; Pavone, V.; Puma Pagliarello, C.; et al. The epidemiology of proximal femur fractures during COVID-19 emergency in Italy: A multicentric study. *Acta Biomed.* **2021**, *92*, e2021398. [CrossRef] [PubMed]
8. Maniscalco, P.; Quattrini, F.; Ciatti, C.; Gattoni, S.; Pagliarello, C.P.; Burgio, V.; Di Stefano, G.; Cauteruccio, M.; Giovanelli, M.; Magro, A.; et al. The Italian Covid-19 Phase 2 in Piacenza: Results of the first semester of 2020 and future prospective of new orthopedics surgical procedures. *Acta Biomed.* **2020**, *91*, e2020159. [CrossRef] [PubMed]
9. Maniscalco, P.; Ciatti, C.; Gattoni, S.; Pagliarello, C.P.; Moretti, G.; Cauteruccio, M.; Carpaneto, D.; Capelli, P.; Gurrieri, L.; Banchini, F.; et al. The impact of COVID-19 pandemic on the Emergency Room and Orthopedic Departments in Piacenza: A retrospective analysis. *Acta Biomed.* **2020**, *91*, e2020028. [CrossRef] [PubMed]
10. Corman, V.M.; Landt, O.; Kaiser, M.; Molenkamp, R.; Meijer, A.; Chu, D.K.W.; Bleicker, T.; Brünink, S.; Schneider, J.; Schmidt, M.L.; et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance* **2020**, *25*, 2000045. [CrossRef] [PubMed]
11. Corman, V.; Bleicker, T.; Brünink, S.; Drosten, C.; Land, O.; Koopmans, M.; Zambon, M. *Diagnostic Detection of 2019-nCoV by Real-Time RT-RCR*; Incl. Workflow Protocoll on the Last 3 Pages; Carité: Berlin, Germany, 2020; p. 13.
12. Pasta, G.; Polizzi, A.; Annunziata, S.; Klersy, C.; Fenech, L.; Farahani, M.R.D.; Ghiara, M.; Castelli, A.; Jannelli, E.; Grassi, F.A.; et al. Patients with Musculoskeletal Disorders Presenting to the Emergency Department: The COVID-19 Lesson. *Int. J. Environ. Res. Public Health* **2022**, *19*, 5891. [CrossRef] [PubMed]

13. Hernigou, P. Bone marrow in orthopaedics (part II): A three hundred and seventy million-year saga from the Devonian to the coronavirus disease 2019 pandemic—osteonecrosis; transplantation; "human chimera"; stem cells, bioreactors, and coronavirus disease. *Int. Orthop.* **2020**, *44*, 2787–2805. [CrossRef] [PubMed]
14. Xia, J.; Tong, J.; Liu, M.; Shen, Y.; Guo, D. Evaluation of coronavirus in tears and conjunctival secretions of patients with SARS-CoV-2 infection. *J. Med. Virol.* **2020**, *92*, 589–594. [CrossRef] [PubMed]
15. Grassi, M.; Giorgi, V.; Nebuloni, M.; Zerbi, P.; Gismondo, M.R.; Salaffi, F.; Sarzi-Puttini, P.; Rimoldi, S.G.; Manzotti, A. SARS-CoV-2 in the knee joint: A cadaver study. *Clin. Exp. Rheumatol.* **2022**, *40*, 608–612. [CrossRef] [PubMed]
16. Raghavan, R.; Middleton, P.R.; Mehdi, A. Minimising aerosol generation during orthopaedic surgical procedures—Current practice to protect theatre staff during Covid-19 pandemic. *J. Clin. Orthop. Trauma* **2020**, *11*, 506–507. [CrossRef] [PubMed]
17. Dexter, F.; Parra, M.C.; Brown, J.R.; Loftus, R.W. Perioperative COVID-19 Defense: An Evidence-Based Approach for Optimization of Infection Control and Operating Room Management. *Anesthesia Analg.* **2020**, *131*, 37–42. [CrossRef] [PubMed]
18. Karachalios, T.; Maasalu, K.; Felländer-Tsai, L. Personal protection equipment for orthopaedic and trauma surgery during the COVID-19 pandemic: The results of an EFORT survey initiative. *EFORT Open Rev.* **2022**, *7*, 122–128. [CrossRef] [PubMed]
19. Lin, C.; Ye, R.; Xia, Y. A meta-analysis to evaluate the effectiveness of real-time PCR for diagnosing novel coronavirus infections. *Genet. Mol. Res.* **2015**, *14*, 15634–15641. [CrossRef] [PubMed]
20. Uchida, K.; Mukai, M.; Miyagi, M.; Fukushima, K.; Uchiyama, K.; Nakayama, A.; Matsumoto, M.; Takahira, N.; Urabe, K.; Takaso, M.; et al. Management of regional bone bank during declaration of a state of emergency concerning the COVID-19 in Japan. *Cell Tissue Bank.* **2021**, *22*, 703–709. [CrossRef] [PubMed]
21. Paggiaro, A.O.; Conceição, R.O.; Bianchi, M.P.; Gemperli, R. COVID-19 and risk management in a tissue bank. *Clinics* **2020**, *75*, e2132. [CrossRef] [PubMed]

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Assessing the Efficacy of Early Therapies against SARS-CoV-2 in Hematological Patients: A Real-Life Study from a COVID-19 Referral Centre in Northern Italy

Marta Colaneri [1], Teresa Chiara Pieri [1], Silvia Roda [1], Alessandra Ricciardi [1], Manuel Gotti [2], Jacqueline Ferrari [2], Luca Arcaini [2,3], Sara Rattotti [2], Antonio Piralla [4], Federica Giardina [4], Guglielmo Ferrari [4], Paolo Sacchi [1], Valentina Zuccaro [1], Fausto Baldanti [5,4] and Raffaele Bruno [5,1,*]

[1] Division of Infectious Diseases, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy
[2] Division of Hematology, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy
[3] Department of Molecular Medicine, University of Pavia, 27100 Pavia, Italy
[4] Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy
[5] Department of Medical, Surgical, Diagnostic and Pediatric Science, University of Pavia, 27100 Pavia, Italy
* Correspondence: raffaele.bruno@unipv.it

Abstract: Early therapies to prevent severe COVID-19 have an unclear impact on patients with hematological malignancies. The aim of this study was to assess their efficacy in this group of high-risk patients with COVID-19 in preventing hospitalizations and reducing the SARS-CoV-2 shedding. This was a single-center, retrospective, observational study conducted in the Fondazione IRCSS Policlinico San Matteo of Pavia, Northern Italy. We extracted the data of patients with hematologic malignancies and COVID-19 who received and did not receive early COVID-19 treatment between 23 December 2021, and May 2022. We used a Cox proportional hazard model to assess whether receiving any early treatment was associated with lower rates of hospitalization and reduced viral shedding. Data from 88 patients with hematologic malignancies were extracted. Among the patients, 55 (62%) received any early treatment, whereas 33 (38%) did not. Receiving any early therapy did not significantly reduce the hospitalization rate in patients with hematologic malignancies (HR 0.51; SE 0.63; *p*-value = 0.28), except in the vaccinated non-responders subgroup of patients with negative anti SARS-CoV-2 antibodies at the time of infection, who benefited from early therapies against SARS-CoV-2 (HR 0.07; SE 1.04; *p*-value = 0.001). Moreover, no difference on viral load decay was observed. In our cohort of patients with hematologic malignancies infected with SARS-CoV-2, early treatment were not effective in reducing the hospitalization rate due to COVID-19, neither in reducing its viral shedding.

Keywords: COVID-19; early remdesivir; molnupiravir; ritonavir-boosted nirmatrelvir; sotrovimab; hematological patients; hospitalizations rate; prolonged viral shedding

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1. Introduction

Patients with hematological malignancies or who underwent hematopoietic stem-cell transplantation (HSCT) are considered at high risk of developing severe COVID-19 [1]. COVID-19-related mortality in patients with hematologic malignancies is higher than in the general population, being approximately 30% in several studies performed both in the pre- and in the post-vaccine era [1,2].

These patients are at higher risk of severe COVID-19, due to the long-lasting immunodeficiency resulting from malignancy itself, anticancer treatments, or HSCT [3,4]. Moreover, there is evidence of an impaired humoral immune or cellular response after anti-SARS-CoV-2 vaccination among patients with hematologic malignancies and HSCT patients [5], and a lower post-vaccination immunogenicity [6].

Furthermore, patients with hematologic malignancies and HSCT patients may have a prolonged viral shedding [7] compared to the roughly 10-days average duration usually reported for the general population [8]. Hence, plenty of studies have demonstrated a prolonged shedding duration of active virus, up to months after symptom onset [9–12].

Currently, there are valid options for symptomatic outpatients with COVID-19 that are at a high risk for progression to severe disease. Among those, the oral combination of nirmatrelvir/ritonavir is the recommended option [13], since it has been shown to reduce the risk for hospitalization by 89% [14]. Remdesivir has a similar efficacy and is an alternative option, but its use is impractical in some outpatient settings since it requires parenteral administration over 3 days [15]. A third option is anti-SARS-CoV-2 monoclonal antibodies which have variable activity against the different SARS-CoV-2 variants. Among them, Sotrovimab was the only one that retained some activity against BA.1/BA.1.1 sub-lineages of the Omicron variant [16], but is currently no longer effective against BA.2 [17]. Molnupiravir is another possible option. However, since its lower efficacy, which was roughly 30% in reducing COVID-19-related hospitalization by 28 days [18], the COVID-19 Treatment Guidelines Panel recommended its use only when the other options are contraindicated [19]. Together with COVID-19 related hospitalization and mortality rate reduction, these drugs might also lead to a significant reduction in viral load [20].

Although clinical trials generally exclude patients with hematologic malignancies, the European Conference on Infections in Leukemia recently recommended treating patients with hematologic malignancies with mild COVID-19 with these drugs [21].

The aim of this study was to assess the impact of early therapies in reducing the hospitalization rate and the 28-days mortality due to COVID-19 in patients with hematologic malignancies in our Hospital Fondazione IRCCS Policlinico San Matteo in Pavia, Northern Italy. We also aimed to evaluate the time length of viral shedding in patients with hematologic malignancies and HSCT patients who were and were not treated with early therapies.

2. Materials and Methods

2.1. Study Design

This study was a retrospective, single-center analysis of patients with a confirmed diagnosis of COVID-19 referred to our hospital. The study was approved by our Institutional Review Board (n.prot.0031226/22).

The medical records of all the adult patients with hematologic malignancies who tested positive for real-time reverse-transcription polymerase chain reaction (RT-PCR) from nasal swabs for SARS-CoV-2 and were consequently evaluated for early treatment in our clinic, were anonymized and abstracted on standardized data collection forms. In particular, patients suffering with myeloma, Hodgkin and non-Hodgkin lymphoma, chronic and acute leukemia, paroxysmal nocturnal hemoglobinuria, amyloidosis, and myelodysplastic syndrome/myeloproliferative neoplasms were included.

Only patients with mild to moderate COVID19 diseases were considered eligible for a therapy. Specifically, they did not present with any of the following features: oxygen saturation of <94% on room air; respiratory rate of >30 breaths/min; PaO_2/FiO_2 < 300 mmHg; and lung infiltrates > 50%.

We only extracted the data of patients evaluated between 23 December 2021 and 30 of April 2022, when the vast majority of COVID-19 cases were due to the Omicron variant.

The following exclusion criteria were applied: patients hospitalized for COVID-19 and/or requiring oxygen therapy for COVID-19 at the first clinical evaluation; asymptomatic patients.

2.2. Study Setting

One of the Infectious Diseases outpatients' clinics of our hospital was allocated to the early treatment of COVID-19 outpatients from 23 December 2021. In this clinic, an infectious disease (ID) specialist was in charge of receiving daily e-mails from general practitioners

and specialists of other units who promptly notified the cases of SARS-CoV-2 positive high-risk patients, both outpatients and patients admitted for reasons other than COVID-19.

The appropriate therapy for each notified patient was chosen by the ID specialist, according to both the inclusion and exclusion criteria and the availability of each drug's pilot sheet. After signing an informed consent form, the patient was then examined and informed about the selected therapy.

Among these, ritonavir-boosted nirmatrelvir was selected as the first oral medication, but it was available only from 20 February 2022. If an intravenous (IV) drug was selected, remdesivir was administered as an IV infusion over 30 min at the recommended dosage of 200 mg for the loading dose on day 1, followed by a 100 mg maintenance dose administered on days 2 and 3. As regard with sotrovimab, it was given as a single 500 mg IV infusion, but it was used from the arrival of the Omicron BA.2 subvariant, at the end of April 2022. Patients were monitored during each infusion and observed for at least one hour after for signs and symptoms of hypersensitivity.

As a last resort, molnupiravir was administered to the individuals who were not eligible to any other drug.

2.3. Patients' Characteristics

The demographic data included sex and age. Clinical data included symptoms at presentation, comorbidities (history of cancer, heart disease, hypertension, diabetes, chronic kidney disease, lung disease, and obesity), vaccination status, and anti-spike IgG antibodies for SARS-CoV-2 (results greater than or equal to the cut-off value 50.0 AU/mL were reported as positive). Type of hematological disease; ongoing chemotherapy; type and time of HSCT if performed.

The Italian Agency of Drugs (AIFA)s guidelines for excluding patients from one treatment rather than another was strictly followed.

2.4. SARS-CoV-2 RNA Detection

Total RNA was extracted on the MGISP-960 automated workstation using the MGI Easy Magnetic Beads Virus DNA/RNA Extraction Kit (MGI Technologies, Shenzhen, China). Detection of SARS-CoV-2 RNA was performed using the SARS-CoV-2 variants ELITe MGB® kit (ELITechGroup, Puteaux, France; cat. no. RTS170ING) on the CFX96 Touch Real-time PCR detection system (BioRad, Mississauga, ON, Canada).

2.5. Outcomes

The primary outcome was to evaluate the impact of early therapies, such as remdesivir, molnupiravir, ritonavir-boosted nirmatrelvir, and sotrovimab, in preventing the hospitalization due to COVID-19 of patients with hematologic malignancies infected by SARS-CoV-2 by day 28.

In particular, we considered the progression of COVID-19 as the presence of clinical manifestations which are consistent with the categories of moderate, severe, and critical illness defined by the National Institute of Health Guidelines [22].

We also evaluated admission to the intensive care unit (ICU) of our hospital and the intra-hospital mortality by day 28.

The secondary outcomes were to evaluate the effect of the single drug in preventing the 28 days hospitalization due to COVID-19, to evaluate the length of SARS-CoV-2 viral shedding of patients receiving early therapies versus those who did not receive them, and finally, to evaluate the impact of the early therapies in patients with hematologic malignancies with negative SARS-CoV-2 antibodies at the time of evaluation.

2.6. Statistical Analysis

Data for continuous variables were presented as means and standard deviations.

Categorical variables were presented as frequencies and percentages. Comparisons between the treated and non-treated groups of patients with hematologic malignancies

were performed using chi-square tests for categorical variables and Mann–Whitney tests for non-normal continuous data.

The log-rank test was used to estimate the difference between the 28-day Kaplan–Meier hospitalization curves of patients who received and did not receive early therapies. The duration of viral shedding was calculated by using the Kaplan–Meier curves and tested by the log-rank test for survival curve comparison. When viral clearance could not be determined, the duration was censored with the last positive sample. A Cox proportional hazard model was performed controlling for sex, age, number of underlying comorbidities, and number of anti-SARS-CoV-2 vaccinations performed. A multivariable Cox proportional-hazard regression model was also performed to evaluate the impact of each drug on the hospitalization rate compared to no drugs.

Finally, a multivariable Cox proportional-hazard regression model was performed to evaluate the impact of early therapies in patients with hematologic malignancies with negative anti SARS-CoV-2 antibodies at the time of evaluation.

The results were reported as hazard ratios (HRs) and 95% confidence intervals (CIs). Statistical analyses were conducted using R (version 4.1.2).

3. Results

Data from 88 patients were extracted. A total of 55 (62%) received early therapy and 33 (38%) did not. Demographic, clinical, and treatment characteristics are presented in Table 1.

Table 1. Demographic and clinical characteristics.

		All Patients (88)	Treated (55)	Non-Treated (33)	*p*-Value
Sex, *n* (%)	Female	47 (53)	27 (31)	20 (23)	
	Male	41 (47)	28 (32)	13 (15)	0.41
Age, Median (IQR)		63 (49.0, 71.2)	62 (52.5, 70.0)	63 (48, 72)	0.89
Vaccination doses, Mean (sd)		2.7 (0.7)	2.6 (0.8)	2.7 (0.5)	0.69
Days from last vaccination, Mean (sd)		124.1 (65)	128.1(64.3)	116.9 (67.1)	0.51
Remdesivir, *n* (%)		-	15 (27)		-
Ritonavir-boosted Nirmatrelvir, *n* (%)		-	10 (18)		-
Sotrovimab, *n* (%)		-	15 (27)		-
Molnupiravir, *n* (%)		-	15 (27)		
Bone marrow transplantation, *n* (%)		24 (27)	18 (75)	6 (25)	0.22
Days from bone marrow transplantation, Mean (sd)		1307.4 (1793.8)	1390.3 (1981.8)	1009 (929.2)	0.68
Type of Bone marrow transplantation, *n* (%)	Autologous	20 (22)	14 (25)	6 (18)	
	Allogenic	4 (4)	4 (7)	0 (0)	0.28
Hematological disease, *n* (%)	Myeloma	26 (29)	17 (31)	9 (27)	
	Hodgkin Lymphoma	8 (9)	3 (5)	5 (15)	
	High-Grade Non-Hodgkin Lymphoma	12 (14)	10 (18)	2 (6)	
	Acute Myeloid Leukemia	4 (4)	3 (5)	1 (3)	

Table 1. *Cont.*

		All Patients (88)	Treated (55)	Non-Treated (33)	*p*-Value
	Low-Grade Non-Hodgkin Lymphoma	16 (18)	7 (13)	9 (27)	
	Chronic Lymphocytic Leukemia	4 (4)	3 (5)	1 (3)	
	Chronic Myeloid Leukemia	8 (9)	7 (13)	1 (3)	
	MDS/MPN	3 (3)	2 (4)	1 (3)	
	Paroxysmal Nocturnal Hemoglobinuria	1 (1)	0 (0)	1 (3)	
	Acute Lymphocytic Leukemia	4 (4)	2 (4)	2 (6)	
	Amyloidosis AL	1 (1)	1 (2)	0 (0)	0.25
Immunosuppressive therapies, *n* (%)	Rituximab	20 (23)	13 (24)	7 (21)	1.00
	Obinutuzumab	5 (6)	3 (6)	2 (6)	1.00
	Methotrexate	10 (11)	5 (9)	5 (15)	0.60
	CHOP	15 (17)	12 (22)	3 (9)	0.21
	CHOEP	1 (1)	0 (0)	1 (3)	0.79
	ABVD	4 (4)	1 (2)	3 (9)	0.29
	Poli chemotherapy (VCR, Ara-C, Ida, EDX, Cisplatin, Bendamustine)	21 (24)	13 (4)	8 (24)	1.00
	VD (Bortezomib-Dexamethasone)	12 (14)	8 (14)	4 (12)	1.00
	Eculizumab	1 (1)	0 (0)	1 (3)	0.80
	Tyrosine kinase inhibitors (TKIs)	13 (15)	10 (18)	3 (9)	0.37
	Others (Daratumumab, Isatuximab, IMIDs, Brentuximab, Ab anti-PD1-PDL1)	30 (34)	17 (31)	13 (39)	0.60
Days between last therapy and examination, mean (sd)		3205.2 (11,379.2)	2902.1 (10,844.7)	3799 (12,582.5)	0.75
Positive anti SARS-CoV-2 antibodies, *n* (%)		44 (50)	20 (36)	24 (73)	<0.01
Viral decay (sd)		26.3 (21.6)	25.4 (18.0)	27.7 (24)	0.63
Comorbidities	NPL, *n* (%)	59 (69)	32 (63)	25 (78)	0.22
	CKD, *n* (%)	8 (10)	3 (7)	5 (15)	
	CVD, *n* (%)	14 (16)	8 (15)	6 (18)	0.90
	HTN, *n* (%)	34 (39)	21 (39)	13 (39)	1.00
	DM, *n* (%)	10 (11)	5 (9)	5 (15)	0.62
	LD, *n* (%)	10 (11)	7 (13)	3 (9)	0.83
	HCV, *n* (%)	2 (3)	2 (4)	0 (0)	0.70
	Obesity, *n* (%)	1 (1)	0 (0)	1 (3)	0.80

Table 1. *Cont.*

		All Patients (88)	Treated (55)	Non-Treated (33)	*p*-Value
	Smoke, *n* (%)	10 (13)	5 (12)	5 (16)	0.90
	Number of comorbidities, mean (sd)	1.5 (1.2)	1.4 (1.1)	1.7 (1.3)	0.24
Mortality, *n* (%)		2 (2)	0 (0)	2 (6)	0.27
Hospital admission, *n* (%)		12 (14)	6 (11)	6 (18)	0.52
ICU admission, *n* (%)		1 (1)	0 (0)	1 (3)	0.79
Stay at Home, *n* (%)		78 (87)	50 (91)	28 (85)	0.60
Symptoms, *n* (%)					
	Asymptomatic	10 (12)	1 (2)	9 (30)	<0.01
	Fever	39 (48)	30 (57)	9 (32)	0.06
	Cough	32 (39)	24 (45)	8 (29)	0.2
	Pharyngodinia	25 (31)	16 (30)	9 (32)	1.00
	Dyspnea	10 (13)	3 (6)	7 (25)	0.04
	Diarrhea	2 (2)	2 (4)	0 (0)	0.77
	Asthenia	15 (18)	10 (19)	5 (17)	1.00
Pneumonia		12 (14)	6 (11)	6 (21)	0.39
Oxygen therapy		11 (13)	5 (9)	6 (19)	0.33

Notes: MDS/MPN, Myelodysplastic syndrome/Myeloproliferative neoplasms; ABVD, Adriamycin/bleomycin/vinblastine/dacarbazine; VCR, vincristine; EDX, 4′-epidoxorubicin; IMIDs, immunomodulatory drugs; NPL, Neoplasia; CKD, Chronic Kidney Disease; CVD, Cardiovascular Disease; HTN, Hypertension; DM, Diabetes Mellitus; LD, lung disease; Obesity considered as Body Mass Index (BMI) > 30 kg/m^2; ICU, intensive care unit; Positive anti-SARS-CoV-2 antibodies was considered when IgG anti-trimeric SARS-CoV-2 spike protein were ≥50 AU/mL; HCV, presence of antibodies against HCV. Data are reported as absolute number and percentage and mean with standard deviation.

Most patients were vaccinated against SARS-CoV-2 (94%). However, among them, only 44 (50%) patients had positive IgG anti- SARS-CoV-2 spike protein.

Regarding the treatment, 55 (62%) patients received an early treatment for SARS-CoV-2. Fifteen (27%) were treated with remdesivir, 10 (18%) with ritonavir-boosted nirmatrelvir, 15 (27%) with sotrovimab, and 15 (27%) with molnupiravir.

Globally, the length of PCR positivity for SARS-CoV-2 on nasal swab had a mean of 26.3 (±21.6) days, 25.4 (±18.0) and 27.7 (±24.0) for the treated and untreated group, respectively. Among the treated patients, six (11%) developed COVID-19 related pneumonia, with five of them requiring oxygen therapy and hospitalization. None of the treated patients required ICU admission. Moreover, six untreated patients were hospitalized for COVID-19 related pneumonia. Among them, one was admitted to the ICU, while two died.

3.1. Impact of Early Therapies on the Outcomes

Regarding our primary outcome, treatment with any considered early therapy did not significantly reduce hospital admission by 28 days (Figure 1).

Similarly, after accounting for potential confounders, the multivariable Cox proportional-hazard regression model showed that an early treatment with any of the considered drugs did not significantly reduce the hospitalization rate (HR: 0.51; SE 0.63; $p = 0.28$) (Table 2).

Additionally, the multivariable Cox proportional-hazard regression model showed that none of the early treatments did significantly reduce the hospitalization at day 28 compared with no treatment (Table 3).

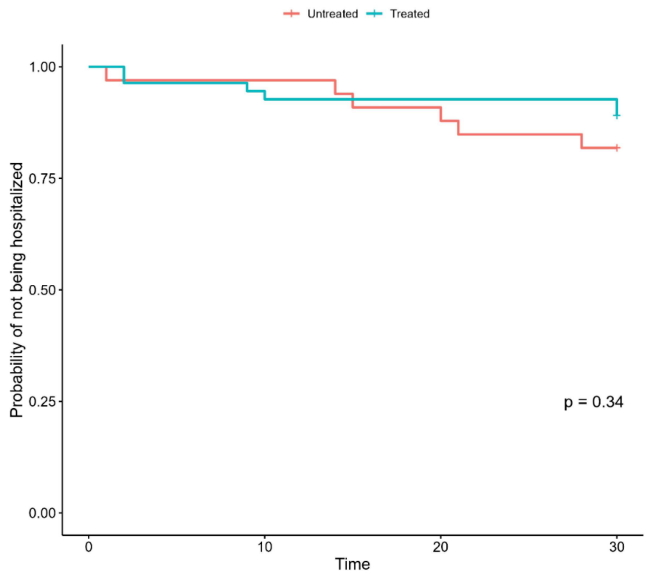

Figure 1. Kaplan–Meier curves of hospitalization in untreated and treated patients with hematologic malignancies and HSCT patients.

Table 2. Multivariate Cox regression for 28-day hospital admission.

Variable	HR	SE	*p*-Value
Treatment	0.51	0.63	0.28
Sex	0.29	0.68	0.07
Age	1.01	0.02	0.73
Number of vaccinations	1.42	0.61	0.56
Comorbidities	1.63	0.26	0.06

Table 3. Multivariate Cox regression for 28-day hospital admission considering the impact of each treatment.

Variable	HR	SE	*p*-Value
Paxlovid	0.51	1.10	0.55
Remdesivir	1.16	0.71	0.83
Molnupiravir	0.28	1.09	0.24
Sotrovimab	0.24	1.09	0.19
Sex	0.32	0.62	0.07
Age	1.03	1.41	1.41
Number of vaccinations	1.43	0.56	0.57

Finally, the multivariable Cox proportional-hazard regression model showed that patients with hematologic malignancies with negative anti SARS-CoV-2 antibodies at the time of infection were at a significantly increased risk of hospitalization if not treated in a timely fashion with early therapies.

Specifically, after accounting for sex, age, number of vaccinations, and comorbidities, being untreated was significantly associated with an increased risk of hospitalization among patients with hematologic malignancies with negative anti SARS-CoV-2 antibodies (Table 4) (Figure 2).

Table 4. Multivariate Cox regression for 28-day hospital admission of patients with hematologic malignancies with negative anti SARS-CoV-2 antibodies.

Variable	HR	SE	*p*-Value
Treatment	0.07	1.04	0.001
Sex	0.37	0.98	0.31
Age	1.00	0.04	0.91
Number of vaccinations	1.05	0.74	0.93
Comorbidities	1.63	0.35	0.16

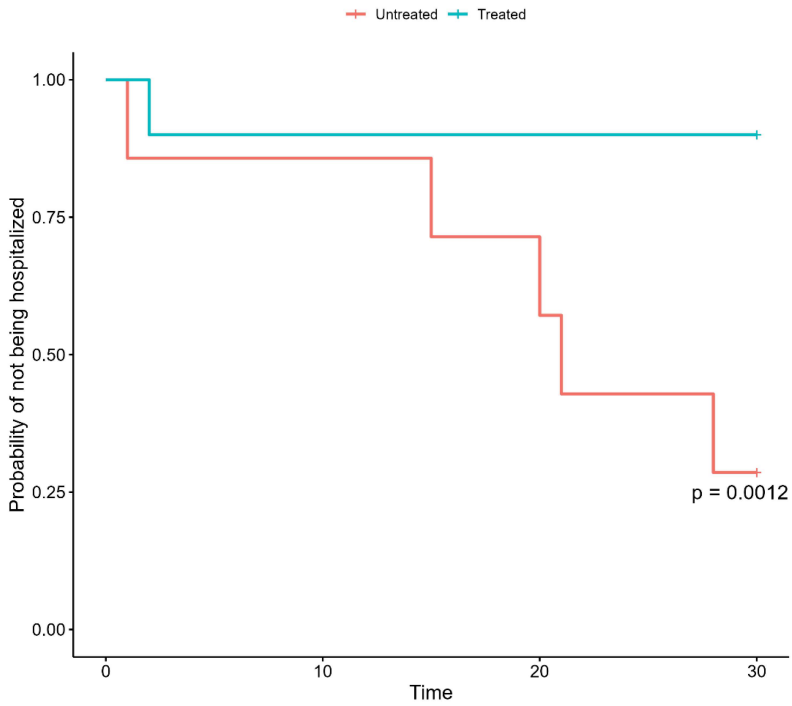

Figure 2. Kaplan–Meier curves of hospitalization in untreated and treated patients with hematologic malignancies and HSCT patients with negative anti-SARS-CoV-2 antibodies.

3.2. SARS-CoV-2 RNA Load Kinetics

In a subset of patients (49/79; 62.0%), the duration of viral load was available, and the median duration was 15 days (range 8–87 days) for untreated, 21 days (range 8–31 days) for Remdesivir, 17 days (6–46 days) for sotrovimab, and 17 days (8–27 days, log-rank test $p = 0.48$) for molnupiravir (Figure 3A). Only one patient treated with ritonavir-boosted nirmatrelvir had data on viral load duration (8 days censored). Among the untreated group, the more prolonged infection was observed in a patient with RNA detected at 87 days after first positivity, while in the treated patients' group, the more prolonged shedding was observed in one case treated with Sotrovimab with detectable RNA at 52 days after first positivity.

In addition, in a subset of patients (43/79; 54.4%) Ct values were available and used to calculate viral load decay normalized per day (Ct/day). No difference in viral load decay was observed between the groups of patients. However, the highest reduction in SARS-CoV-2 RNA was observed in untreated patients (median 1.27, range 0.50–3.25 Ct/day) as compared to Remdesivir (median 0.78, range 0.40–1.60 Ct/day),

sotrovimab (median 0.75, range 0.29–2.22 Ct/day) and Molnupiravir (median 1.00, range 0.61–1.88 Ct/day) (Figure 3B). Only one patient treated with ritonavir-boosted nirmatrelvir had data on viral load decay (2.13 Ct/day).

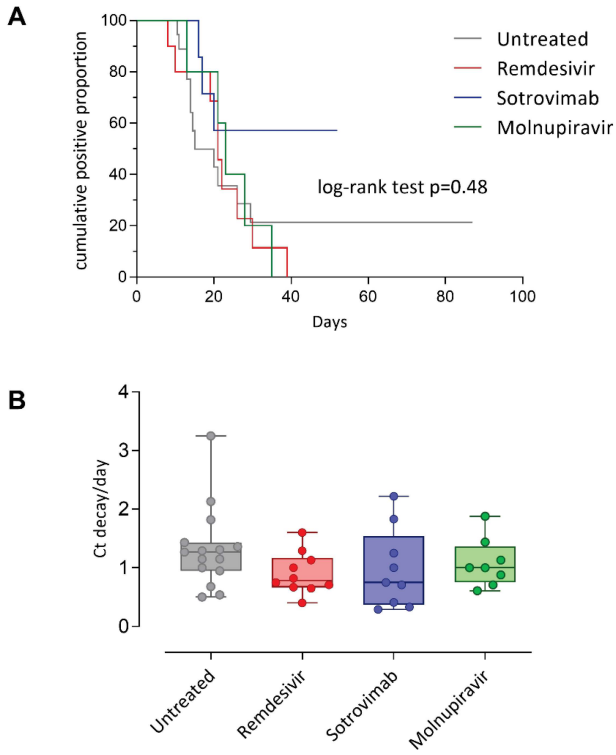

Figure 3. Kaplan–Meier curves of viral shedding duration in untreated and treated patients (**A**). SARS-CoV-2 RNA load clearance in different patients' categories (**B**).

4. Discussion

In the present study, we did not notice a significant impact of early anti-SARS-CoV-2 treatments on the COVID-19-related 28-day hospitalization rate and SARS-CoV-2 load decay in patients with hematological malignancy or HSCT. However, untreated patients with negative anti-SARS-CoV-2 antibodies had a significantly higher risk of being hospitalized than treated ones.

Patients with hematologic malignancies and HSCT might experience a relatively slow viral decay and, as a result, the duration of RT-PCR positivity in these patients was longer than that of other patients [7]. Based on previous studies, a beneficial impact of early therapies on hastening the SARS-CoV-2 viral decay was expected [14,23,24]. Interestingly, our data did not confirm this hypothesis. This result should be taken with caution since the absence of a significant effect could also be explained by a lack of statistical power due to the relatively small sample size Although a prolonged duration of RT-PCR positivity does not indicate higher severity of COVID-19 [24], the fact that the viral load in these patients is long-lasting has serious healthcare implications. In fact, RT-PCR positivity in these patients generally prevents the implementation of specific treatments for their underlying disease, and access to outpatients' care services.

In summary, the clinical and therapeutic management of hematologic malignancies and HSCT represent a major challenge for physicians. In this regard, and especially because of the constant surfacing of new SARS-CoV-2 variants of concern, we should reflect on the need of patients with hematologic malignancies or HSCT for updated vaccination

strategies, such as prompt additional vaccine doses, which might be an effective choice to enhance immunity response [25]. Even though it has been reported that the severity of the Omicron SARS-CoV-2 variant is attenuated [26,27], this is likely due to population immunity rather than to a characteristic of the virus. Therefore, despite the ongoing trend of gradually relaxing epidemic containment measures, these patients should be instructed to maintain infection control measures, such as aerosol and contact full isolation, social distancing, and wide use of masks and personal hygiene measures.

We believe that it is extremely valuable to perform real-life studies on these patients, because of their high risk of mortality and morbidity due to COVID-19 [28–30], and their low response to anti-SARS-CoV-2 vaccines [6] due to their specific illness, chemotherapy, and other immunosuppressive treatments. Our data confirm this unfortunate trend, as only slightly more than half of the subgroup of fully immunized patients with hematologic malignancies were serologically positive for IgG anti-SARS-CoV-2 spike protein. The fact that patients with hematologic malignancies who have failed to mount an adequate SARS-CoV-2 vaccine response encounter poor outcomes is well known [26], and our data support the relevance of providing a timely treatment to these patients using early therapies against COVID-19.

We have to mention some limitations of our study, such as its retrospective and monocentric nature, and the relatively small sample size. Moreover, due to the real-life experience, we did not exclude those patients treated with molnupiravir, which is less effective than the other treatments [18]. Finally, since our sample only includes patients who were infected by the Omicron variant, the generalization of our results to patient affected by other variants should be executed with caution. However, to the best of our knowledge, no previous data supporting the use of early drugs in patients with hematologic malignancies or HSCT are available. Therefore, we believe that this study fills this literature gap with real-life daily practice findings.

In conclusion, we believe that reporting these real-life data may still be the most appropriate approach to appreciate how to focus our full consideration of patients with hematologic malignancies and HSCT patients from different perspectives. However, more data are needed to understand the best way to manage the SARS-CoV-2 infection in this particularly fragile population.

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References

1. Pagano, L.; Salmanton-García, J.; Marchesi, F.; Busca, A.; Corradini, P.; Hoenigl, M.; Klimko, N.; Koehler, P.; Pagliuca, A.; Passamonti, F.; et al. COVID-19 infection in adult patients with hematological malignancies: A European Hematology Association Survey (EPICOVIDEHA). *J. Hematol. Oncol.* **2021**, *14*, 168. [CrossRef] [PubMed]
2. Vijenthira, A.; Gong, I.Y.; Fox, T.A.; Booth, S.; Cook, G.; Fattizzo, B.; Martín-Moro, F.; Razanamahery, J.; Riches, J.C.; Zwicker, J.; et al. Outcomes of patients with hematologic malignancies and COVID-19: A systematic review and meta-analysis of 3377 patients. *Blood* **2020**, *136*, 2881–2892. [CrossRef] [PubMed]
3. El-Sharkawi, D.; Iyengar, S. Haematological cancers and the risk of severe COVID-19: Exploration and critical evaluation of the evidence to date. *Br. J. Haematol.* **2020**, *190*, 336–345. [CrossRef] [PubMed]
4. García-Suárez, J.; de la Cruz, J.; De La Cruz, J.; Cedillo, Á.; Llamas, P.; Duarte, R.; Jiménez-Yuste, V.; Hernández-Rivas, J.Á.; Gil-Manso, R.; Kwon, M.; et al. Impact of hematologic malignancy and type of cancer therapy on COVID-19 severity and mortality: Lessons from a large population-based registry study. *J. Hematol. Oncol.* **2020**, *13*, 133. [CrossRef] [PubMed]
5. Mittelman, M.; Magen, O.; Barda, N.; Dagan, N.; Oster, H.S.; Leader, A.; Balicer, R. Effectiveness of the BNT162b2mRNA COVID-19 vaccine in patients with hematological neoplasms in a nationwide mass vaccination setting. *Blood* **2022**, *139*, 1439–1451. [CrossRef]
6. Rotshild, V.; Hirsh-Raccah, B.; Miskin, I.; Muszkat, M.; Matok, I. Comparing the clinical efficacy of COVID-19 vaccines: A systematic review and network meta-analysis. *Sci. Rep.* **2021**, *11*, 22777. Available online: https://www.nature.com/articles/s41598-021-02321-z (accessed on 11 July 2022). [CrossRef]
7. Garcia-Vidal, C.; Puerta-Alcalde, P.; Mateu, A.; Cuesta-Chasco, G.; Meira, F.; Lopera, C.; Monzo, P.; Santos-Bravo, M.; Duenas, G.; Chumbita, M.; et al. Prolonged viral replication in patients with hematologic malignancies hospitalized with COVID-19. *Haematologica* **2022**, *107*, 1731–1735. [CrossRef]
8. Tarabay, J.; Babiker, A.; Adelman, M.W.; Stittleburg, V.D.; Varkey, J.; Pouch, S.M.; Waggoner, J.; Piantadosi, A. 278. Immunocompromised Patients with Prolonged Viral Shedding of SARS-COV-2. *Open Forum Infect. Dis.* **2021**, *8* (Suppl. S1), S244–S245. [CrossRef]
9. Nakajima, Y.; Ogai, A.; Furukawa, K.; Arai, R.; Anan, R.; Nakano, Y.; Kurihara, Y.; Shimizu, H.; Misaki, T.; Okabe, N. Prolonged viral shedding of SARS-CoV-2 in an immunocompromised patient. *J. Infect. Chemother.* **2021**, *27*, 387–389. [CrossRef]
10. Nakamura, S.; Kanemasa, Y.; Atsuta, Y.; Fujiwara, S.; Tanaka, M.; Fukushima, K.; Kobayashi, T.; Shimoyama, T.; Omuro, Y.; Sekiya, N.; et al. Characteristics and outcomes of coronavirus disease 2019 (COVID-19) patients with cancer: A single-center retrospective observational study in Tokyo, Japan. *Int. J. Clin. Oncol.* **2020**, *26*, 485–493. [CrossRef]
11. Levi, G.; Rocchetti, C.; Magri, R.; Uccelli, S.; Bottone, D.; Quadri, F.; Novali, M.; Santin, A.D.; Bezzi, M. Hyperimmune plasma infusion in an immunocompromised COVID-19 patient previously treated for follicular lymphoma. *Monaldi Arch. Chest Dis.* **2021**, *91*, 1–10. [CrossRef] [PubMed]
12. Zimmerli, A.; Monti, M.; Fenwick, C.; Eckerle, I.; Beigelman-Aubry, C.; Pellaton, C.; Jaton, K.; Dumas, D.; Stamm, G.-M.; Infanti, L.; et al. Case Report: Stepwise Anti-Inflammatory and Anti-SARS-CoV-2 Effects Following Convalescent Plasma Therapy with Full Clinical Recovery. *Front. Immunol.* **2021**, *12*, 613502. Available online: https://www.frontiersin.org/articles/10.3389/fimmu.2021.613502 (accessed on 11 July 2022). [CrossRef] [PubMed]
13. Ritonavir-Boosted Nirmatrelvir (Paxlovid). COVID-19 Treatment Guidelines. Available online: https://www.covid19treatmentguidelines.nih.gov/therapies/antiviral-therapy/ritonavir-boosted-nirmatrelvir--paxlovid-/ (accessed on 28 June 2022).
14. Hammond, J.; Leister-Tebbe, H.; Gardner, A.; Abreu, P.; Bao, W.; Wisemandle, W.; Baniecki, M.; Hendrick, V.M.; Damle, B.; Simón-Campos, A.; et al. Oral Nirmatrelvir for High-Risk, Nonhospitalized Adults with COVID-19. *N. Engl. J. Med.* **2022**, *386*, 1397–1408. Available online: https://www.nejm.org/doi/full/10.1056/NEJMoa2118542 (accessed on 11 July 2022). [CrossRef] [PubMed]
15. Gottlieb, R.L.; Vaca, C.E.; Paredes, R.; Mera, J.; Webb, B.J.; Perez, G.; Oguchi, G.; Ryan, P.; Nielsen, B.U.; Brown, M.; et al. Early Remdesivir to Prevent Progression to Severe COVID-19 in Outpatients. *N. Engl. J. Med.* **2022**, *386*, 305–315. [CrossRef] [PubMed]
16. Chen, P.; Nirula, A.; Heller, B.; Gottlieb, R.L.; Boscia, J.; Morris, J.; Huhn, G.; Cardona, J.; Mocherla, B.; Stosor, V.; et al. SARS-CoV-2 Neutralizing Antibody LY-CoV555 in Outpatients with COVID-19. *N. Engl. J. Med.* **2021**, *384*, 229–237. Available online: https://www.nejm.org/doi/full/10.1056/nejmoa2029849 (accessed on 11 July 2022). [CrossRef]
17. Cao, Y.; Wang, J.; Jian, F.; Xiao, T.; Song, W.; Yisimayi, A.; Huang, W.; Li, Q.; Wang, P.; An, R.; et al. Omicron escapes the majority of existing SARS-CoV-2 neutralizing antibodies. *Nature* **2022**, *602*, 657–663. Available online: https://www.nature.com/articles/s41586-021-04385-3 (accessed on 11 July 2022). [CrossRef]
18. Jayk Bernal, A.; Gomes da Silva, M.M.; Musungaie, D.B.; Kovalchuk, E.; Gonzalez, A.; Delos Reyes, V.; Martín-Quirós, A.; Caraco, Y.; Williams-Diaz, A.; Brown, M.L.; et al. Molnupiravir for Oral Treatment of COVID-19 in Nonhospitalized Patients. *N. Engl. J. Med.* **2022**, *386*, 509–520. Available online: https://www.nejm.org/doi/full/10.1056/NEJMoa2116044 (accessed on 11 July 2022). [CrossRef]
19. Molnupiravir. COVID-19 Treatment Guidelines. Available online: https://www.covid19treatmentguidelines.nih.gov/therapies/antiviral-therapy/molnupiravir/ (accessed on 28 June 2022).
20. Kunal, S.; Sakthivel, P.; Malhotra, N.; Ish, P. Newer oral antivirals for COVID-19: Are they the real game changer? *Hear. Lung* **2022**, *52*, 200–203. [CrossRef]

21. Cesaro, S.; Ljungman, P.; Mikulska, M.; Hirsch, H.H.; von Lilienfeld-Toal, M.; Cordonnier, C.; Meylan, S.; Mehra, V.; Styczynski, J.; Marchesi, F.; et al. Recommendations for the management of COVID-19 in patients with haematological malignancies or haematopoietic cell transplantation, from the 2021 European Conference on Infections in Leukaemia (ECIL 9). *Leukemia* **2022**, *36*, 1467–1480. [CrossRef] [PubMed]
22. Information on COVID-19 Treatment, Prevention and Research. COVID-19 Treatment Guidelines. Available online: https://www.covid19treatmentguidelines.nih.gov/ (accessed on 11 July 2022).
23. Bai, Y.; Shen, M.; Zhang, L. Antiviral Efficacy of Molnupiravir for COVID-19 Treatment. *Viruses* **2022**, *14*, 763. [CrossRef]
24. Biancofiore, A.; Mirijello, A.; Puteo, M.A.; Di Viesti, M.P.; Labonia, M.; Copetti, M.; De Cosmo, S.; Lombardi, R. CSS-COVID-19 Group Remdesivir significantly reduces SARS-CoV-2 viral load on nasopharyngeal swabs in hospitalized patients with COVID-19: A retrospective case–control study. *J. Med. Virol.* **2022**, *94*, 2284–2289. [CrossRef] [PubMed]
25. Grewal, R.; Kitchen, S.A.; Nguyen, L.; Buchan, S.A.; Wilson, S.E.; Costa, A.P.; Kwong, J.C. Effectiveness of a fourth dose of COVID-19 mRNA vaccine against the omicron variant among long term care residents in Ontario, Canada: Test negative design study. *BMJ* **2022**, *378*, e071502. [CrossRef] [PubMed]
26. Abdullah, F.; Myers, J.; Basu, D.; Tintinger, G.; Ueckermann, V.; Mathebula, M.; Ramlall, R.; Spoor, S.; de Villiers, T.; Van der Walt, Z.; et al. Decreased severity of disease during the first global omicron variant covid-19 outbreak in a large hospital in tshwane, south africa. *Int. J. Infect. Dis.* **2021**, *116*, 38–42. [CrossRef] [PubMed]
27. Early assessment of the clinical severity of the SARS-CoV-2 omicron variant in South Africa: A data linkage study. *Lancet* **2022**, *399*, 437–446. Available online: https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(22)00017-4/fulltext (accessed on 20 October 2022). [CrossRef] [PubMed]
28. Williamson, E.J.; Walker, A.J.; Bhaskaran, K.; Bacon, S.; Bates, C.; Morton, C.E.; Curtis, H.J.; Mehrkar, A.; Evans, D.; Inglesby, P.; et al. Factors associated with COVID-19-related death using OpenSAFELY. *Nature* **2020**, *584*, 430–436. [CrossRef]
29. Haeusler, G.M.; Ammann, R.A.; Carlesse, F.; Groll, A.H.; Averbuch, D.; Castagnola, E.; Agyeman, P.K.; Phillips, B.; Gilli, F.; Solopova, G.; et al. SARS-CoV-2 in children with cancer or after haematopoietic stem cell transplant: An analysis of 131 patients. *Eur. J. Cancer* **2021**, *159*, 78–86. [CrossRef]
30. Zaki, A.; Soomar, S.M.; Khan, D.H.; Sheikh, H.S.; Iftikhar, R.; Mir, A.; Aziz, Z.; Bano, K.; Naseer, H.; Chaudhry, Q.U.; et al. Outcomes of COVID-19 infection in patients with hematological malignancies—A multicenter analysis from Pakistan". *PLoS ONE* **2022**, *17*, e0267139. Available online: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0267139 (accessed on 11 July 2022). [CrossRef]

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Characteristics and outcomes of vaccinated and nonvaccinated patients hospitalized in a single Italian hub for COVID-19 during the Delta and Omicron waves in Northern Italy

Francesca Rovida[1,2], Giuliana Lucia Esposito[1,3], Marco Rissone[4,5], Viola Novelli[4], Sara Cutti[4], Alba Muzzi[4], Claudia Rona[4], Emanuela Bertoli[4], Marinella Daglio[4], Antonio Piralla[1], Stefania Paolucci[1], Giulia Campanini[1], Guglielmo Ferrari[1,3], Federica Giardina[1], Federica Zavaglio[1], Daniele Lilleri[1,*], Anna Maria Grugnetti[6], Giuseppina Grugnetti[6], Anna Odone[5], Carlo Marena[4], Fausto Baldanti[1,2]

[1] *Microbiology and Virology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy*
[2] *Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy*
[3] *Specialization School in Microbiology and Virology, University of Pavia, Pavia, Italy*
[4] *Medical Direction, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy*
[5] *Department of Public Health, Experimental and Forensic Medicine, Section of Hygiene, University of Pavia, Pavia, Italy*
[6] *Health Professions Direction, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy*

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ABSTRACT

Objective: We compared the characteristics and outcomes of vaccinated and nonvaccinated patients hospitalized with COVID-19.
Design: We analyzed patients hospitalized in a COVID hub during three one-month periods: (i) October 15, 2020-November 15, 2020 (prevaccination peak); (ii) October 15, 2021-November 15, 2021 (Delta wave); (iii) December 15, 2021-January 15, 2022 (Omicron wave). To define the epidemiologic context, SARS-CoV-2 infection in healthcare workers was analyzed.
Results: SARS-CoV-2 infection incidence in healthcare workers was 146 cases per 1000 persons in 2020 (prevaccination) and 67 in 2021 (postvaccination, when the Omicron variant caused most infections). There were 420 hospitalized patients in the prevaccination period, 51 during the Delta wave (52.1% vaccinated) and 165 during the Omicron wave (52.9% vaccinated). During the Delta wave, a significantly higher number of nonvaccinated (29.2%) than vaccinated patients (3.7%) were admitted to the intensive care unit (ICU) ($p = 0.019$). Nonvaccinated patients were younger and had a lower rate of concomitant medical conditions (53.2% vs 83.7%; $p < 0.001$) during the Omicron wave when 80% of patients admitted to ICU and all those who died were still infected by the Delta variant.
Conclusions: Vaccine effectiveness in fragile individuals appears to be lower because of a faster immunity decline. However, the Omicron variant seems to cause less severe COVID-19.

1. Introduction

Since the emergence of SARS-CoV-2 pandemic that causes COVID-19, several efforts have been made to contain and prevent the spread of infection and disease. Among the available interventions, population-based vaccination campaigns have been implemented worldwide after the development of highly effective vaccines (Baden et al., 2021; Polack et al., 2020; Sadoff et al., 2021; Voysey et al., 2021). In Italy, the vaccination campaign started on

Abbreviations: S, Spike; ICUs, Intensive care units; IQR, Interquartile range; CI, Confidence interval.

* Corresponding author: Daniele Lilleri, Microbiology and Virology Unit, Fondazione IRCCS Policlinico San Matteo, Viale Golgi 19, 27100 Pavia, Italy.
E-mail address: d.lilleri@smatteo.pv.it (D. Lilleri).

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December 27, 2020, and the following vaccines were adopted for immunization: BNT162b2 (Pfizer-BioNTech), mRNA-1273 (Moderna), ChAdOx1 (AstraZeneca), and Ad26.COV2.S (Janssen). The available vaccines showed high efficacy in protection from infection or disease in the original clinical trials, and their effectiveness has been confirmed at the population level by real-life postauthorization studies (Angel et al., 2021; Dagan et al., 2021; Haas et al., 2021; Hall et al., 2021; Lopez Bernal et al., 2021; Rovida et al., 2021b; Vasileiou et al., 2021). However, several issues are to be fully elucidated, such as the duration of the protective effectiveness and the impact of the diffusion of new viral variants on protection against severe disease. In particular, although the vaccines have been designed on the Spike (S) protein of the original wild-type strain, several viral variants arose before the vaccine implementation. Particularly, those defined as variants of concern are characterized by mutations conferring potentially higher transmissibility, immune evasiveness, or severity (https://www.ecdc.europa.eu/en/covid-19/variants-concern). At the end of 2020, the Alpha variant (B.1.1.7) was identified in the United Kingdom and became dominant in several countries, including Italy. Subsequently, the Delta variant (B.1.617.2) emerged and replaced the Alpha variant worldwide, and, more recently, the Omicron variant (B.1.1.529) was identified in South Africa and became predominant. Although vaccine effectiveness was still high against the Alpha variant (Rovida et al., 2021b), Delta and especially Omicron harbored mutations on the S protein associated with vaccine evasion (https://www.who.int/docs/default-source/coronaviruse/2022-01-07-global-technical-brief-and-priority-action-on-omicron---corr2.pdf?sfvrsn=918b09d_26). Meanwhile, on February 10, 2022, full vaccination vaccine coverage in the Italian population reached 88.61% of individuals older than 12 years and 21.47% of individuals younger than 12 years (https://www.governo.it/it/cscovid19/report-vaccini/). However, considering that vaccine effectiveness is below 100%, waning immunity, and immune escape of the new variants, the occurrence of breakthrough infections is expected to increase. However, the ratio between infections in vaccinated versus unvaccinated individuals is still unclear. The objective of this study was to compare the characteristics and outcomes of vaccinated and nonvaccinated patients hospitalized with COVID-19 in a single Italian hub, at the Fondazione IRCCS Policlinico San Matteo Hospital in Pavia, Northern Italy (Lombardy region, 10 million inhabitants), during the Delta and Omicron waves. The San Matteo Hospital is an Italian research hospital affiliated with the University of Pavia. In 2021, there were nearly 83,500 emergency visits, 30,000 admissions, 20,000 surgical procedures, 190 transplant procedures, and more than 2,500,000 outpatient clinic visits. Data were compared with those of patients hospitalized during the second pandemic wave before the implementation of the vaccination campaign. As an indicator of the epidemiological context, we analyzed the incidence of SARS-CoV-2 infection and the relevant virus genotype among healthcare workers in the same hospital.

2. Materials and methods

2.1. Study design

We analyzed the characteristics of patients admitted for COVID-19 at the San Matteo Hospital in Pavia, Northern Italy, during three one-month periods: (i) October 15, 2020-November 15, 2020 (prevaccination), corresponding to the peak of the second epidemic wave in Italy, which was sustained by the ancestral virus strain (with the D614G mutation); (ii) October 15, 2021-November 15, 2021, when >70% of the population was fully vaccinated, and the Delta variant accounted for almost all cases of infections (https://www.epicentro.iss.it/coronavirus/pdf/sars-cov-2-monitoraggio-varianti-rapporti-periodici-10-dicembre-2021.pdf), this period was defined as “Delta wave” in this study; (iii) December 15, 2021-January 15, 2022, when the Omicron variant accounted for most of the circulating strains (https://www.epicentro.iss.it/coronavirus/pdf/sars-cov-2-monitoraggio-varianti-indagini-rapide-3-gennaio-2022.pdf), this period was defined as “Omicron wave” in this study. Patients hospitalized for other diagnoses who were found positive for SARS-CoV-2 during recurring screening performed in hospital wards with an asymptomatic infection were excluded from the analysis. Patients’ data are collected within the routine Regional Healthcare system surveillance of SARS-CoV-2 infection; therefore, informed consent was not required. The study was approved by the Medical Direction of Fondazione IRCCS Policlinico, San Matteo.

2.2. Data collection

Patients’ data were retrieved and anonymized from electronic medical records and the Regional vaccination registry. The following information was collected: age, sex, vaccination status, concomitant chronic medical conditions (hypertension, cardiovascular disease, pulmonary disease, nephropathy, diabetes mellitus, obesity, neoplastic disease, immune depression), admission to intensive care units (ICUs), and death. For healthcare workers with SARS-CoV-2 infection, previous SARS-CoV-2 infection and vaccination status were collected.

2.3. Surveillance of SARS-CoV-2 infection in healthcare workers

Data on the occurrence of SARS-CoV-2 infection were available for 3832 healthcare workers of Fondazione IRCCS Policlinico, San Matteo, in 2020 and 4066 healthcare workers in 2021 (Rovida et al., 2021a; Rovida et al., 2021b; Lilleri et al., 2022). Naso-pharyngeal swabs were collected and tested for SARS-CoV-2 RNA positivity in subjects with symptoms suggestive of SARS-CoV-2 infection or in case of contact with infected subjects, as previously reported (Giardina et al., 2021). Moreover, in compliance with the local healthcare workers’ surveillance protocol, personnel working in clinical wards dedicated to fragile patients undergo routine screening for SARS-CoV-2 infection every 14 days, whereas monitoring was scheduled every 30 days for healthcare workers in the other wards. The health condition of all workers was regularly monitored, and data on symptoms were collected during an interview by a physician and inserted into a specific database.

2.4. Virus genotyping and sequencing

SARS-CoV-2 variants were determined in samples from vaccinated healthcare workers with breakthrough infections and as part of a national surveillance program by the Istituto Superiore di Sanità. Multiplex real-time reverse transcription–PCR tests specific for mutations characteristic of Delta (478K and 452R) and Omicron (501Y and 484A) were performed.

In addition, whole-genome sequencing was performed in selected samples using next-generation sequencing as previously reported (Rovida et al., 2021a). Viral variants were also determined in patients admitted to ICU during the Omicron wave period.

2.5. Statistical analysis

The incidence of SARS-CoV-2 infection in healthcare workers was expressed as number of cases per 1000 persons. The annual incidence rate was calculated for the entire 2020 and 2021. Since July 2020, when routine, standardized surveillance of the personnel of the hospital was implemented, data on monthly incidence rates were also calculated. Age was reported as median and range

or interquartile range (IQR) and was compared with the Mann-Whitney U-test when two groups were compared or the Kruskal-Wallis test and Dunn's post-test with correction for multiple comparisons when more than two groups were compared.

Categorical variables were expressed as percentage and compared by the Fisher's exact test or the chi-square test when more than two groups were compared.

2.6. Role of the funding source

This work was partially supported by the European Union's Horizon 2020 Research and Innovation Program (ATAC, No. 101003650). The funding source had no role in the study design, conduct, and report.

3. Results

3.1. SARS-CoV-2 infections in healthcare workers as an indicator of the epidemiological context

Data relevant to the surveillance of SARS-CoV-2 infection in healthcare workers of San Matteo Hospital are partially reported in previous work (Rovida et al., 2021a; Rovida et al., 2021b; Lilleri et al., 2022). For 2020, data relevant to the occurrence of SARS-CoV-2 infection are available for 3832 healthcare workers. A serological screening conducted during April 29, 2020-June 30, 2020, showed that 334 subjects (87 per 1000 persons; 95% confidence interval [CI]: 79-97) were infected during the first pandemic wave. Subsequently, during July 2020-December 2020, monitoring of SARS-CoV-2 infection by testing of nasal swabs showed that an additional 237 subjects (10 SARS-CoV-2-seropositive after the first wave and 227 seronegative) were infected during the second wave, for a total annual incidence of 561 SARS-CoV-2 infected subjects (146 per 1000 persons; 95% CI: 136-158). Among the 237 healthcare workers infected during the second wave, three subjects (0.8 per 1000 persons, 95% CI: 0.2-2.3) were infected in September, 85 (22 per 1000 persons; 95% CI: 18-27) in October, 148 (39 per 1000 persons; 955 CI: 33-45) in November, and one (0.3 per 1000 persons; 95% CI: 0.0-1.5) in December 2020. The peak of infections occurred during October 15th, 2020-November 15, 2020, when 167 subjects (44 per 1000 persons; 95% CI: 38-51) were infected. In 2021, after the implementation of the vaccination campaign, we analyzed the occurrence of SARS-CoV-2 infections among 4066 healthcare workers at San Matteo Hospital, along with the genotype of the infecting virus (Figure 1). These data were considered as proxy of the variants circulation in the general population of the territory of Pavia. A significantly lower incidence of SARS-CoV-2 infection ($p < 0.001$) was observed in 2021 compared to 2020: 271 of 4066 healthcare workers (67 per 1000 persons; 95% CI: 59-75) were positive for SARS-CoV-2 RNA with nasal swab testing during the entire 2021. The number of infected subjects per month increased from January to March 2021, when a peak of 21 cases was observed (5 per 1000 persons; 95% CI: 3-8), and subsequently decreased, maintaining a low, steady state between May and October 2021. The incidence of infection started increasing again in November 2021, when 21 cases were detected (5 per 1000 persons; 95% CI: 3-8), reaching the maximum level in December 2021, with 182 cases detected (45 per 1000 persons; 95% CI: 39-52; Figure 1A). Regarding the vaccination status, we already reported the differential incidence of infections in vaccinated and nonvaccinated subjects during January-May 2021(Rovida et al., 2021a), showing a vaccine effectiveness of 83% in protecting from infection with the Alpha variant. Subsequently, all the study population was vaccinated; therefore, we could not compare the incidence of infection in vaccinated versus nonvaccinated subjects thereafter. Since October 15 2021, healthcare workers have received a third dose of BNT162b2 or mRNA-1273 vaccine. At the end of the year, most healthcare workers had received three vaccine doses, and most infections in December 2021 occurred in three-dose vaccinated subjects (Figure 1A). No vaccinated healthcare worker developed pneumonia or required hospitalization for COVID-19. Among the 271 SARS-CoV-2-infected subjects, identification of the infecting variant was available for 127 subjects (Figure 1B). Between February and May 2021, the Alpha variant was detected in all the subjects whose viral RNA content was sufficient for genotyping analysis. Between July and November 2021, only the Delta variant was detected, whereas, in December 2021, the Omicron variant appeared and accounted for 89% of the genotyped strains, whereas the remaining 11% of cases harbored the Delta variant.

3.2. Characteristics of vaccinated and nonvaccinated patients hospitalized for COVID-19

In the prevaccination period, 420 patients were hospitalized, whereas 51 patients were hospitalized during the Delta wave and 165 during the Omicron wave. Patients' characteristics are listed in Table 1. Among the 51 patients of the Delta wave, 27 (52.9%) were vaccinated, and among the 165 patients of the Omicron wave, 86 (52.1%) were vaccinated. Nonvaccinated patients of the Delta wave (median age 66 years; IQR 55-74 years) were younger than vaccinated patients (median age 78 years; IQR 71-86 years; $p = 0.003$). Similarly, nonvaccinated patients of the Omicron wave (62, IQR 54-73 years) were younger than vaccinated patients (median age 74 years; IQR 63-84 years, $p < 0.001$). In addition, patients of the prevaccination period (median age 68 years; IQR 59-79 years) were younger than vaccinated patients of both the Delta ($p = 0.004$) and Omicron waves ($p = 0.024$). In the Delta and Omicron waves, there were more nonvaccinated patients than vaccinated patients in the age group of 61-70 years and the younger age groups, whereas more vaccinated than nonvaccinated patients were observed in the older age groups (Figure 2). Concomitant chronic medical conditions (Figure 2B) were present in 375 (89.3%) patients in the prevaccination period. Among patients of the Delta wave, concomitant medical conditions were present in 18 (75%) nonvaccinated patients and 24 (88.9%) vaccinated patients ($p = 0.276$). Among patients of the Omicron wave, a significantly lower number of nonvaccinated ($n = 42$; 53.2%) than vaccinated patients ($n = 72$; 83.7%) had concomitant medical conditions ($p < 0.001$).

3.3. Outcome of COVID-19 in vaccinated and nonvaccinated hospitalized patients

In the prevaccination period, 32 (7.6%) patients were admitted to ICU wards (Figure 2C). Among patients of the Delta wave, a significantly higher number of nonvaccinated ($n = 7$; 29.2%) than vaccinated patients ($n = 1$; 3.7%) were admitted to the ICU ($p = 0.019$). Among patients of the Omicron wave, 17 (21.5%) nonvaccinated and 11 (12.8%) vaccinated patients were admitted to the ICU ($p = 0.151$). During the prevaccination period, 90 (21.4%) patients died because of COVID-19 (Table 2). A similar rate of death was observed among patients of the Delta wave ($n = 10$, 19.6%). No difference was observed between vaccinated and nonvaccinated patients. Nonvaccinated patients who died had a median age of 74 (IQR 65-83) years and were younger than vaccinated patients (median age 86 years, IQR 71-92 years; $p = 0.071$). Among patients of the Omicron wave, 41 (24.8%) died because of COVID-19. In all the periods analyzed, most patients who died had concomitant chronic medical conditions, except for nonvaccinated patients who died during the Omicron wave, when about half of them were not affected by other medical conditions (Table 2).

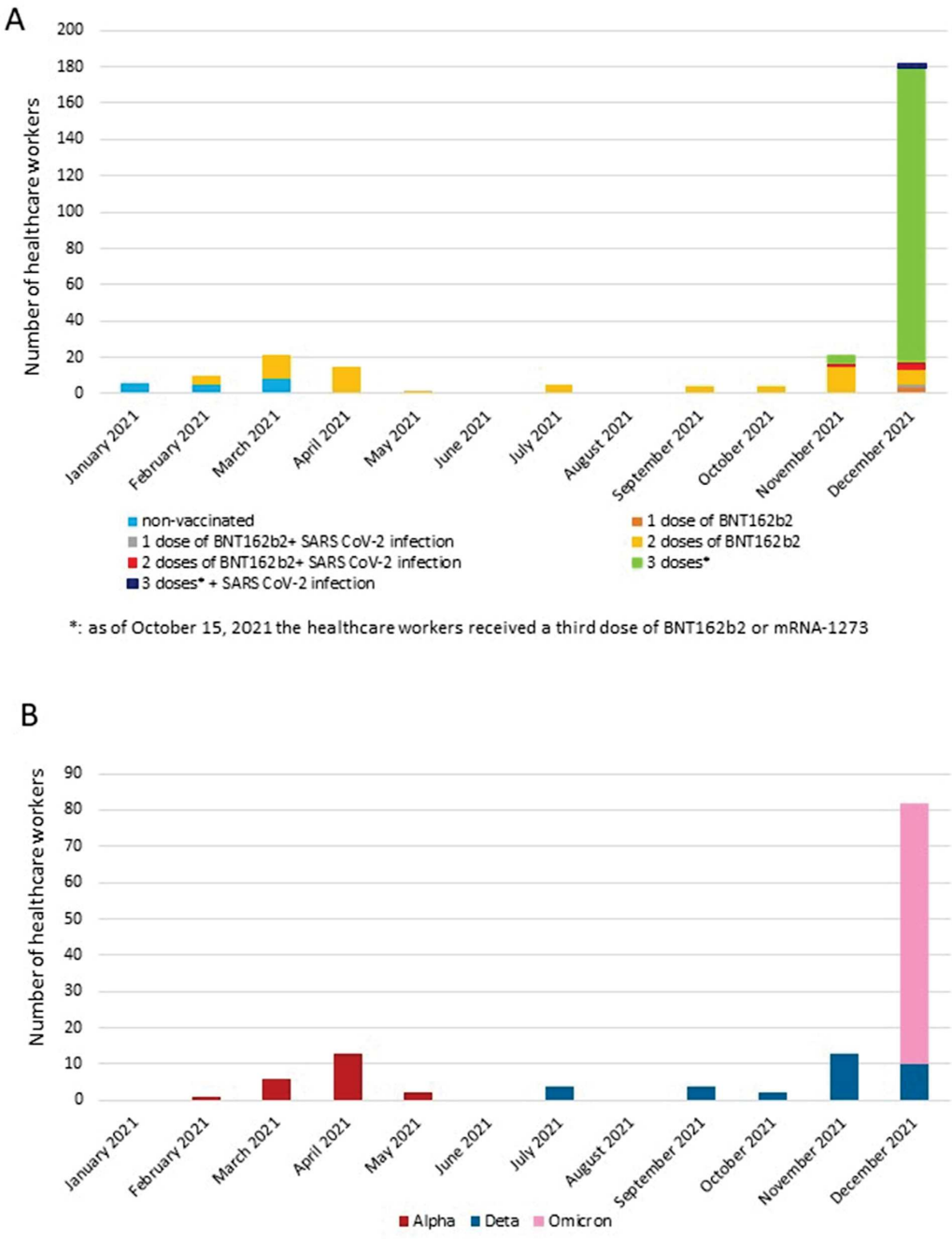

Figure 1. SARS-CoV-2 infections in healthcare workers of Fondazione IRCCS Policlinico San Matteo, Pavia, in 2021. (A) Monthly number of infections and vaccination status. (B) SARS-CoV-2 variants detected.

Table 1
Characteristics of the study population

Characteristics	Period of hospitalization of COVID-19 patients		
	Prevaccination[a] (n = 420)	Delta wave[b] (n = 51)	Omicron wave[c] (n = 165)
Age, median (IQR), years	68 (59-79)	71 (62-82)	69 (58-80)
Sex, M/F n. (%)	294/126 (70/30)	30/21 (59/41)	98/67 (68/32)
Patients without chronic medical conditions, n. (%)	45 (10.7)	9 (17.6)	51 (30.9)
Patients with chronic medical conditions, n. (%)	375 (89.3)	42 (82.4)	114 (69.1)
Hypertension	270 (72.0)	30 (71.4)	83 (74.5)
Cardiovascular disease	120 (32.0)	15 (35.7)	39 (34.2)
Pulmonary disease	84 (22.5)	9 (21.4)	14 (12.2)
Nephropathy	60 (16.0)	14 (33.3)	16(14.0)
Diabetes	101 (26.9)	10(23.8)	28 (24.5)
Obesity	71 (18.9)	8 (19.0)	13 (11.4)
Neoplastic disease	67 (17.8)	11 (26.1)	9 (7.8)
Immune depression	24 (6.4)	2 (4.7)	11 (9.6)
Patients with one chronic medical conditions, n. (%)	53 (14.1)	4 (9.5)	25 (21.9)
Patients with two chronic medical conditions, n. (%)	63 (16.8)	6 (14.3)	38 (33.3)
Patients with ≥3 chronic medical conditions, n. (%)	259 (69.1)	32 (76.2)	51 (44.7)
Vaccinated, n. (%)	0 (0)	27 (52.9)	86 (52.1)

[a] October 15, 2020-November 15, 2020
[b] October 15, 201-November 15, 2021
[c] December 15, 2021-January 15, 2022F = female; M = male; IQR = interquartile range.

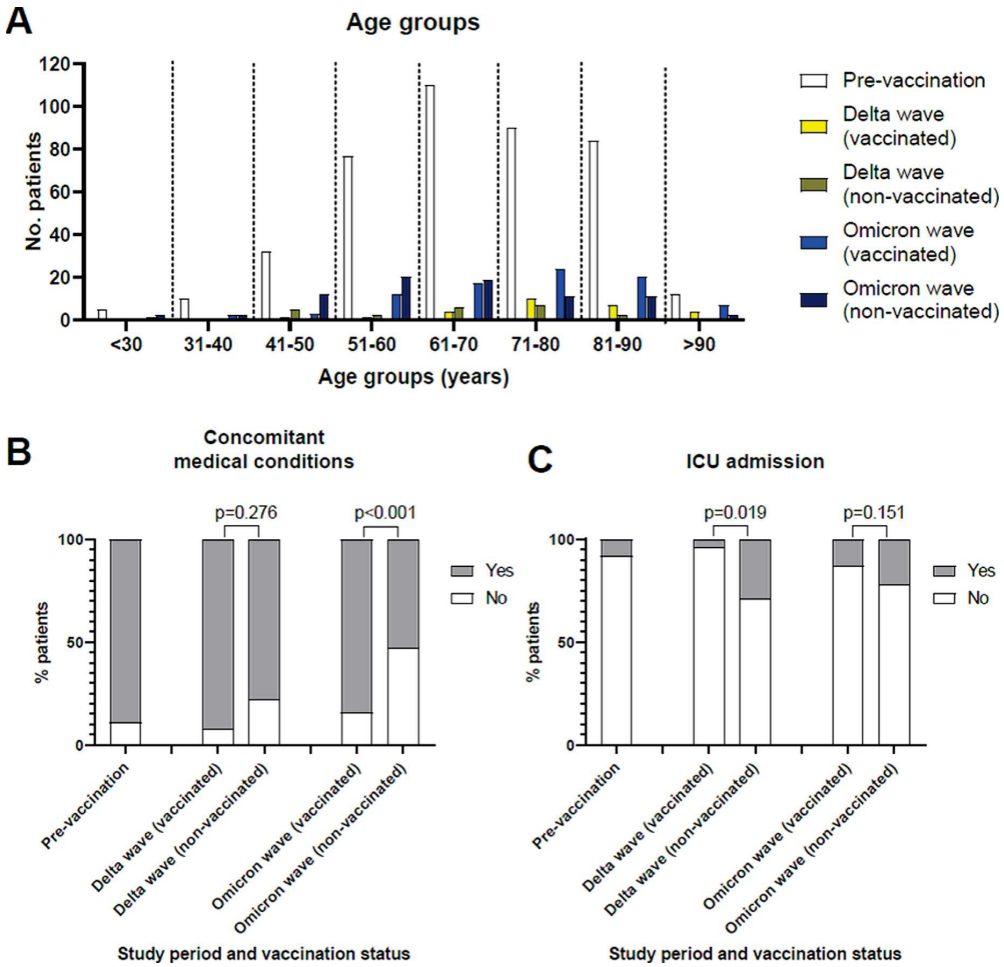

Figure 2. Characteristics of vaccinated and nonvaccinated patients with SARS-CoV-2 infection in the three study periods. (A) Number of patients in the different age groups. (B) Rate of patients with concomitant chronic medical conditions. (C) Rate of patients requiring admission to the intensive care unit (ICU). Prevaccination: October 15, 2020-November 15, 2020; Delta: October 15, 2021-November 15, 2021; Omicron: December 15, 2021-January 15, 2022.

Table 2
Outcome of COVID-19 in vaccinated and nonvaccinated patients

Period of hospitalization of COVID-19 patients	n. (%) deceased patients	Median age, years (range)	n. (%) deceased patients with chronic medical conditions
Prevaccination[a] (n = 420)	90 (21.4)	80 (39-95)	87 (96.7)
Delta wave[b] (n = 51)	10 (19.6)	79 (65-92)	8 (80.0)
-vaccinated (n = 27)	5 (18.5)	86 (71-92)	4 (80.0)
-nonvaccinated(n = 24)	5 (20.8)	74 (65-83)	4 (80.0)
Omicron wave[c] (n = 165)	41 (24.8)	81 (47-95)	30 (73.2)
-vaccinated (n = 86)	26 (30.2)	78 (58-95)	23 (88.5)
-nonvaccinated (n = 79)	15 (19.0)	77 (8-92)	7 (46.7)

[a] October 15, 2020-November 15, 2020
[b] October 15, 2021-November 15, 2021
[c] December 15, 2021-January 15, 2022

3.4. SARS-CoV-2 variant in patients admitted to ICU during the Omicron wave

The SARS-CoV-2 variant in 20 of 28 patients admitted to ICU during the Omicron period was successfully determined. The Omicron variant was detected in four (20%) cases, whereas the Delta variant was detected in the remaining 16 (80%) cases. The virus genotype was available for 9 of 41 patients who died during the Omicron wave (i.e., patients who were admitted to ICU), and in all nine cases, the Delta variant was detected. Notwithstanding the predominance of the Omicron variant among the general population, most patients required ICU admission, and all patients who died were infected with the Delta variant.

4. Discussion

Results of this study show that in our study population, a resurgence of SARS-CoV-2 infection in vaccinated individuals occurred with the Omicron but not with the Delta variant. However, a lower number of patients was hospitalized during both the Delta and Omicron waves, when >70% of the population was vaccinated, than in the prevaccination era. Vaccinated patients hospitalized for COVID-19 were older than nonvaccinated patients hospitalized during the Delta and Omicron waves and in the prevaccination era. In addition, vaccinated patients hospitalized during the Omicron wave were more frequently affected by concomitant chronic medical conditions than nonvaccinated patients. Finally, vaccinated patients had a significantly lower rate of admission to ICU than nonvaccinated patients during the Delta wave. Some real-life studies showed only a modest reduction in the effectiveness of BNT162b2 and ChAdOx1 against Delta compared to the Alpha variant (Lopez Bernal et al., 2021; Sheikh et al., 2021), especially in protection from severe infections requiring hospitalization (Sheikh et al., 2021), whereas other studies documented a major reduction in vaccine effectiveness (Keehner et al., 2021; Rosenberg et al., 2022). Waning of immunity with time after vaccination is another factor contributing to reduced protection (Collier et al., 2021; Goldberg et al., 2021; Khoury et al., 2021; Pouwels et al., 2021; Thomas et al., 2021; Wall et al., 2021), which is difficult to differentiate from reduced effectiveness against the Delta or Omicron variants. Notwithstanding the decline of the antibody response observed six months after vaccination and the partial immune evasiveness of the Delta variant, we did not observe a resurgence of SARS-CoV-2 infections and hospitalizations during the Delta wave, as it was observed elsewhere (Keehner et al., 2021; Rosenberg et al., 2022). The peak monthly incidence of SARS-CoV-2 infection in vaccinated healthcare workers was similar during periods dominated by the Alpha or Delta variants. The high vaccination coverage at the population level, coupled with nonpharmacological measures, such as the persistence of indoor masking requirements, may have contributed to the reduction of virus circulation in the general population during the Delta wave, avoiding a significant resurgence of infections both in vaccinated and nonvaccinated individuals. A modeling study showed that increasing the rate of vaccination could have prevented substantial hospitalizations and deaths, even in the Delta-driven wave (Vilches et al., 2022). In addition, in our study, infections with the Delta variant appeared more severe in nonvaccinated patients because a higher proportion of nonvaccinated than vaccinated patients required intensive care, as also reported in other studies (Taylor et al., 2021; Tenforde et al., 2021). The vaccine evasion of the Omicron variant was even higher, as reflected by the increased breakthrough infections in vaccinated healthcare workers and the number of hospitalized patients. However, our data support the lower pathogenicity of this variant, as was suggested in early studies from South Africa (Maslo et al., 2022; Wolter et al., 2022). An indirect data is the fact that, although the monthly incidence of infection in vaccinated healthcare workers increased by almost 10 times from the Delta to the Omicron wave, the number of hospitalized patients only increased by about three times. Moreover, during the Omicron wave, most of the ICU-admitted patients were infected with the Delta variant, and the Delta variant was detected in all the patients with fatal outcomes, although Omicron accounted for more than 80% of the strains circulating in the general population. The viral variants detected in the hospitalized population may reflect those circulating in the general population about one to two weeks before hospitalization. Nevertheless, the prevalence of Omicron among patients admitted to the ICU appears to be lower than expected. Finally, hospitalized vaccinated patients during the Delta and Omicron waves were older than nonvaccinated patients admitted to hospital in both the pre- and postvaccination eras. In addition, during the Omicron wave, a higher proportion of vaccinated patients had concomitant chronic medical conditions than nonvaccinated patients. Also, among patients who died during the Omicron wave, a higher proportion of vaccinated patients had concomitant chronic medical conditions than nonvaccinated patients. These observations confirm lower vaccine effectiveness and faster waning of immunity in older individuals and those with underlying clinical conditions (Andrews et al., 2022) and more sustained protection in younger subjects. Limitations of this study reside in its observational retrospective nature, the lack of a noninfected control group to estimate vaccine effectiveness, the relatively low number of patients examined, and the partial availability of virus genotype. In addition, different behaviors and containment measures in the population occurring during the three study periods have influenced virus circulation. However, the stringency of containment measures was lower in the vaccination era, whereas a partial lockdown was implemented during the second wave of the prevaccination era. This may have potentially increased the number of hospitalizations concerning what may have occurred if, instead, the same measures applied during the second wave were maintained in the vaccination era. In conclusion, vaccinated patients hospital-

ized for COVID-19 during the Delta and Omicron waves are older and more fragile; the risk of developing more severe COVID-19 is lower in vaccinated individuals with Delta variant breakthrough infections than in nonvaccinated subjects, and the Omicron variant seems to cause less severe COVID-19. Vaccine effectiveness in fragile individuals appears to be lower because of a faster immunity decline and may take advantage of periodical vaccine boosters, which could be adjourned on the newly identified viral variants.

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Ethical approval statement

Patients' data are collected within the routine Regional Healthcare system surveillance of SARS-CoV-2 infection; therefore, informed consent was not required. The study was approved by the Medical Direction of Fondazione IRCCS Policlinico, San Matteo.

Author contributions

Conceptualization: FB.
Methodology: DL.
Formal analysis: FR, GLE, and DL.
Investigation: FR, GLE, MR, CR, EB, MD, AP, SP, GC, GF, FG, and FZ.
Resources: AMG, GG, and AO.
Data curation: FR, MR, VN, SC, and AM.
Writing – original draft preparation: DL.
Writing – review, and editing: FR and FZ.
Visualization: FR and DL.
Supervision: CM and FB.
Funding acquisition: FB.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available because of privacy or ethical restrictions.

Conflicts of interest

The authors have no competing interests to declare.

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References

Andrews N, et al. Duration of protection against mild and severe disease by COVID-19 vaccines. N Engl J Med 2022;386:340–50.
Angel Y, et al. Association between vaccination with BNT162b2 and incidence of symptomatic and asymptomatic SARS-CoV-2 infections among health care workers. JAMA 2021;325:2457–65.
Baden LR, et al. Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine. N Engl J Med 2021;384:403–16.
Collier AY, et al. Differential kinetics of immune responses elicited by COVID-19 vaccines. N Engl J Med 2021;385:2010–12.
Dagan N, et al. BNT162b2 mRNA COVID-19 vaccine in a nationwide mass vaccination setting. N Engl J Med 2021;384:1412–23.
Giardina F, et al. No evidence of SARS-CoV-2 circulation in the framework of influenza surveillance between October 2019 and February 2020 in Lombardy, Italy. Travel Med Infect Dis 2021;40.
Goldberg Y, et al. Waning immunity after the BNT162b2 vaccine in Israel. N Engl J Med 2021;385:e85.
Haas EJ, et al. Impact and effectiveness of mRNA BNT162b2 vaccine against SARS–CoV-2 infections and COVID-19 cases, hospitalisations, and deaths following a nationwide vaccination campaign in Israel: an observational study using national surveillance data. Lancet 2021;397:1819–29.
Hall VJ, et al. COVID-19 vaccine coverage in health-care workers in England and effectiveness of BNT162b2 mRNA vaccine against infection (SIREN): a prospective, multicentre, cohort study. Lancet 2021;397:1725–35.
Keehner J, et al. Resurgence of SARS-CoV-2 infection in a highly vaccinated health system workforce. N Engl J Med 2021;385:1330–2.
Khoury DS, et al. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. Nat Med 2021;27:1205–11.
Lilleri D, et al. SARS-CoV-2 infection in vaccinated health care workers. N Engl J Med 2022;386:199–200.
Lopez Bernal J, et al. Effectiveness of the Pfizer-BioNTech and Oxford-AstraZeneca vaccines on covid-19 related symptoms, hospital admissions, and mortality in older adults in England: test negative case-control study. BMJ 2021;373:n1088.
Lopez Bernal J, et al. Effectiveness of COVID-19 vaccines against the B.1.617.2 (Delta) variant. N Engl J Med 2021;385:585–94.
Maslo C, et al. Characteristics and outcomes of hospitalized patients in South Africa during the COVID-19 omicron wave compared with previous waves. JAMA 2022;327:583–4.
Polack FP, et al. Safety and efficacy of the BNT162b2 mRNA COVID-19 vaccine. N Engl J Med 2020;383:2603–15.
Pouwels KB, et al. Effect of Delta variant on viral burden and vaccine effectiveness against new SARS-CoV-2 infections in the UK. Nat Med 2021;27:2127–35.
Rosenberg ES, et al. COVID-19 vaccine effectiveness in New York State. N Engl J Med 2022;386:116–27.
Rovida F, et al. Incidence of SARS-CoV-2 infection in health care workers from Northern Italy based on antibody status: immune protection from secondary infection- a retrospective observational case-controlled study. Int J Infect Dis 2021a;109:199–202.
Rovida F, et al. SARS-CoV-2 vaccine breakthrough infections with the alpha variant are asymptomatic or mildly symptomatic among health care workers. Nat Commun 2021b;12:6032.
Sadoff J, et al. Safety and efficacy of single-dose Ad26.COV2.S vaccine against COVID-19. N Engl J Med 2021;384:2187–201.
Sheikh A, et al. SARS-CoV-2 Delta VOC in Scotland: demographics, risk of hospital admission, and vaccine effectiveness. Lancet 2021;397:2461–2.
Taylor CA, et al. Severity of disease among adults hospitalized with laboratory-confirmed COVID-19 before and during the period of SARS-CoV-2 B.1.617.2 (delta) predominance - COVID-NET, 14 states, January–August 2021. MMWR Morb Mortal Wkly Rep 2021;70:1513–19.
Tenforde MW, et al. Association between mRNA vaccination and COVID-19 hospitalization and disease severity. JAMA 2021;326:2043–54.
Thomas SJ, et al. Safety and efficacy of the BNT162b2 mRNA COVID-19 vaccine through 6 months. N Engl J Med 2021;385:1761–73.
Vasileiou E, et al. Interim findings from first-dose mass COVID-19 vaccination roll-out and COVID-19 hospital admissions in Scotland: a national prospective cohort study. Lancet 2021;397:1646–57.
Vilches TN, et al. COVID-19 hospitalizations and deaths averted under an accelerated vaccination program in northeastern and southern regions of the USA. Lancet Reg Health Am 2022;6.
Voysey M, et al. Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. Lancet 2021;397:99–111.
Wall EC, et al. Neutralising antibody activity against SARS-CoV-2 VOCs B.1.617.2 and B.1.351 by BNT162b2 vaccination. Lancet 2021;397:2331–3.
Wolter N, et al. Early assessment of the clinical severity of the SARS-CoV-2 omicron variant in South Africa: a data linkage study. Lancet 2022;399:437–46.

Rapid communication

Fulminant echovirus 11 hepatitis in male non-identical twins in northern Italy, April 2023

Antonio Piralla[1,*] , Alessandro Borghesi[2,*] , Amelia Di Comite[2] , Federica Giardina[3] , Guglielmo Ferrari[1] , Simona Zanette[2] , Tiziana Angelica Figar[2] , Micol Angelini[2] , Camilla Pisoni[2] , Antonino Maria Guglielmo Pitrolo[1] , Stefania Paolucci[1] , Francesca Rovida[1,3] , Isabella Pellicioli[4] , Ezio Bonanomi[4] , Fausto Baldanti[1,3,] , Stefano Ghirardello[2,**]**

1. Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
2. Neonatal Intensive Care Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
3. Department of Clinical, Surgical, Diagnostic and Paediatric Sciences, University of Pavia, Pavia, Italy
4. Paediatric Intensive Care Unit, Ospedale Papa Giovanni XXIII, Bergamo, Italy
5. These authors contributed equally to this work and share first authorship
6. These authors contributed equally to the work and share the last authorship

Correspondence: Fausto Baldanti (f.baldanti@smatteo.pv.it)

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Echovirus 11 (E11) has recently been associated with a series of nine neonatal cases of severe hepatitis in France. Here, we present severe hepatitis caused by E11 in a pair of twins. In one of the neonates, the clinical picture evolved to fulminant hepatitis. The E11 genome showed 99% nucleotide identity with E11 strains reported in the cases in France. Rapid genome characterisation using next generation sequencing is essential to identify new and more pathogenetic variants.

The World Health Organization (WHO) has recently reported an increasing number of severe neonatal infection associated with a specific species B enterovirus type, echovirus 11 (E11) [1]. By July 2022, nine cases of neonatal sepsis with liver disease and multi-organ failure had been reported in France from three metropolitan regions [2]. The European Centre for Disease Prevention and Control (ECDC) has recently included this virus in the ECDC Communicable Disease Threat Report [3]. According to the WHO, the public health risk for the general population is low, but the increase in reported cases remains a concern. Here, we report life-threatening E11 infection in a pair of dichorionic twins with a presentation and clinical course closely resembling those reported in the French cases [1,2].

Description of cases and laboratory investigations

In April 2023, two non-identical, male, late-preterm twin brothers, P1 and P2, were transferred from the nursery to the neonatal intensive care unit (NICU) due to episodes of apnoea requiring respiratory support. They were later diagnosed with life-threatening E11 infection.

Pregnancy history

The two infants were born to a healthy pregnant woman at 35 weeks and 3 days gestational age, following a spontaneous pregnancy. During pregnancy, their mother was given acetylsalicylic acid up to 35 weeks of gestation due to high risk of pre-eclampsia as assessed at the Bi-test, and enoxaparin sodium due to three previous spontaneous abortions. No other complications of the pregnancy were reported. All serological and biochemical laboratory tests during pregnancy were unremarkable. No episodes of diarrhoea were reported before partum by the mother or father. At 35 weeks and 1 day of gestation, the mother was admitted to the obstetrics unit for onset of labour with increased C-reactive protein (63 mg/L, norm: < 5.0), and betamethasone for the prophylaxis of neonatal respiratory distress and antibiotics were administered. A vaginal swab performed at admission later proved negative for group B streptococcus colonisation. One single fever episode with spontaneous resolution was registered at 35 weeks and 2 days of gestational age. The mother underwent a Caesarean section because of podalic presentation of P1 and progression of labour. Birth weights were 2,610 g (54th percentile) and 2,660 g (59th percentile), and Apgar scores 9 and 10 and 9 and 9 at 1 and 5 min for P1 and P2, respectively. No further virological investigations were performed on clinical specimens from the mother (i.e milk, plasma or stool).

Neonate P1

On day 4 of life, P1 presented with episodes of apnoea and cyanosis and was transferred to the NICU on the following day for respiratory support with high flow nasal cannula (HFNC, maximum flow: 2 L/min). The infant underwent blood withdrawal for blood culture,

Table

Laboratory testing results on day 9 of life, twin neonates with echovirus 11 infection, Italy, April 2023

Laboratory test	P1	P2	Reference range
Aspartate transaminase (mU/mL)	294	2,740	11.0–39.0
Alanine transaminase (mU/mL)	291	11,925	11.0–34.0
Gamma-GT (mU/mL)	191	202	11.0–53.0
Creatinine (mg/dL)	0.39	0.46	0.30–0.70
Total bilirubin (mg/dL)	2.11	11.28	0.2–1.1
Direct bilirubin (mg/dL)	Not available	2.73	0.00–0.25
Plasma prothrombin (%)	94.00	Undetectable	70.00–120.00
Thromboplastin time (s)	42.90	Undetectable	20.00–32.00
Fibrinogen (mg/dL)	374	Undetectable	170–410
International normalised ratio	1.03	Unmeasurable	0.90–1.20
Hepatic cholinesterase (mU/mL)	5,656	2,986	5,300–12,900
Plasma ammonium (µg/dL)	160	192	19.0–94.0
Ferritin (ng/mL)	2,542	124,801	8–398

later proven negative (5 days of monitoring), and was started on antibiotics. On day 6, a nasal swab for virological analyses, performed for the appearance of fever despite administration of broad-spectrum antibiotic, demonstrated the presence of enterovirus by specific real-time RT-PCR. Chest X-ray imaging on day 7 was normal. Antibiotics were stopped on day 7. Following clinical improvement, the respiratory support was stopped on day 10 of life, with further management of the infant in room air.

Further virological testing on day 9 demonstrated the presence of enteroviral RNA in plasma (2.6×10^3 RNA copies/mL) and urine (4.9×10^3 RNA copies/mL). Laboratory testing was consistent with acute hepatitis with elevated liver enzymes and normal renal function, bilirubin levels and blood clotting values; the laboratory values at day 9 of life are reported in the Table. In the following days, the infant showed progressive clinical improvement with normalisation of liver enzymes on day 18 and was discharged home in good clinical condition on day 25 of life.

Neonate P2

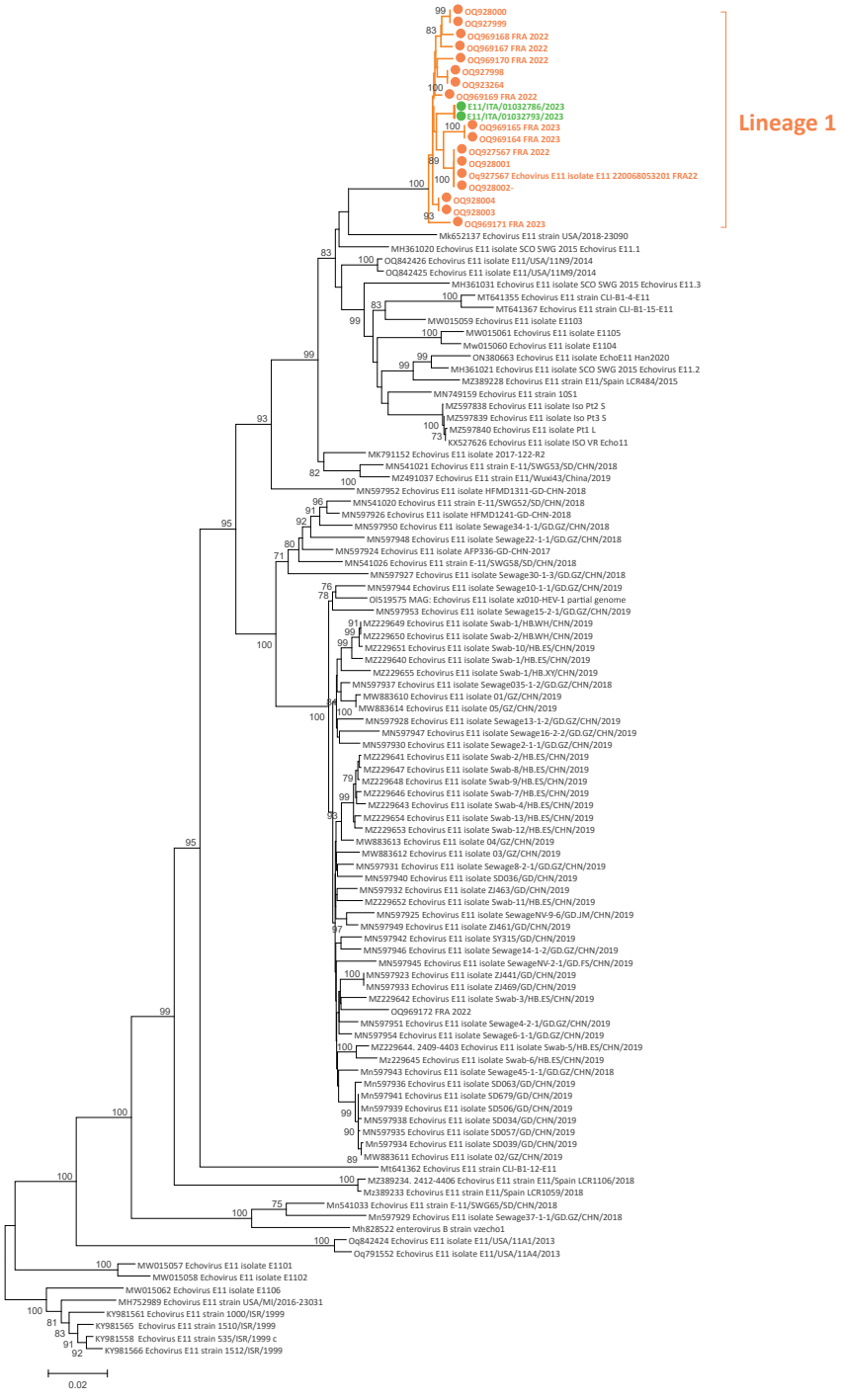
At the clinical disease onset, on day 6 of life, the infant presented with hypoxaemic respiratory distress requiring respiratory support on HFNC. On day 12, because his respiratory condition worsened and pleural effusion appeared, the infant received nasal continuous positive airway pressure with a maximum fraction of inspired oxygen (FiO2) of 0.25. The infant was weaned from any respiratory support on day 18.

On day 9 of life, a blood count revealed thrombocytopenia (12,000/µl) with an extremely severe coagulopathy, undetectable clotting factors and fibrinogen, low haemoglobin (7.5 g/dL) and severely altered liver function; the laboratory values at days 9 of life are reported in the Table. EV RNA was detected in plasma (5.2×10^5 RNA copies/mL) and urine (6.3×10^3 RNA copies/mL). The infant underwent intensive daily transfusion treatment including 1–3 fresh frozen plasma and 1–2 platelet units per day, red blood cells (on days 10, 12 and 15) (transfusion volumes of 15–20 mL/kg), intravenous immunoglobulins (0.5 g/kg/day for 4 days) and albumin transfusion. Despite slow but progressive decrease in the liver enzymes and increase in cholinesterase levels, the infant remained transfusion-dependent until day 21. A first exchange transfusion due to high bilirubin levels (total bilirubin: 25.74 mg/dL; direct bilirubin: 12.35 mg/dL) was performed on day 17, and a second exchange transfusion due to persistently high bilirubin levels (total bilirubin: 26.7 mg/dL; direct bilirubin: 12.3 mg/dL) and elevated plasma ammonium (300 µg/dL) was performed on day 20 of life. Lumbar puncture was not performed due to the high risk of bleeding. The cerebral ultrasound on day 7 of life revealed enlarged lateral ventricles and two hyperechoic cerebellum lesions of haemorrhagic origin. No seizures were detected on electroencephalographic monitoring.

On day 21, the infant was transferred to another paediatric intensive care unit for further management and possible consideration for liver transplantation. He was breathing spontaneously in room air and still transfusion-dependent. At the time of this report, the patient is still hospitalised, intubated in weaning from mechanical ventilation; thrombocytopenia persists without the need for platelet transfusion; the liver enzymes have normalised, but bilirubin concentration is still high (› 20 mg/dL, norm: 0.2–1.1) and due to coagulopathy, the infant requires plasma and cryoprecipitate transfusions at occurrence. Due to the persistence of severe hepatic insufficiency with ascites, the patient will be evaluated for liver transplantation.

Figure

Phylogenetic tree of echovirus 11 complete genome sequences (n = 112)



Green: sequences from the two neonates reported in this manuscript; orange: sequences from neonatal cases reported in France [2].

Phylogenetic and molecular analysis of the E11 strains

Enterovirus typing was performed in urine for P1 and in plasma samples for P2 by whole genome sequencing (WGS) and showed the presence of E11 strains. For WGS, we used the metagenomic approach as previously described by Kufner et al. [4]. Reads were mapped to the reference genome OQ927998 using the INSaFLU pipeline (https://insaflu.insa.pt) [5]. Phylogenetic analysis was performed on complete genome sequences obtained from urine of P1 (E11/ITA/01032786/2023) and plasma samples of P2 (E11/ITA/01032793/2023) (Figure). A maximum likelihood phylogenetic tree was inferred using the IQ-TREE web server (v1.6.8) [6], and the robustness of branches was evaluated using the Shimodaira–Hasegawa approximate likelihood-ratio test (SH-aLRT) and ultrafast bootstrap approximation tests. The Italian strains clustered with French strains collected in 2023 [2] (Figure), which together composed a divergent lineage. The average nucleotide identity based on the complete genome sequence was 99.3% (range: 98.9–99.6) between Italian and French E11 strains belonging to lineage 1, and it was 81.6% (range: 72.7–89.9) between the Italian strains and all other E11 genomes of good quality that were available and retrieved from GenBank (n = 93).

Discussion

An increase in the incidence and severity of acute and fulminant hepatitis associated with an emerging lineage of E11 in neonates, with marked prevalence in male twins, is currently observed in France [1,2]. The E11 and other non-polio enteroviruses have been circulating continuously in the European Union [7]. Several E11 lineages were circulating in the same geographical region at the same time [8]. A recombinant origin of the strain found in France was hypothesised by Grapin et al. [2], however, further analyses are needed to elucidate the origin of this divergent lineage as previously done for other E11 lineages [8].

Manifestations of neonatal E11 infections may range from asymptomatic to severe fatal multi-system disease [9]. In accordance with some other studies, severe E11 infection in our neonates was associated with a haemorrhage-hepatitis syndrome that causes hepatitis with liver dysfunction and coagulopathy as previously reported [2,10]. The clinical course of the twin brothers described here was consistent with that reported in the French cohort: fulminant, with sudden onset, quick worsening of the clinical conditions and liver function and development of life-threatening hepatitis, although with different severity and duration in the two infants. For both infants, survival was strictly dependent on rapid recognition of the infection and timely administration of intensive care. According to the French report and ours, a host genetic predisposition in male and twin categories might be hypothesised [2].

Conclusion

The present report together with data of recent E11 cases in France direct public health attention to non-polio enteroviruses and their rare but severe clinical presentations. The risk factors for a severe course and the underlying causative mechanisms still need to be elucidated. The hospital-based enterovirus surveillance in France and Italy is voluntary, but these two reports highlight the need for active surveillance protocols in all cases with unexpected clinical presentations. In addition, complete genome sequencing could help with precise typing and molecular characterisation of emerging and re-emerging pathogenic variants, including identifying recombinant strains.

Ethical statement

Ethical approval was not needed for this retrospective study because the study was part of routine management and treatments for children. Nevertheless, the children's parents have been informed and consented to this report.

Funding statement

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Data availability

Data of this study are available in the manuscript.

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Conflict of interest

None declared.

Authors' contributions

AP and AB wrote the first draft of the manuscript. AB, ADC, SZ, TAF, MA, CP, IP and EB took care of the children/mothers during illness. FG, GF and AMGP were involved in the analysis of the epidemiological and virological data of enterovirus infections and in the sequence analyses. SP and FR analysed the clinical specimens and collected virological data of the neonates. EB, FB and SG revised the manuscript and supervise. All authors were involved in the study group on severe neonatal enterovirus infections, contributed to the manuscript and approved the final version.

References

1. World Health Organization (WHO). Enterovirus infection – France. Geneva: WHO; 2023Available from: https://www.who.int/emergencies/disease-outbreak-news/item/2023-DON469

2. Grapin M, Mirand A, Pinquier D, Basset A, Bendavid M, Bisseux M, et al. Severe and fatal neonatal infections linked to a new variant of echovirus 11, France, July 2022 to April 2023. Euro Surveill. 2023;28(22):2300253. https://doi.org/10.2807/1560-7917.ES.2023.28.22.2300253 PMID: 37261730
3. European Centre for Disease Prevention and Control (ECDC). Weekly bulletin. Communicable Disease Threats Report. Week 18, 30 April–6 May 2023. Stockholm: ECDC; 2023. Available from: https://www.ecdc.europa.eu/en/publications-data/communicable-disease-threats-report-30-april-6-may-2023-week-18
4. Kufner V, Plate A, Schmutz S, Braun DL, Günthard HF, Capaul R, et al. Two years of viral metagenomics in a tertiary diagnostics unit: evaluation of the first 105 cases. Genes (Basel). 2019;10(9):661. https://doi.org/10.3390/genes10090661 PMID: 31470675
5. Borges V, Pinheiro M, Pechirra P, Guiomar R, Gomes JP. INSaFLU: an automated open web-based bioinformatics suite "from-reads" for influenza whole-genome-sequencing-based surveillance. Genome Med. 2018;10(1):46. https://doi.org/10.1186/s13073-018-0555-0 PMID: 29954441
6. Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, et al. IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. Mol Biol Evol. 2020;37(5):1530-4. https://doi.org/10.1093/molbev/msaa015 PMID: 32011700
7. Bubba L, Broberg EK, Jasir A, Simmonds P, Harvala H, Redlberger-Fritz M, et al. Circulation of non-polio enteroviruses in 24 EU and EEA countries between 2015 and 2017: a retrospective surveillance study. Lancet Infect Dis. 2020;20(3):350-61. https://doi.org/10.1016/S1473-3099(19)30566-3 PMID: 31870905
8. McWilliam Leitch EC, Cabrerizo M, Cardosa J, Harvala H, Ivanova OE, Kroes AC, et al. Evolutionary dynamics and temporal/geographical correlates of recombination in the human enterovirus echovirus types 9, 11, and 30. J Virol. 2010;84(18):9292-300. https://doi.org/10.1128/JVI.00783-10 PMID: 20610722
9. Chuang YY, Huang YC. Enteroviral infection in neonates. J Microbiol Immunol Infect. 2019;52(6):851-7. https://doi.org/10.1016/j.jmii.2019.08.018 PMID: 31607572
10. Wang P, Xu Y, Liu M, Li H, Wang H, Liu Y, et al. Risk factors and early markers for echovirus type 11 associated haemorrhage-hepatitis syndrome in neonates, a retrospective cohort study. Front Pediatr. 2023;11:1063558. https://doi.org/10.3389/fped.2023.1063558 PMID: 37090924

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Original article

Impact of SARS-CoV-2 Omicron and Delta variants in patients requiring intensive care unit (ICU) admission for COVID-19, Northern Italy, December 2021 to January 2022

Antonio Piralla[a,*], Francesco Mojoli[b,c], Laura Pellegrinelli[d], Ferruccio Ceriotti[e], Antonia Valzano[e], Giacomo Grasselli[f], Maria Rita Gismondo[g], Valeria Micheli[g], Antonio Castelli[h], Claudio Farina[i], Marco Arosio[i], Ferdinando Luca Lorini[j], Diana Fanti[k], Andrea Busni[k], Matteo Laratta[l], Fabrizio Maggi[m], Federica Novazzi[m], Luca Cabrini[n], Anna Paola Callegaro[o], Roberto Keim[p], Giuseppe Remuzzi[q], Annalisa Cavallero[r], Sergio Maria Ivano Malandrin[r], Roberto Rona[s], Federica Giardina[a], Guglielmo Ferrari[a], Federica Zavaglio[a], Piera D'angelo[a], Cristina Galli[d], Laura Bubba[d], Sandro Binda[d], Massimo Oggioni[e], Sara Colonia Uceda Renteria[e], Patrizia Bono[e], Andreina Baj[m], Francesca Drago Ferrante[m], Davide Guarneri[i], Marco Tonelli[i], Gavino Napolitano[i], Alice Nava[k], Lorenzo Romeo[t], Elena Nicolini[t], Rea Valaperta[o], Ludovica Varisano[o], Caterina Mele[u], Lucia Liguori[u], Monica Raggi[r], Silvia Mongodi[b], Michele Pagani[b], Paolo Severgnini[v], Dario Gasberti[n], Ezio Bonanomi[j], Paolo Gritti[j], Francesco Marrazzo[l], Ilaria Giovannini[l], Noemi Sacchi[p], Orlando Sagliocco[p], Danilo Cereda[w], Sabrina Buoro[w], Fausto Baldanti[a,c], Elena Pariani[d]

[a] *Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy*
[b] *Anaesthesia and Intensive Care, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy*
[c] *Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy*
[d] *Department of Biomedical Sciences for Health, University of Milan, Milan, Italy*
[e] *Clinical Laboratory, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy*
[f] *Department of Anesthesia, Critical Care and Emergency, Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico, Milan, Italy*
[g] *Laboratory of Clinical Microbiology, Virology and Bioemergencies, ASST Fatebenefratelli Sacco, Milan, Italy*
[h] *Division of Anesthesiology and Intensive Care, ASST Fatebenefratelli Sacco, Milan, Italy*
[i] *Microbiology and Virology Laboratory, ASST "Papa Giovanni XXIII", Bergamo, Italy*
[j] *Department of Emergency and ICU, ASST "Papa Giovanni XXIII", Bergamo, Italy*
[k] *Laboratory of Clinical Microbiology, ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy*
[l] *Department of Anesthesia and Intensive Care Ospedale Niguarda, Università degli studi Milano Bicocca, Milan, Italy*
[m] *Department of Medicine and Surgery, University of Insubria, Varese, Italy*
[n] *Intensive Care and Anesthesia Unit, Ospedale di Circolo e Fondazione Macchi, Varese, Italy*
[o] *Laboratory Medicine Unit, ASST Bergamo Est, Italy*
[p] *Intensive Care Unit, ASST Bergamo Est, Italy*
[q] *Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Italy*
[r] *Microbiology Laboratory San Gerardo Hospital, Monza, Italy*
[s] *Department of Anesthesia and Intensive Care Medicine, San Gerardo Hospital ASST Monza, Monza, Italy*
[t] *Residency in Microbiology and Virology, Università degli Studi di Milano, Milan*
[u] *Laboratory of Immunology and Genetics of Rare Diseases, Istituto di Ricerche Farmacologiche Mario Negri IRCCS*
[v] *Department of Biotechnology and Life, Ospedale di Circolo e Fondazione Macchi, University of Insubria, Varese, Italy*
[w] *Direzione Generale Welfare, Regione Lombardia, Milan, Italy*

* Corresponding author.
E-mail address: a.piralla@smatteo.pv.it (A. Piralla).

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ABSTRACT

This multicenter observational study included 171 COVID-19 adult patients hospitalized in the ICUs of nine hospitals in Lombardy (Northern Italy) from December, 1st 2021, to February, 9th 2022. During the study period, the Delta/Omicron variant ratio of cases decreased with a delay of two weeks in ICU patients compared to that in the community; a higher proportion of COVID-19 unvaccinated patients was infected by Delta than by Omicron whereas a higher rate of COVID-19 boosted patients was Omicron-infected. A higher number of comorbidities and a higher comorbidity score in ICU critically COVID-19 inpatients was positively associated with the Omicron infection as well in vaccinated individuals. Although people infected by Omicron have a lower risk of severe disease than those infected by Delta variant, the outcome, including the risk of ICU admission and the need for mechanical ventilation due to infection by Omicron versus Delta, remains uncertain. The continuous monitoring of the circulating SARS-CoV-2 variants remains a milestone to counteract this pandemic.

Introduction

Assessing the clinical severity of infection with new SARS-CoV-2 variants is crucial to ensure the public health response in terms of control measures and mitigation strategies and to support clinicians in patients' management - particularly in case of severe disease. Since its emergence in November 2021, the SARS-CoV-2 Omicron variant of concern (VOC) (lineage B.1.1.529) has rapidly spread, replacing SARS-CoV-2 Delta (lineage B.1.617.2) and its AY.xx sub-lineages in most European countries [1]. Preliminary findings have indicated that the effectiveness of COVID-19 vaccines is significantly lower against Omicron compared to the Delta variant [2,3]. On the contrary, recent studies have suggested that Omicron infection is associated with less severe disease as it has a lower replication efficiency in lungs than other SARS-CoV-2 variants [4,5]. A reduction of 36–73% in the risk of hospitalization for Omicron compared with Delta has been reported [5,6].

This study aimed at comparing the epidemiological features of Omicron and Delta variants in patients tested positive for SARS-CoV-2 RNA who required intensive care unit (ICU) for COVID-19 in Lombardy (Northern Italy, nearly 10 million inhabitants) during the period of transition from Delta (December 2021) to Omicron dominance (January 2022).

Material and methods

Study population

This multicentre study included adult patients (> 18 years old) hospitalized in the ICUs of nine hospitals in Lombardy from 1st December 2021 to 9th February 2022. Respiratory specimens were collected for SARS-CoV-2 diagnosis and processed at the Microbiology and Virology laboratory of each hospital using commercial multiplex real-time RT-PCR assays. Genotyping of SARS-CoV-2 VOCs was carried out by means of real-time RT-PCR screening tests targeting specific single nucleotide polymorphisms in the S gene, or by whole genome sequencing or partial sequencing of the S gene.

For each patient, the following information were collected: (1) age and gender; (2) date of hospitalization; (3) COVID-19 vaccination status (i.e. no vaccinated, completed vaccination with two doses ≥120 days before, completed vaccination with two doses <120 days before, completed vaccination with booster doses ≥7 days before); (5) presence or absence of underlying comorbidities defined to increase the risk of severe COVID-19 (6). These underlying comorbidities were divided into: (i) medium risk (individual with diseases or other conditions that cause a moderate risk of severe COVID-19) and including diabetes mellitus, obesity (BMI ≥ 30 kg/m^2), autoimmune diseases, chronic cardiac diseases and hypertension, chronic lung disease; (ii) high risk (individuals with diseases or conditions that carry a high risk of severe COVID-19, also in younger people) and including chronic renal diseases, solid organ transplant, active malignancy, and immunocompromised patients.

Statistical analyses

Categorical variables were summarized by use of frequency distributions and compared by use of Pearson's χ^2 test and Fisher Exact tests and *t*-test and one-way ANOVA were used for comparing continuous independent variables. The frequency was expressed as crude proportion with corresponding 95% confidence interval (95% CI) calculated by Mid-P exact test assuming a normal distribution. The risk of infection was expressed as the number of individuals with Omicron or Delta laboratory-confirmed infection out of the total number of individuals. The conditional maximum-likelihood estimate (CMLE) of odds ratios (OR) with corresponding 95% CI were calculated.

The study population was categorized according to the age in the following age groups (years): age groups (years): 18–29, 30–44, 45–54, 55–64, 65–74, 75–84. An overall comorbidity score weighting the impact of comorbidities was assigned and summed one point for each comorbidity of medium risk and two points for each comorbidity of high risk [6].

The proportion of males in the study population was compared with those of the Italian population by using exact probability binomial level [7]); Differences were considered significant at a p-value <0.05. Statistical analysis was performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, CA, USA). This study did not require ethical approval as it is based on routine surveillance data on COVID-19.

Results

A total of 205 adult patients were included in the study and in 171/205 (83.4%) of these patients, the identification of SARS-CoV-2 VOC was successfully performed whereas in 34/205 (16.6%) this information was not available due to low viral load or unavailability of the respiratory samples, thus excluded from further analyses. Table 1 shows the characteristics of the study patients. Overall, 119 (78.9%) Delta/Delta-like and 36 (21.1%) Omicron cases were observed. The median age of patients was 62 years (range 21–84 years: Inter Quartile Range [IQR]: 16 years), and no difference ($p = 0.39$) in age was observed between patients infected by Delta (median 62 years, range 38–84 years; IQR: 17 years) and those infected by Omicron (median 61.5 years, range 21–79 years; IQR: 16.7 years). The 65–74 years age group was the most represented for both Delta ($n = 46$; 34.1%) and Omicron ($n = 9$; 30%) (Table 1). Overall, the proportion of

Table 1
Characteristics of patients hospitalized in ICU with detected SARS-CoV-2 variants in Lombardy (Italy), 1st December 2021 - 9th February 2022.

Categories Median age (range; IQR*)		All 171; 100% 62 (21–84;16)	Delta 135; 78.9% 62 (38–84;17)	Omicron 36; 21.1% 61.5 (21–79;16.7)
Age group (years)	18–29	1 (0.6%)	0 (0%)	1 (2.8%)
	30–44	5 (2.9%)	4 (3%)	1 (2.8%)
	45–54	41 (24%)	31 (23%)	7 (23.3)
	55–64	48 (28.1%)	39 (28.9%)	8 (26.7)
	65–74	58 (33.9%)	46 (34.1%)	9 (30.0)
	75–84	18 (10.5%)	15 (11.1%)	3 (10.0)
Gender	Female	51 (29.8%)	44 (32.6%)	7 (19.4%)
	Male	120 (70.2%)	91 (67.4%)	29 (80.6%)
Week of surveillance	48–2021	7 (4.1%)	7 (5.2%)	0 (0)
	49–2021	18 (10.5%)	18 (13.3%)	0 (0)
	50–2021	18 (10.5%)	18 (13.3%)	0 (0)
	51–2021	29 (17%)	28 (20.7%)	1 (3.3%)
	52–2021	30 (17.5%)	29 (21.5%)	1 (2.8%)
	1–2022	17 (9.9%)	11 (8.1%)	6 (16.7%)
	2–2022	23 (13.5%)	15 (11.1%)	8 (22.2%)
	3–2022	18 (10.5%)	6 (4.4%)	12 (33.3%)
	4–2022	7 (4.1%)	2 (1.5%)	5 (8.3%)
	5–2022	3 (1.8%)	0 (0%)	3 (3.3%)
	7–2022	1 (0.6%)	1 (0.7%)	1 (2.8%)
Comorbidities	No	52 (30.4%)	44 (32.6%)	8 (22.2%)
	Yes	119 (69.6%)	91 (67.4%)	28 (77.8%)
	1	45 (26.3%)	38 (28.1%)	7 (19.4%)
	2–3	65 (34.5%)	44 (32.6%)	15 (41.7%)
	>3	14 (8.8%)	9 (6.7%)	5 (16.7%)
COVID-19 vaccination	No	98 (57.3%)	83 (61.5%)	15 (41.7%)
	Yes	69 (40.4%)	48 (35.6%)	21 (58.3%)
	Unknown	4 (2.3%)	4 (3.0%)	0 (0)
	1 dose	4 (5.8%)	3 (6.3%)	1 (4.8%)
	2 doses <120 days	6 (8.7%)	4 (8.3%)	2 (9.5%)
	2 doses >120 days	41 (59.4%)	34 (70.8%)	7 (33.3%)
	3 doses	18 (26.1%)	7 (14.6%)	11 (35.6%)
Intubated	No	27 (15.8%)	21 (15.6%)	6 (16.7%)
	Yes	144 (84.2%)	114 (84.4%)	30 (83.3%)

males (120/171; 70.2%) was significantly higher than that of the Italian population (48.7%; $p < 0.001$) a similar proportion of males was observed in ICU patient groups infected by Delta (67.4%) and Omicron (80.6%; $p = 0.13$) (Table 1).

In order to compare the prevalence of VOCs infecting COVID-19 patients requiring ICU with variants circulating in the general population during the same period, VOCs point-prevalence data from national/regional surveys were considered [8]. According to temporal distribution Delta cases were overcame by Omicron cases since week 1 of 2022 (Fig. 1A). Delta cases peaked at week 51 of 2021 (29 cases; 21.5% of all Delta cases) while the peak of Omicron cases was at week 3–2022 (12 cases, 33.3% of all Omicron cases) (Table 1). No Omicron cases were observed in ICU inpatients hospitalized from week 48–2021 to week 50–2021. The Delta/Omicron ratio of cases decreased more rapidly in the general population than in our series of ICU patients. In detail, the Delta/Omicron ratio dropped below the value of 0.5 between week 52 of 2021 and 01 of 2022 in the general population, while in our ICU patients between week 2 and 3–2022 with a delay of at least two weeks (Fig. 1B).

Overall, 52 out of 171 (30.4%) patients had no comorbidities, whereas of the remaining 119 (66.9%) patients with comorbidities, 26.3% ($n = 45$) had one comorbidity, 34.5% ($n = 65$) two/three comorbidities, and 8.8% ($n = 14$) showed more than three comorbidities (Table 1).

The percentage of ICU inpatients with more than three comorbidities was statistical higher in the Omicron-infected group than that observed in the Delta-infected group (16.7% vs 6.7%; $p < 0.001$). By estimating the comorbidity score the mean score of comorbidities in ICU patients infected by Omicron was statistically higher than that observed in patients infected by Delta (1.8 vs 0.8; $p = 0.004$). The mean score of comorbidities in ICU patients with vaccination (at least

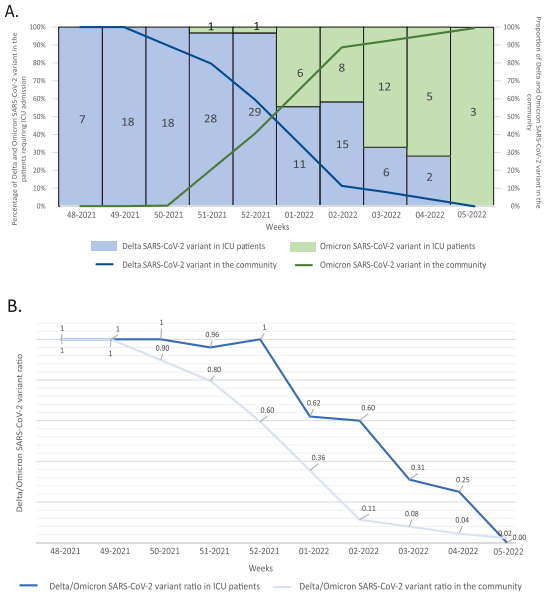

Fig. 1. (A) Percentage of ICU cases by infecting SARS-CoV-2 VOC (left) and proportion of Delta and Omicron cases by week of sampling (right, data obtained from national/regional point-prevalence flash survey (8)) Lombardy (Italy), 1st December 2021 – 9th February 2022. Number of ICU cases is reported within each bar. (B) Delta/Omicron ratio in our series of COVID-19 ICU patients as compared to the general population (data from national/regional point-prevalence surveys (8)).

one dose) was significantly higher than that identified in ICU patients with no vaccination (1.4 vs 0.8, $p = 0.005$). No difference in the mean score of comorbidities was observed in patients aged less than 64 years and those older than 65 years (0.9 vs 1.2; $p = 0.16$). No difference in the risk of infection by Delta (OR: 1.5; 95%CI: 0.9−2.4) neither Omicron (OR: 0.5; 95% CI: 0.2−1.3) was observed between individuals 64 years old or younger and those over 65 years.

Overall, only 69/171 (40.4%) ICU patients were COVID-19 vaccinated (Table 1). This proportion is significantly lower ($p < 0.001$) than the percentage of the Italian adult population receiving at least two doses of vaccine (88.4%) [9]. Among ICU Delta-positive inpatients, 61.5% (83/135) was COVID-19 unvaccinated whereas among ICU Omicron-positive inpatients, 41.7% (15/36) was COVID-19 unvaccinated ($p = 0.2$) (Table 1). The percentage of ICU COVID-19 boosted individuals was statistically higher in the group of Omicron-infected inpatients than that observed in the Delta-infected group (35.6% vs 14.6%; $p < 0.001$).

Overall, 144 out of 171 (84.2%) patients had been intubated requiring invasive mechanical ventilation with no correlation related to the Delta (114/135; 84.4%) and Omicron (30/36; 83.3%) infection.

Discussion

In most European countries, the epidemiological scenario of SARS-CoV-2 VOCs has rapidly changed since the beginning of December 2021 [1], when the Delta variant - which was largely predominant in the last six-eight months − was entirely replaced by the Omicron [5,6,10]. The mutational pattern of Omicron exhibited a greater genetic diversity compared to other VOCs that had been circulating previously [11], thus raising major concerns on its transmissibility, severity, and immune response escape ability. This swift and complete replacement of circulating VOCs led to an unpredicted rising in the number of cases primarily due to the higher transmissibility of Omicron, which is estimated to be 2.7−3.7 times more infectious than Delta variant in vaccinated and boosted people [12]. As expected, in the present study the consequence of the Omicron variant in COVID-19 patients requiring ICU were delayed by about two weeks compared to its circulation in the community, reflecting the timing for SARS-CoV-2 worsening symptoms and ICU admission (10−14 days).

A greater proportion of unvaccinated patients was infected by Delta than by Omicron; interestingly, in our ICU inpatients, the rate of ICU COVID-19 boosted individuals was statistically higher in the group of Omicron-infected inpatients than that observed in the Delta-infected group, in line with data uncovered the Omicron immune-evasive properties [2,3,12].

The percentage of ICU inpatients with more than three comorbidities was statistical higher in the Omicron-infected group than that observed in the Delta-infected group; moreover, the mean score of comorbidities was higher in patients infected by Omicron compared to Delta patients as well as in vaccinated compared to unvaccinated patients; similarly, a recent study has underlined that total number of comorbidities in critically COVID-19 hospitalized patients was positively associated with the Omicron group [14].

It has been demonstrated that people infected by Omicron have a lower risk of severe disease than those infected by Delta variant: in fact, both the incidence of ICU admission [15] and the mortality [16] decrease with the Omicron variant in comparison to Delta. Nevertheless, the outcome, including the risk of ICU admission and the need for mechanical ventilation due to infection by Omicron versus Delta remains uncertain. The outcome from SARS-CoV-2 infection however could be related to individual characteristics such as age, comorbidities, and prior immunity from vaccination or could be driven by viral markers of pathogenicity or individual biomarkers [13]. Among the limitations of the present study is that no follow-up data were available for the patients analyzed. Additionally, no comparisons with data obtained from patients admitted to general hospital wards (different from ICU) were performed.

Conclusions

Despite the not increased pathogenicity of breakthrough SARS-CoV-2 Omicron VOC infections from vaccinated individuals, the exceptionally high transmission levels of this variant have resulted in a significant increase in hospitalization, continuing to pose overwhelming demands on health care systems in most countries, and possibly leading to significant morbidity, particularly in vulnerable populations. The circulation of this more transmissible but less severe variant in countries with high vaccination coverage has endorsed the best clinical scenario in the worst epidemiological situation (nearly 90 million cases in less than one month worldwide). The continuous monitoring of the circulating SARS-CoV-2 variants remains a milestone to counteract this pandemic.

Author's contributions

AP, EP and FB designed the paper. LP, FC, AV, MRG, VM, CF, MA, DF, AB, FM, FN, APC, GR, AC, SMIM carried out the analysis. FM, GG, AC, FLL, ML, LC, RK, RR acquired and manage clinical data. AP, LP and EP conducted statistical analyses and created graphics. AP, LP, and EP wrote the manuscript, which was then reviewed and approved by the other authors. The members of the *COVID-19 laboratory group* collected the samples and performed SARS-CoV-2 typing while the *ICU clinicians' group* collected and managed clinical data. AP and EP had full access to all data in the study, and the corresponding author had final responsibility for the decision to submit for publication.

Declaration of Competing Interest

None declared.

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References

[1] European Centre for Disease Prevention and Control. Weekly epidemiological update: omicron variant of concern (VOC) – week 2 (data as of 20 January 2022) EU/EEA https://www.ecdc.europa.eu/en/news-events/weekly-epidemiological-update-omicron-variant-concern-voc-week-2-data-20-january-2022.

[2] Andrews N, Stowe J, Kirsebom F, Toffa S, Rickeard T, Gallagher E, Gower C, Kall M, Groves N, O'Connell AM, Simons D, Blomquist PB, Zaidi A, Nash S, Iwani Binti Abdul Aziz N, Thelwall S, Dabrera G, Myers R, Amirthalingam G, Gharbia S, Barrett JC, Elson R, Ladhani SN, Ferguson N, Zambon M, Campbell CNJ, Brown K, Hopkins S, Chand M, Ramsay M, Lopez Bernal J. Covid-19 vaccine effectiveness against the Omicron (B.1.1.529) variant. N Engl J Med 2022;386(16):1532–46 Apr 21. doi: 10.1056/NEJMoa2119451.

[3] Dejnirattisai W, Huo J, Zhou D, Zahradník J, Supasa P, Liu C, et al. SARS-CoV-2 Omicron-B.1.1.529 leads to widespread escape from neutralizing antibody responses. Cell 2022;185(3):467–84 Feb 3e15. doi: 10.1016/j.cell.2021.12.046.

[4] Iuliano AD, Brunkard JM, Boehmer TK, Peterson E, Adjei S, Binder AM, et al. Trends in disease severity and health care utilization during the early Omicron variant period compared with previous SARS-CoV-2 high transmission periods - United States, December 2020-January 2022. MMWR Morb Mortal Wkly Rep 2022;71(4):146–52 Jan 28. doi: 10.15585/mmwr.mm7104e4.

[5] Lewnard JA, Hong VX, Patel MM, Kahn R, Lipsitch M, Tartof SY. Clinical outcomes associated with SARS-CoV-2 Omicron (B.1.1.529) variant and BA.1/BA.1.1 or BA.2 subvariant infection in Southern California. Nat Med 2022;28(9):1933–43 Sep. doi: 10.1038/s41591-022-01887-z.

[6] Veneti L, Bøås H, Bråthen Kristoffersen A, Stålcrantz J, Bragstad K, Hungnes O, et al. Reduced risk of hospitalisation among reported COVID-19 cases infected with the SARS-CoV-2 Omicron BA.1 variant compared with the Delta variant,

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A new case of Echovirus 11 neonatal fulminant hepatitis involving male twins in a Northern Italy Tertiary University Hospital: Insight on a possible immunological clue

Federica Novazzi[1,2,*], Antonio Piralla[3], Simona Perniciaro[4], Angelo Paolo Genoni[1,2], Francesca Drago Ferrante[2], Federica Giardina[5], Guglielmo Ferrari[3], Laura Pellegrinelli[6], Nicola Clementi[7], Elena Pariani[6], Ivan Zanoni[8], Fausto Baldanti[3,5], Massimo Agosti[9], Nicasio Mancini[1,2]

[1] University of Insubria, Department of Medicine and Technological Innovation Varese, Varese, Italy
[2] ASST Sette Laghi, Laboratory of Medical Microbiology and Virology, Varese, Italy
[3] Fondazione IRCCS Policlinico San Matteo, Microbiology and Virology Department, Pavia, Italy
[4] Ospedale Filippo del Ponte Ostetricia e Ginecologia, Neonatology and NICU, Del Ponte Hospital, Varese, Italy
[5] University of Pavia, Department of Clinical, Surgical, Diagnostic and Pediatric Sciences Pavia, Pavia, Italy
[6] University of Milan, Department of Biomedical Sciences for Health Milano, Milano, Italy
[7] IRCCS San Raffaele Hospital, Milan, Italy, Department of Biomedical Sciences for Health, Milano, Italy
[8] Harvard University, Boston Children's Hospital Cambridge, Cambridge, USA
[9] University of Insubria, Department of Medicine and Surgery, Varese, Italy

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ABSTRACT

The European Center for Disease Prevention and Control has reported 19 cases of severe echovirus 11 infections in neonates since 2022, nine of which were fatal. We report a new fatal neonatal case that occurred in a male twin for which we evaluated the respiratory and intestinal mucosal innate immune response.

Introduction

Infections caused by enteroviruses (EV) are a significant warning in the neonatal disease burden and public health [1–5]. The main clinical signs of EV infections are fever, lethargy, and lack of appetite. EV infection usually resolves without complications. However, in a small percentage of cases, it can cause serious diseases such as hepatitis [1–5]. In this report, we describe a fatal neonatal case of congenital echovirus 11 (EV11) infection and the mucosal expression of innate immune mediators in the respiratory and intestinal mucosa of the infected twins.

Case series

Case description and microbiological investigation

In December 2023, two male dizygotic twins (T1 and T2) were born to a healthy mother at 35 weeks of gestation by cesarean section at the University Hospital of Varese (Italy). T1 was born with a birth weight of 2.710 g and an Apgar score of 10 at 5 minutes but, after 1 week, was transferred to the neonatal intensive care unit (NICU) and intubated because of respiratory distress and meningeal signs. In contrast, T2 (birth weight: 2.000 g; Apgar score 9 at 5 minutes) did not show any clinical signs of infection.

The clinical course of T1 was complicated by coagulopathy and thrombocytopenia requiring plasma and platelet concentrates infusion. X-ray done showed bi-basal accentuation of lung interstitium compatible with transient tachypnea of the newborn. Clear laboratory signs of massive hepatic involvement were reported (aspartate transaminase >7000 mU/ml, alanine transaminase 729 mU/ml) with concurrent multi-organ involvement (troponin 279 ng/l, creatinine 1.27 mg/dl). Cerebrospinal fluid (CSF) and bronchoalveolar lavage (BAL) were sent to the laboratory for culture-based and molecular assays. CSF white blood cell count was 20 cells/μl, protein concentration was 61 mg/dl and CSF glucose was 65 mg/dl. CSF was tested using the FilmArray Meningitis/Encephalitis panel (BioMerieux, Marcy l'Étoile, France) detects 14 of the most common pathogens in encephalitis, including bacteria, fungi

* Corresponding author: Tel.: +39 3337979234.
E-mail address: federica.novazzi@uninsubria.it (F. Novazzi).

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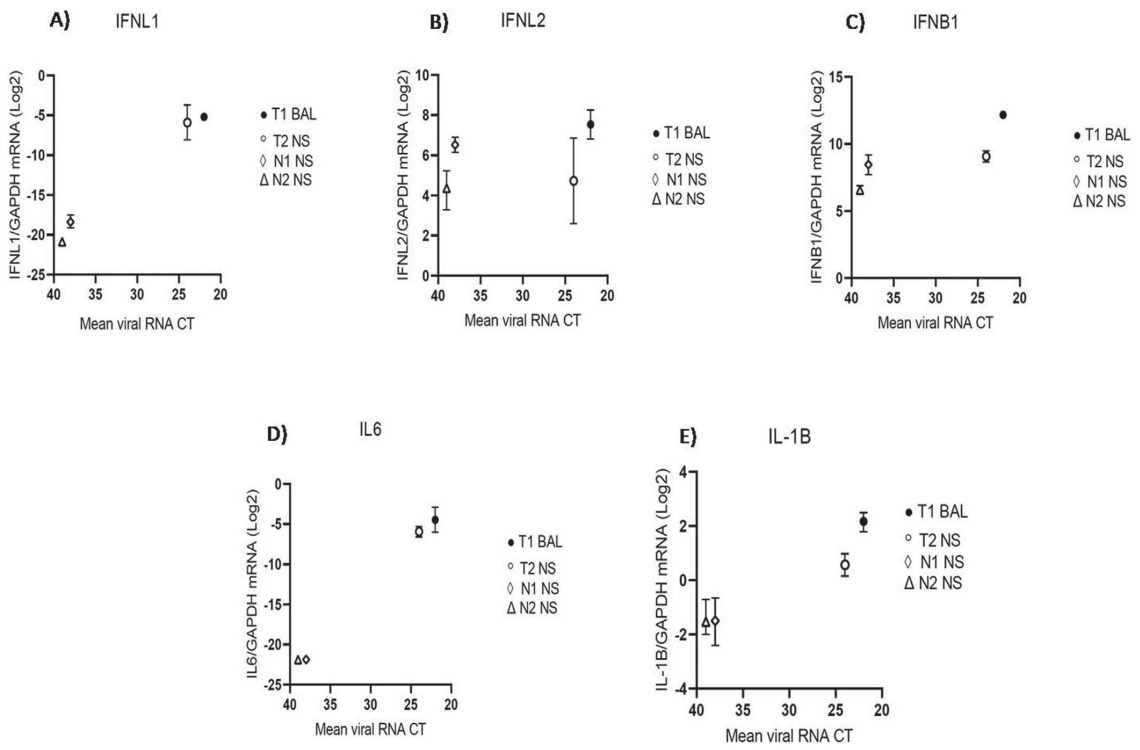

Figure 1(a). The mucosal expression of innate immune mediators parallels EV load in respiratory samples of infected newborns.
IFNL1 (A), IFNL2 (B), IFNB1 (C), IL 6 (D), and IL1B (E) mRNA expression was evaluated in respiratory samples of EV-positive newborns described in this report. Each symbol represents a newborn. The median with range is depicted. Cytokine mRNA expression is plotted against mean viral RNA CT as log2 (gene/GAPDH mRNA + 0.5 x gene-specific minimum).

and viruses, while the BAL was processed with the FilmArray Respiratory Panel 2 plus panel (BioMerieux, Marcy l'Étoile, France) recognizing 34 targets of which certain antimicrobial resistance genes as well as the most common causes of bacterial, viral, and yeast pneumonia.

Both samples tested positive for EV. The clinician in charge was immediately notified and EV genotyping was performed. Antibiotic treatment with ceftazidime and vancomycin plus antiviral acyclovir was started. EV RNA was also detected in rectal swab (RS) and plasma from T1 using the quantitative EV ELITe MGB reverse transcription-polymerase chain reaction test (ELITech Group, Turin, Italy) featuring EV RNA viral load of 83.803 copies/mL (corresponding to cycle threshold [CT] 28) and 676.241 copies/ml (CT 25), respectively. Interestingly, at the respiratory level, a preferential involvement of the lower tract was observed in T1, featuring a negative nasal swab (NS) notwithstanding the strongly positive BAL (2.245.180 copies/ml - CT 22).

NS and RS samples were collected also from T2, despite the absence of any sign of ongoing infection. Both samples tested positive, featuring 5.557 copies/ml (CT 32) in RS and 1.708.215 copies/ml (CT 24) in NS. The asymptomatic mother (M) of T1 and T2 was also tested for EV at the nasal, rectal, and vaginal (VS) level, resulting negative in NS but positive in both RS (693 copies/ml; CT 35) and VS (<500 copies/ml; CT 38).

At the same time, EV surveillance was initiated in the NICU, including 24 NS and 24 paired RS from each little inpatient and 28 NS from healthcare workers. Two newborns (N1 and N2) admitted to the NICU tested positive for low-grade EV (<500 copies/ml - CT 38) in the NS, whereas none of the NS from healthcare workers was positive. Neither N1 nor N2 showed any clinical sign of infection and both tested negative within a few days.

Methods

Enterovirus molecular subtyping

EV genotyping was performed by Sanger sequencing of conserved genomic regions (5′UTR, 2 C, $3D^{pol}$) [6] on all EV-positive samples, including CSF, BAL, RS, and blood for T1, NS and RS for T2, RS and VS for M, and NS for N1 and N2. All samples were positive for E11 featuring a very high nucleotide identity with the strains described in France (97.73%) and in Italy (97.28%). The E11-positive samples were sent to the regional reference laboratories (Fondazione IRCCS Policlinico San Matteo, Pavia and The Department of Biomedical Sciences for Health, University of Milan) for whole genome sequencing, (accession numbers PP498690 and PP498691), evidencing an average nucleotide identity of 98.9% with other Italian strains [5].

Evaluation of respiratory and rectal mucosal innate immune response

It is well known that a balanced mucosal innate immune response is fundamental to properly control viral replication and prevent more severe complications, as repeatedly confirmed by the most severe COVID-19 cases driven by SARS-CoV-2 infection [7–11]. Based on this evidence, we assessed the levels of a panel of innate immune mediators in the respiratory and intestinal mucosa. In particular, RS and NS, or BAL for T1, were utilized to assess multiple inflammatory mediators by quan-

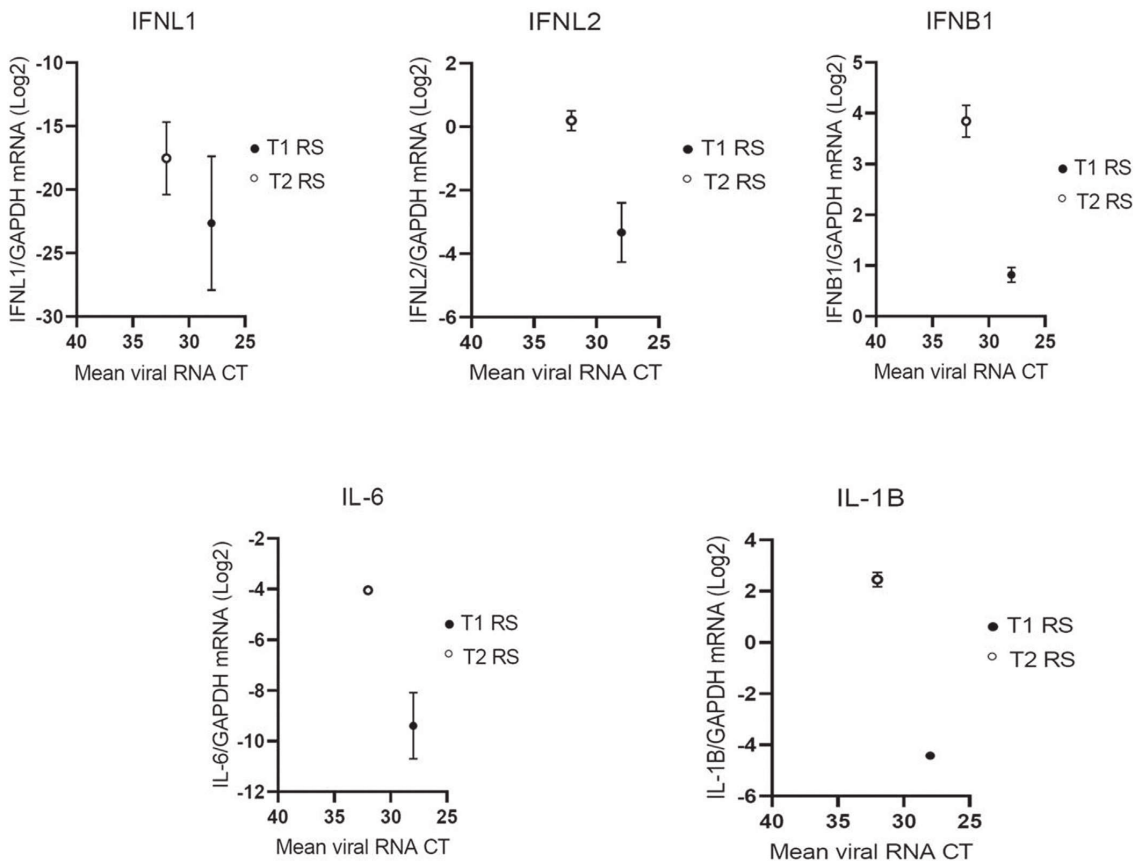

Figure 1(b). The mucosal expression of innate immune mediators is lower in the newborn with a fatal outcome, suggesting the ineffective primary control of EV replication at the gastrointestinal level.

IFNL1 (A), IFNL2 (B), IFNB1 (C), IL 6 (D), and IL1B (E) mRNA expression was evaluated in rectal swabs of EV-positive newborns described in this report. Each symbol represents a newborn. The median with range is depicted. Cytokine mRNA expression is plotted against mean viral RNA CT as log2 (gene/GAPDH mRNA + 0.5 x gene-specific minimum). Only positive EV-11 positive rectal swabs were reported. BAL, bronchoalveolar lavage; CT, cycle threshold; EV, enterovirus; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; mRNA, messenger RNA; NS, nasal swab; RS, rectal swab.

titative polymerase chain reaction. Based on our previous studies in patients with severe COVID-19 [9], we included both antiviral (interferon beta-1 [IFN-B1] and IFN lambda -1 and -2 [IFNL1 and IFNL2]) and pro-inflammatory (interleukin-1-beta [IL1B] and IL-6) molecules in our analysis. The level of each cytokine was then compared to the local E11 load for all the positive newborns described in our report (T1, T2, N1, and N2).

At the respiratory level, the expression of both antiviral and pro-inflammatory cytokines was apparently driven by E11 replication and local viral load (Figure 1a).

Interestingly, an opposite trend was observed when analyzing the RS, in which we found very low cytokine levels in T1, the newborn with the highest E11 load, which was also the patient experiencing the worst outcome (Figure 1b).

Discussion

EV, including echoviruses such as E11, are a common cause of pediatric infections often with seasonal occurrence and a self-limiting clinical evolution, although newborns may present a higher risk of severe complications. Recently, fatal neonatal cases with massive liver failure caused by a new variant of E11 have been described, with a peculiar involvement of male non-homozygotic twins [2,3]. Our report confirms that this variant is still circulating in Europe and deserves attention to control and limit its spread. No specific evidence regarding viral factors justifying the increased pathogenicity of the new E11 variants has been described to date but, among other factors, the severity of the infection may also be determined by a differential immune control of the viral replication at the mucosal level. Local viral replication may then influence the risk of systemic spread. Most EVs infect preferentially the gastrointestinal tract (and/or the upper respiratory tract) with a possible subsequent spread to several organs, such as the central nervous system, the heart, or, as in the case of E11, the liver [10]. In the reported cases, we observed a massive local interferon response both in the airways and intestine in the newborn with no clinical complications. On the contrary, the sibling undergoing the fatal outcome was characterized by a potent antiviral response in the airways, but by a very low response at the gastrointestinal level associated with a very high viral load in the rectum. We, thus, speculate that the lack of viral control in the intestinal mucosa is a key factor favoring the systemic dissemination of E11 and, possibly, its massive spread from the gut to the liver and to other organs.

Conclusion

As also recently described by ongoing surveillance [4,5], our report confirms that E11 is still circulating in Italy and can be fatal in newborns. Awareness must be maintained via control and prevention measures to minimize the spread of EV infections in neonatal units. The elements leading to the increased pathogenicity of the circulating E11 strain are not clear yet but, beyond still unidentified viral factors, host-related factors certainly deserve attention. No definitive conclusions may be drawn by our report, but the low levels of the antiviral innate immune response observed at the gastrointestinal level in the newborn with the fatal outcome support the hypothesis of viral spread from mucosal tissue to other organs, starting from the liver. Multi-organ involvement upon E11 infection, and stochastically upon EV infection, is associated with severe cases and the factors favoring it warrant to be studied in larger cohorts. These observations may strengthen the importance of several proposed antiviral approaches (potentially including all viruses with a pivotal phase of mucosal replication) focused on the importance of potentiating the local antiviral innate containment in at-risk categories of patients [11].

Declarations of competing interest

The authors have no competing interests to declare.

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Ethical statement

Ethical approval was not needed for this retrospective study because the study was part of routine management and treatments for children. Nevertheless, parents of children have been informed and consented to this report.

Author contributions

All the authors contributed significantly to this manuscript. Federica Novazzi wrote the first draft; Angelo Paolo Genoni, Francesca Drago Ferrante, Federica Maria Giardina, Guglielmo Ferrari, and Laura Pellegrinelli analyzed the clinical specimens and processed sequencing; Fausto Baldanti, Antonio Piralla, Elena Pariani, Ivan Zanoni, Nicola Clementi, contributed to the critical analysis of the data and revised the manuscript; Massimo Agosti and Simona Perniciaro collected the clinical data and revised the manuscript; Antonio Piralla and Elena Pariani were involved in the sequence analyses; Nicasio Mancini designed the study and supervised the manuscript. All the authors reviewed and approved the final submission.

Data availability

The partial viral genome sequence is available in GenBank under accession number PP256153 (Tween1). The total viral genome sequences are available in GenBank under accession numbers PP498690 (Tween2) and PP498691 (Tween1).

References

[1] European Centre for Disease Prevention and Control (ECDC). Epidemiological update: echovirus 11 infections in neonates 19 July 2023, https://www.ecdc.europa.eu/en/news-events/epidemiological-update-echovirus-11-infections-neonates; 2023 [accessed 19 July 2023].

[2] Grapin M, Mirand A, Pinquier D, Basset A, Bendavid M, Bisseux M, et al. Severe and fatal neonatal infections linked to a new variant of echovirus 11, France, July 2022 to April 2023. *Euro Surveill* 2023;**28**:2300253. doi:10.2807/1560-7917.ES.2023.28.22.2300253.

[3] Piralla A, Borghesi A, Di Comite A, Giardina F, Ferrari G, Zanette S, et al. Fulminant echovirus 11 hepatitis in male non-identical twins in northern Italy, April 2023. *Euro Surveill* 2023;**28**:2300289. doi:10.2807/1560-7917.ES.2023.28.24.2300289.

[4] Pellegrinelli L, Galli C, Giardina F, Ferrari G, Uceda Renteria SCU, Ceriotti F, et al. Increased circulation of echovirus 11 in the general population and hospital patients as elicited by the non-polio enterovirus laboratory-based sentinel surveillance in northern Italy, 2023. *Int J Infect Dis* 2024;**142**:106998. doi:10.1016/j.ijid.2024.106998.

[5] Piralla A, Giardina F, Ferrari G, Gaiarsa S, Romano G, Pellegrinelli L, et al. Molecular characterization of emerging Echovirus 11 (E11) shed light on the recombinant origin of a variant associated with severe hepatitis in neonates. *J Med Virol* 2024;**96**:e29658. doi:10.1002/jmv.29658.

[6] Genoni A, Canducci F, Rossi A, Broccolo F, Chumakov K, Bono G, et al. Revealing enterovirus infection in chronic human disorders: an integrated diagnostic approach. *Sci Rep* 2017;**7**:5013. doi:10.1038/s41598-017-04993-y.

[7] Bastard P, Rosen LB, Zhang Q, Michailidis E, Hoffmann HH, Zhang Y, et al. Auto antibodies against type I IFNs in patients with life-threatening COVID-19. *Science* 2020;**370**:eabd4585. doi:10.1126/science.abd4585.

[8] Wang EY, Mao T, Klein J, Dai Y, Huck JD, Jaycox JR, et al. Diverse functional autoantibodies in patients with COVID-19. *Nature* 2021;**595**:283–8. doi:10.1038/s41586-021-03631-y.

[9] Sposito B, Broggi A, Pandolfi L, Crotta S, Clementi N, Ferrarese R, et al. The interferon landscape along the respiratory tract impacts the severity of COVID-19. *Cell* 2021;**184**:4953–68. doi:10.1016/j.cell.2021.08.016.

[10] Tapparel C, Siegrist F, Petty TJ, Kaiser L. Picornavirus and enterovirus diversity with associated human diseases. *Infect Genet Evol* 2013;**14**:282–93. doi:10.1016/j.meegid.2012.10.016.

[11] Walker FC, Sridhar PR, Baldridge MT. Differential roles of interferons in innate responses to mucosal viral infections. *Trends Immunol* 2021;**42**:1009–23. doi:10.1016/j.it.2021.09.003.

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Dynamics of viral DNA shedding and culture viral DNA positivity in different clinical samples collected during the 2022 mpox outbreak in Lombardy, Italy

Antonio Piralla [a,1], Davide Mileto [b,1], Alberto Rizzo [b], Guglielmo Ferrari [a], Federica Giardina [c], Stefano Gaiarsa [a], Greta Petazzoni [a], Micol Bianchi [b], Federica Salari [b], Fiorenza Bracchitta [b], Josè Camilla Sammartino [c], Alessandro Ferrari [a], Gloria Gagliardi [b], Alessandro Mancon [b], Claudio Fenizia [d], Mara Biasin [d], Francesca Rovida [c,a], Stefania Paolucci [a], Elena Percivalle [a], Alessandra Lombardi [b], Valeria Micheli [b], Silvia Nozza [e], Antonella Castagna [e], Davide Moschese [f], Spinello Antinori [g], Andrea Gori [h], Paolo Bonfanti [i], Roberto Rossotti [j], Antonella D'Arminio Monforte [k], Federica Attanasi [l], Marcello Tirani [m], Danilo Cereda [m], Fausto Baldanti [c,a,*], Maria Rita Gismondo [b,d], MPXV Lombardy Network

[a] Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
[b] Clinical Microbiology, Virology and Bioemergency Diagnostics, ASST Fatebenefratelli Sacco, Luigi Sacco Hospital, Milan, Italy
[c] Department of Clinical-Surgical, Diagnostic and Pediatric Sciences, Università degli Studi di Pavia, Pavia, Italy
[d] Department of Biomedical and Clinical Sciences "L.Sacco", University of Milan, Italy
[e] Clinic of Infectious Diseases, Vita-Salute University, San Raffaele Scientific Institute, Milan, Italy
[f] I Division of Infectious Diseases, Luigi Sacco Hospital, ASST Fatebenefratelli Sacco, Milan, Italy
[g] Department of Biomedical and Clinical Science, Università degli Studi di Milano, III Division of Infectious Diseases, ASST Fatebenefratelli Sacco, Milan, Italy
[h] Department of Infectious Diseases Ospedale "Luigi Sacco", Centre for Multidisciplinary Research in Health Science (MACH), University of Milan, Italy
[i] Department of Infectious Diseases, San Gerardo Hospital – University of Milano Bicocca, Monza, Italy
[j] Department of Infectious Diseases, ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy
[k] Clinic of Infectious Diseases, Department of Health Sciences, ASST Santi Paolo E Carlo, University of Milan, Milan, Italy
[l] Agenzia per la Tutela della Salute della Brianza, Monza, Italy
[m] Directorate General for Health, Lombardy Region, Milan, Italy

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Multiple samples

ABSTRACT

Background: Mpox virus (MPXV) has recently spread outside of sub-Saharan Africa. This large multicentre study was conducted in Lombardy, the most densely populated Italian region accounting for more than 40% of Italian cases. The present study aims to: i) evaluate the presence and the shedding duration of MPXV DNA in different body compartments correlating the MPXV viability with the time to onset of symptoms; ii) provide evidence of MPXV persistence in different body compartment as a source of infection and iii) characterize the MPXV evolution by whole genome sequencing (WGS) during the outbreak occurred in Italy.

Material and methods: The study included 353 patients with a laboratory-confirmed diagnosis of MPXV infection screened in several clinical specimens in the period May 24th - September 1st, 2022. Viral isolation was attempted from different biological matrices and complete genome sequencing was performed for 61 MPXV strains.

Results: MPXV DNA detection was more frequent in the skin (94.4%) with the longest median time of viral clearance (16 days). The actively-replicating virus in cell culture was obtained for 123/377 (32.6%) samples with a significant higher viral quantity on isolation positive samples (20 vs 31, $p < 0.001$). The phylogenetic analysis highlighted the high genetic identity of the MPXV strains collected, both globally and within the Lombardy region.

* Corresponding author. Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, 27100, Pavia, Italy.
E-mail address: f.baldanti@smatteo.pv.it (F. Baldanti).
[1] These authors have equally contributed.

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Conclusion: Skin lesion is gold standard material and the high viral load and the actively-replicating virus observed in genital sites confirms that sexual contact plays a key role in the viral transmission.

1. Introduction

Since May 2022, *Mpox virus* (MPXV) has been responsible for a global outbreak, the first related to *Orthopoxvirus* (OPXV) after *Smallpox virus* eradication, with more than 86,746 cases and 112 fatalities worldwide, the majority of which in countries where MPXV was never reported before [1]. Until April 12th, 2023, European Union (EU) accounted for 25,874 cases, with a hospitalization rate of 6% [2]. The 2022 MPXV outbreak mainly involves men who have sex with men (MSM), displaying characteristics of a sexually transmitted infection (STI) [3–7]. Mpox virus is a linear double-stranded DNA zoonotic virus belonging to the *Orthopoxvirus* genus *Poxviridae* family, to which the WHO has attributed an epidemic, or even a pandemic potential. Two distinct genetic clades were described: the central African (Congo Basin, Clade I), which causes a more severe disease, and the west African clade (Clade II) causing a milder disease and associated with the 2022 outbreak (named Clade IIb) [3,8,9]. Human-to-human transmission occurs through direct contact with skin lesions and infectious body fluids or from indirect contact with contaminated materials, such as clothing or linens. Furthermore, prolonged face-to-face contact is necessary for human-to-human transmission through large respiratory droplets, sneezing, or coughing [10]. Incubation period lasts 3–17 days, followed by a two-stage disease of 2–4 weeks. In detail, the invasive stage (0–5 days) is characterized by fever, lymphadenopathy, headache, myalgia, and fatigue, while in the second stage, distinctive skin lesions occur, and their number is descriptive of disease severity [10,11]. Severe outcomes are usually rare [12], and the overall case fatality has been assessed up to 3–6%, depending on health conditions, and access to therapies [2,12]. Immune deficiencies or coinfection may lead to a faster progression to a worse clinical picture [13,14]. The recent emergency has stepped up the research on MPXV pathogenesis, transmission, diagnosis, and management, which, according to recent bibliometric analyses, is still too limited [13,14]. New data on route of transmission, the extent of asymptomatic infection, and correspondence between PCR cycle threshold value and, infectivity are recently reported by several studies in France, Spain and Australia [15–17]. Lombardy, the most densely populated Italian region (10 million inhabitants) accounted for more than 40% of national cases [18].

In this epidemiological scenario, the present study described clinical and virological data collected during the monitoring of 353 MPXV-positive subjects. Clinical specimens from different anatomical sites (oropharyngeal, anal, urethral, and skin lesion swabs, together with plasma, urine, and semen) were collected, and analysed to i) evaluate the presence of MPXV DNA in different body compartments; ii) measure the shedding duration of MPXV DNA; iii) correlate the MPXV viability with the time to onset of symptoms iv) provide evidence of MPXV persistence in different body compartment as a source of infection and v) finally, characterize the MPXV evolution by whole genome sequencing (WGS) during the outbreak occurred in Italy.

2. Material and Methods

2.1. Study population

A total of 793 subjects were tested during the Regional Surveillance Program using molecular assays targeting *Orthopoxvirus* and MPXV-specific real-time PCR as confirmation in the period May 24th - September 1st, 2022. The study included 353 individuals with a laboratory-confirmed diagnosis of MPXV infection. Infection was defined as the detection of viral DNA in at least one of tested biological specimens. Demographical, clinical, and epidemiological data were collected at first access and during the follow-up period and reported in a Regional shared database. Follow-up samples were available only for a series of patients included in the present study and described below.

All diagnostic and experimental procedures were performed in the two Regional Mpox virus References Centres: i) Laboratory of Clinical Microbiology, Virology, and Bioemergencies, "L. Sacco" University Hospital (Milan) and ii) Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo (Pavia). This study complies with the Declaration of Helsinki. The institutional review board approved the study and the use of residual clinical specimens for complete genome characterization of both institutions (no.2022/ST/124 and no.44007/2022).

2.2. Molecular diagnosis

The following biological matrices were collected and analysed for diagnostic and follow-up monitoring purpose: swabs from the oropharynx, skin lesion, anus, and urethra in Universal Transport Medium swabs (UTM-RT®; COPAN Diagnostics, Italy), blood, urine, and seminal fluid samples. DNA was extracted with QIAsymphony® DSP Virus/Pathogen Kit on QIAsymphony® SP automated platform (QIAGEN, Germany). OPXV screening was performed by means of RealStar® *Orthopoxvirus* PCR Kit 1.0 (Altona DIAGNOSTICS) real-time PCR, targeting variola virus and non-variola OPXV species (*Cowpox virus*, *Mpox virus*, *Raccoonpox virus*, *Camelpox virus*, *Vaccinia virus*). The presence of MPXV DNA was confirmed using a specific homemade real-time PCR protocol as previously described [19,20]. Results were given as cycle of quantification (Cq) values that inversely reflects viral load. A Cq $\geq$ 40 was set as negative cut-off.

2.3. Viral isolation

A total of 377 MPXV-positive samples were used to attempt viral isolation. An aliquot of 200 μL of each transport medium was plated in duplicate in 24-well plates containing 80–90% confluent Vero E6 cells, adding 800 μL of Dulbecco's Modified Eagle Medium with L-glutamine (Gibco ThermoFisher Scientific) supplemented with 2% of heat-inactivated fetal bovine serum (Gibco ThermoFisher Scientific) and 1% penicillin-streptomycin (5000 U/mL; Pen-Strep, Gibco ThermoFisher Scientific). Plates were incubated at 37 °C and at 5% CO2 atmospheric pressure and checked every 24 h. Wells were monitored daily for virus-induced cytopathic effect (CPE) showing typical monolayer separation and cell rounding and CPE readings were recorded by two independent readers for each sample. In the majority of samples with viable virus, extensive CPE was observed between 2 and 6 days.

2.4. Viral sequencings

In a series of cultured-positive samples, (nearly 10% of total) WGS was performers. In detail, a Nextera XT paired-end library (Illumina) was prepared using 1 ng of DNA extracted from culture supernatants, using a QIAamp DNA mini kit (Qiagen). The library was sequenced on the MiSeq platform using paired-end sequencing, with a read length of 150 nucleotides. Sequencing reads were mapped to a collection of high-quality human Mpox virus genomes. Mapping reads were assembled using Spades [21]. The sequence of all 179 viral genes (reference: NC_063383) was retrieved from the 61 genomes obtained in this study and from a collection of high-quality genomes obtained from GISAID (https://gisaid.org/, N = 523). Genes were aligned, concatenated, and

used to infer a maximum-likelihood phylogeny with the software IQ-TREE [22]. Gene alignments were further analysed to obtain all genic mutations and their nucleotide-substitution pattern. Lastly, the association between all mutations and the topology of the phylogenetic tree was tested using Cramer's V index.

2.5. Genome assembly

All Sequencing reads pairs (N = 61) were quality-filtered using Trimmomatic1, which was also used to trim sequencing primers and low-quality bases at both ends of all reads. High-quality reads were mapped to a collection of 121 MPXV (available in the NCBI Assembly database on May 31, 2022) using Bowtie22. Mapping reads were assembled using SPAdes3 in "careful" mode.

2.6. Phylogenetic analyses

All high-coverage complete genomes with a full collection date available and relative to viral samples collected after January 1, 2018 were retrieved from the GISAID database on October 17, 2022 (gisaid.org; N = 618). The Coding Sequences (CDSs) of genome NC_063383 (N = 179) were retrieved from the NCBI repository and blast-searched on both the 61 genomes of the study and the database genomes. Database genomes from which it was not possible to retrieve all CDSs were excluded from the study. The final dataset included the CDSs of 585 genomes (including 61 study genomes, 523 database genomes, and the reference). All CDSs were aligned using MAFFT4 and concatenated. The resulting alignment of 166090 bp was used as input IQ-TREE5 to infer the phylogeny, using the TN + F + I + I + R6 substitution model (chosen according to BIC within the IQ-TREE internal pipeline).

2.7. Mutation analysis

All CDS alignments were scanned triplet by triplet for mutations from the reference using an in-house python script. Each mutation was classified as synonymous or non-synonymous and the nucleotide substitution pattern was extracted. Lastly, the occurrences of each mutation were counted in the genomes inside and outside the phylogenetic cluster including the 2022 outbreak. The Association of each mutation with the 2022 outbreak cluster was assessed using the Cramer's V index, after the removal of all occurrences of non-standard nucleotides (e.g. "N"s).

2.8. Statistical analysis

Comparisons were calculated using $\chi 2$ or Fisher exact for categorical variables and Kruskal-Wallis tests for continuous variables, since they were not normally distributed. Non-parametric survival analysis presenting the Kaplan-Meier curve was performed to assess the persistence of MPXV-DNA in different clinical samples and curves were compared using the Long Rank test. Spearman's correlation coefficient was used to evaluate the association with Cq and time of onset symptoms. Differences were considered statistically significant at $p < 0.05$ for all tests. All statistical analyses were performed using GraphPad software version 8.3.0 (Prism).

3. Results

3.1. Study population and clinical characteristics

A total of 793 patients were screened for MPXV infection and 353 (44.5%) were confirmed by real-time PCR. A total of 4018 clinical specimens were analysed for diagnosis as well as during follow-up. Of these, 1191 (29.6%) were oropharyngeal swabs, 1166 (29.0%) were vesicular or pustular swab samples, 639 (15.9%) were anogenital swabs,

Table 1
Demographic, clinical characteristics and disease severity of confirmed mpox cases in Lombardy (n = 353).

Categories		overall		vaccinated		unvaccinated		unknown		p value[a]
		No.	%	No.	%	No.	%	No.	%	
Total cases		353	100.0	30	8.5	231	65.4	92	26.1	
Median Age yrs (range)		37 (15–67)		50 (30–58)		37 (15–6)		37 (19–67)		**<0.001**
Age group	0–19	3	0.8	0	0.0	2	0.9	1	1.1	**<0.001**
	20–29	55	15.5	0	0.0	43	18.6	12	13.0	
	30–39	164	46.6	3	10.0	115	49.8	46	50.0	
	40–49	97	27.4	9	30.0	64	27.7	24	26.1	
	50–59	29	8.2	18	60.0	6	2.6	5	5.4	
	>60	5	1.4	0	0.0	1	0.4	4	4.3	
Gender	Male	350	99.2	29	96.7	229	99.1	92	100.0	>0.99
	Female	3	0.8	1	3.3	2	0.9	0	0.0	
HIV status	Positive	37	10.5	3	10.0	22	9.5	12	13.0	>0.99
	Negative	18	5.1	1	3.3	14	6.1	3	3.3	
	Unknown	300	84.7	26	86.7	195	84.4	77	83.7	
Other STIs	Yes	7	2.0	3	10.0	6	2.6	0	0.0	0.08
	No	32	9.0	1	3.3	18	7.8	2	2.2	
	Unknown	315	89.0	26	86.7	207	89.6	90	97.8	
Rash	Yes	331	93.5	27	90.0	218	94.4	85	92.4	0.14
	No	14	4.0	3	10.0	9	3.9	2	2.2	
	Unknown	9	2.5	0	0.0	4	1.7	5	5.4	
Lymphadenopathy	Yes	154	43.5	10	33.3	109	47.2	35	38.0	0.23
	No	190	53.7	19	63.3	118	51.1	52	56.5	
	Unknown	10	2.8	1	3.3	4	1.7	5	5.4	
Fever	Yes	193	54.5	11	36.7	135	58.4	47	51.1	**0.02**
	No	149	42.1	18	60.0	91	39.4	39	42.4	
	Unknown	12	3.4	1	3.3	5	2.2	6	6.5	
Hospitalization	Yes	11	3.1	0	0.0	7	3.0	4	4.3	>0.99
	No	342	96.6	30	100.0	224	97.0	87	94.6	
	Unknown	1	0.3	0	0.0	0	0.0	1	1.1	
Local transmission case	Yes	247	69.8	21	70.0	160	69.3	60	65.2	>0.99
	No	86	24.3	8	26.7	60	26.0	20	21.7	
	Unknown	21	5.9	1	3.3	11	4.8	12	13.0	

[a]p value is referred to comparison between vaccinated and unvaccinated MPXV cases.

431 (10.7%) were blood samples, 251 (6.2%) were urine, 176 (4.4%) were urethral swabs, and 164 (4.1%) were semen. As summarized in Table 1, the median age of MPXV-confirmed cases was 37.0 years (IQR, 32–43 years: range 15–67 years) and the great majority were males (350/353, 99.2%). A minority of patients (11/353; 3.1%) required hospitalization, while the vast majority (342, 96.6%) were managed as outpatients. For one patient no information was available. Clinical reports were available for 345 (97.5%) confirmed cases with cutaneous rash and lymphadenopathy present in 331 (93.5%) and 154 (43.5%) subjects, respectively while 193 (54.5%) complained of fever (Table 1). A total of 329 (93.2%) exposure histories were available and 244 (74.1%) of them were autochthonous cases while transmission likely occurred abroad in 85 (25.8%) cases (mainly in Spain, France Germany, and Great Britain). Smallpox vaccination status was reported in 261 cases, showing that more than half of MPXV-positive patients were unvaccinated (231, 65.4%). Considering that smallpox vaccination was waived in 1980, it was expected that immunized subjects were older than those unvaccinated (median age 50 *vs* 36 years, $p < 0.01$). A great majority of vaccinated patients belonged to 50–59 age group (18/30; 60.0%), while in unvaccinated patients the most represented age group was 30–39 (115/231; 49.8%) (Table 1).

3.2. MPXV load and persistence in clinical specimens

Overall, 1285 samples were collected at the time of diagnosis, with a median time from the onset of symptoms of 6 days (IQR 3–9). MPXV detection was more frequent from the skin (289 of 306, 94.4%), anogenital (155 of 188, 82.4%), oropharyngeal (266 of 345, 78.0%) and plasma/blood (133 of 186, 71.5%) samples, than from urethral (45 of 69, 65.2%), semen (37 of 77, 48.1%) and urine (23 of 114, 20.2%) samples. The MPXV load, inversely reflected by Cq values, was significantly higher from skin lesions (median Cq 20, IQR 17–26) than from anogenital samples (median Cq 24, IQR 19–33), and oropharynx (median Cq 28, IQR 25–23) (Fig. 1A). In the remaining urethral (median Cq 31, IQR 25.5–35), urine (median Cq 32, IQR 25–34), semen (median Cq 34, IQR 30.5–36), and blood (median Cq 34, IQR 32–35) samples, the median viral load was higher than Cq = 30 and significantly lower than detected from the skin, anogenital and oropharyngeal samples (Fig. 1A, $p < 0.001$).

In 302 patients, skin and oropharyngeal samples were simultaneously collected at the median of 6 days from the onset of symptoms (IQR 3–9 days). Among these, in 229 paired samples (both positive), a significant difference in the median MPXV load was observed (median Cq 19, IQR 17–24 *vs* median Cq 28, IQR 24–32; $p < 0.001$). In 56 and 13 paired samples, only skin (median Cq 23 IQR 18.3–32) and oropharyngeal (median Cq 34 IQR 30–35.5) samples were positive, respectively. Finally, in four paired samples MPXV DNA was detected in neither skin nor oropharynx samples and the diagnosis was performed on anogenital samples.

Follow-up samples were collected only in a subset of patients and the MPXV DNA clearance was investigated in the different body compartments using the Kaplan-Meier method (Fig. 1B and C). The median clearance time of MPXV DNA detection was 16 days in the skin (n = 110 patients), 14 days in the oropharynx (n = 167), 13 days in anogenital (n = 116) and urethral samples (n = 30), 9 days in urine (n = 14), 8 days in blood (n = 80) and finally 7 days in semen (n = 24) (Fig. 1B). Persistent shedding defined as duration >21 days was observed in 10.0% (11/110) of skin samples, 6.1% (10/165) of oropharyngeal swabs, and 3.4% (4/116) of anogenital samples. The most prolonged MPXV DNA shedding was observed in one oropharyngeal sample still positive at 56 days. Kaplan-Meier curves of sample type with a more prolonged viral shedding (skin and oropharyngeal samples) when compared with the log-rank test resulted significantly different (median time, 16 *vs* 14 days, HR, 0.56; 95% CI, 0.41 to 0.77; $p < 0.001$, Fig. 1C).

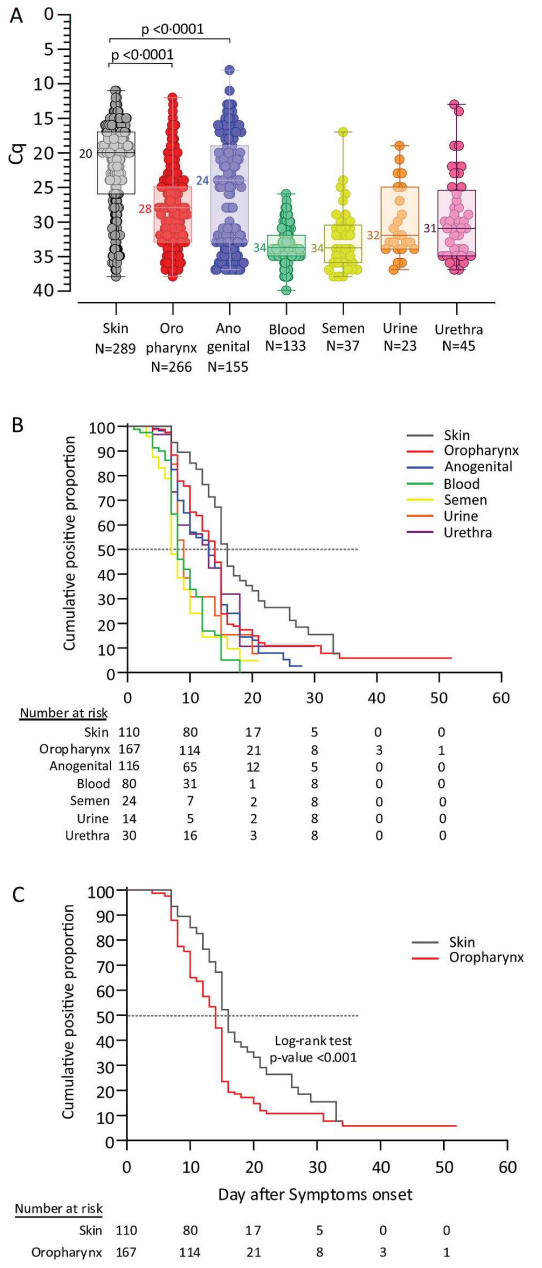

Fig. 1. The cycle of quantification values and viral shedding duration. (A) Cycle of quantification in different clinical specimens (B) Cumulative positive proportion in different clinical specimens. (C) Cumulative positive proportion in the clinical specimens (skin and oropharynx) with the high median MPXV DNA persistence (16 *vs* 14 days). Curves comparison was performed using log rank test analysis.

3.3. Isolation vs cq

MPXV isolation was attempted for 377 samples as summarized in Table 2. An actively-replicating virus was demonstrated for a total of

Table 2
Results of viral isolation assay performed on different MPXV DNA positive biological samples.

Clinical sample	Total (n = 377)		Positive (n = 123)		Negative (n = 248)		Cross-contaminated (n = 6)	
	no.	%[a]	no.	%[b]	no.	%[b]	no.	%
skin	120	31.8	39	32.5	81	67.5	0	0.0
nasopharynx	117	31.0	12	10.3	105	89.7	0	0.0
anogenital	71	18.8	46	64.8	19	26.8	6	8.5
plasma/blood	25	6.6	0	0.0	25	100.0	0	0.0
semen	3	0.8	3	100.0	0	0.0	0	0.0
urine	4	1.1	3	75.0	1	25.0	0	0.0
urethra	37	9.8	20	54.1	17	45.9	0	0.0

[a] calculated based on total samples.
[b] calculated based on total for each sample categories'.

123/377 (32.6%) samples, while it was unsuccessful in 248 and the remaining 6 samples were excluded due to bacterial or fungal contamination. Stratifying isolation results according to sample type, the rate of isolation was as follows: 100% for semen (3/3), 64.8% (46/71) for anogenital swabs, 10.3% (12/117) for oropharynx samples and 0% (0/25) for blood samples (Table 2).

In 359 samples the time between the date of sample collection and the symptoms onset was available and thus results of MPXV isolation were stratified into five categories: 0–7, 8–14, 15–21, 22–28, ≥29 days (Fig. 2A). Higher isolation rate was observed in samples collected between 0 and 7 days (65/158, 41.1%) from the onset of symptoms. The proportion decreased to 31.5% (34/108) for samples collected between 8 and 14 days and below 20% in the other categories (Fig. 2A). Analysing the rate of isolation among each sample category, in skin samples, the rate was over 40.0% for 0-7- and 8-14-day samples and strongly decreased in the 15–21, 22–28, and ≥29 days categories (Fig. 2B). Among oropharyngeal samples, the rate of isolation was less than 20 % in all categories with a maximum rate in samples collected at 0–7 days

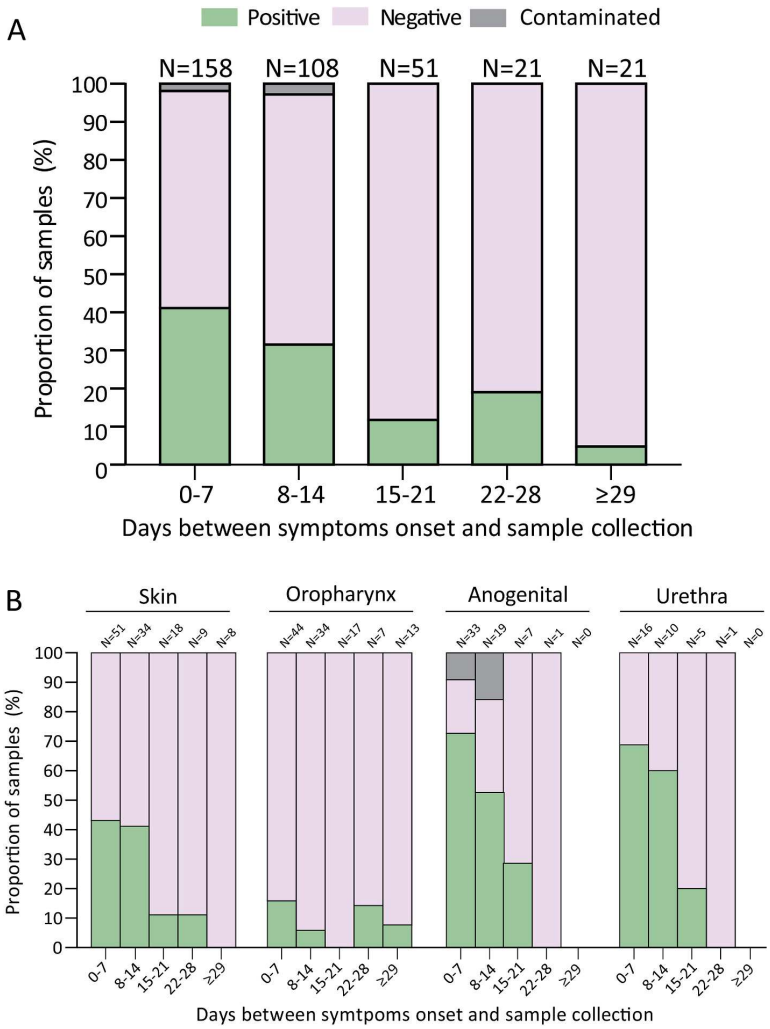

Fig. 2. Proportion of viral culture isolation. (A) Overall proportion of MPXV isolation results stratified by days between symptoms onset and samples collection. (B) Proportion of MPXV isolation results stratified by days between symptoms onset and samples collection according to the clinical sample with at least 30 samples.

from the symptoms onset. Among anogenital samples, the rate of isolation was 72.7% in the 0-7-days, 52.6% in the 8-14-days, and declined to 28.6% in the 15–21-days sample category. Similar findings were observed in the few urethral samples analysed. Overall, the isolation rate was higher than 60.0% for the 0-7- and 8-14-days categories with rates of 68.8% and 60.0%, respectively. For almost all samples (360/377, 95.5%) in which isolation was attempted, Cq values were available. Thus, a correlation between Cq value and isolation success, as well as timing from symptoms onset was assessed (Fig. 3). The median Cq values (Fig. 3A) between isolated and non-isolated samples were significantly different in the 0-7-days (19 vs 30; $p < 0.001$) as well as in 8–14-days (22 vs 29; $p < 0.001$) categories. As for the other categories, including samples collected ≥15 days after the onset of symptoms. Overall, the median Cq of samples with MPXV isolation was lower than other ones (20 vs 31, $p < 0.001$, Fig. 3B). Comparison of Cq values according to isolation success/failure showed a significant difference in the following biological specimens: skin (17 vs 28; $p < 0.001$), oropharyngeal (21 vs 31; $p = 0.0016$), anogenital (20 vs 35, $p < 0.001$) and urethral (28.5 vs 34.0, $p < 0.001$) samples. No comparison on Cq values could be done on plasma/blood, semen and urine samples.

3.4. Genomics and phylogenetic characterization

Sixty-one samples were cultured for virus isolation and their full genome was obtained by MPXV read selection and subsequent de novo assembly. The mean read-depth of the 61 genomes obtained was 118.2, while the mean N50 was 153074 and the mean contig number was 8.1. The 61 novel genomes were included in a Maximum-Likelihood phylogeny, together with 523 genomes available in public database (https://gisaid.org/, Fig. 4). The tree highlights the presence of a large monophylum including all the 2022 outbreak isolates. In addition, bootstrap values do not support the base nodes topology of the outbreak cluster, impeding the identification of outbreak sub-lineages. This result highlights the high genetic identity of the MPXV strains collected, both globally and within the Lombardy region. Nevertheless, it is possible to identify small high-confidence clusters in the most recent nodes of the tree, which are supported by a high bootstrap value and could be used to hypothesize small-scale epidemiological links. The 61 genomes

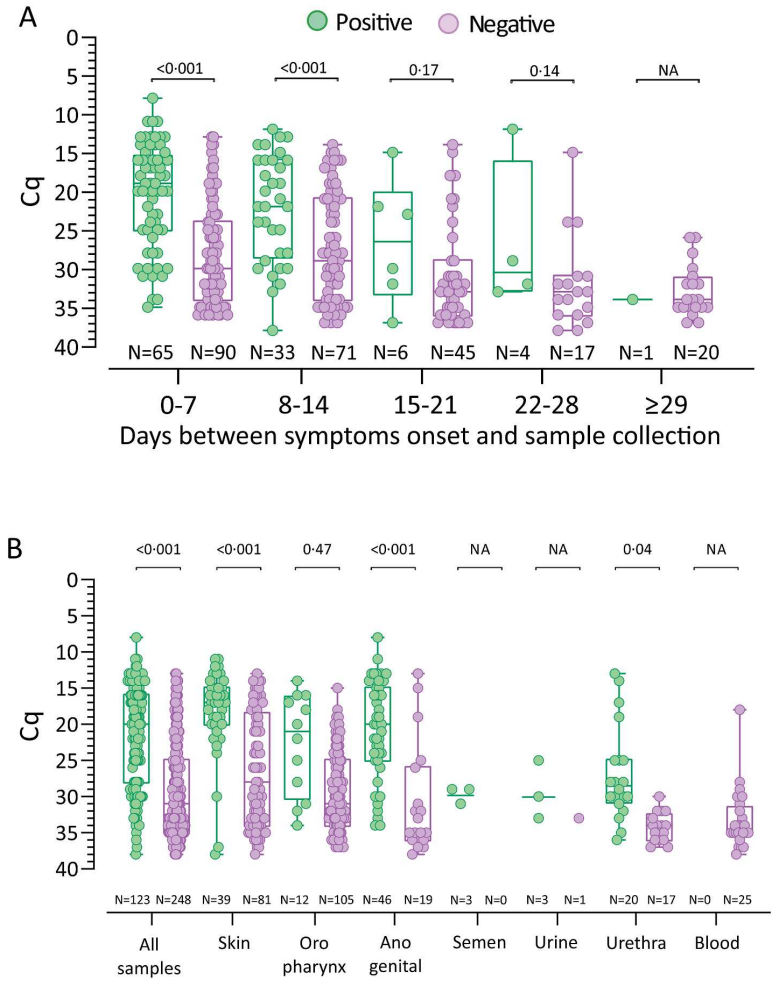

Fig. 3. The cycle of quantification values and viral culture isolation. (A) Comparison of Cq in virus culture positive and negative samples stratified by days between symptoms onset and samples collection. (B) Comparison of Cq in virus culture positive and negative samples stratified by different clinical specimens collected. NA, not applicable.

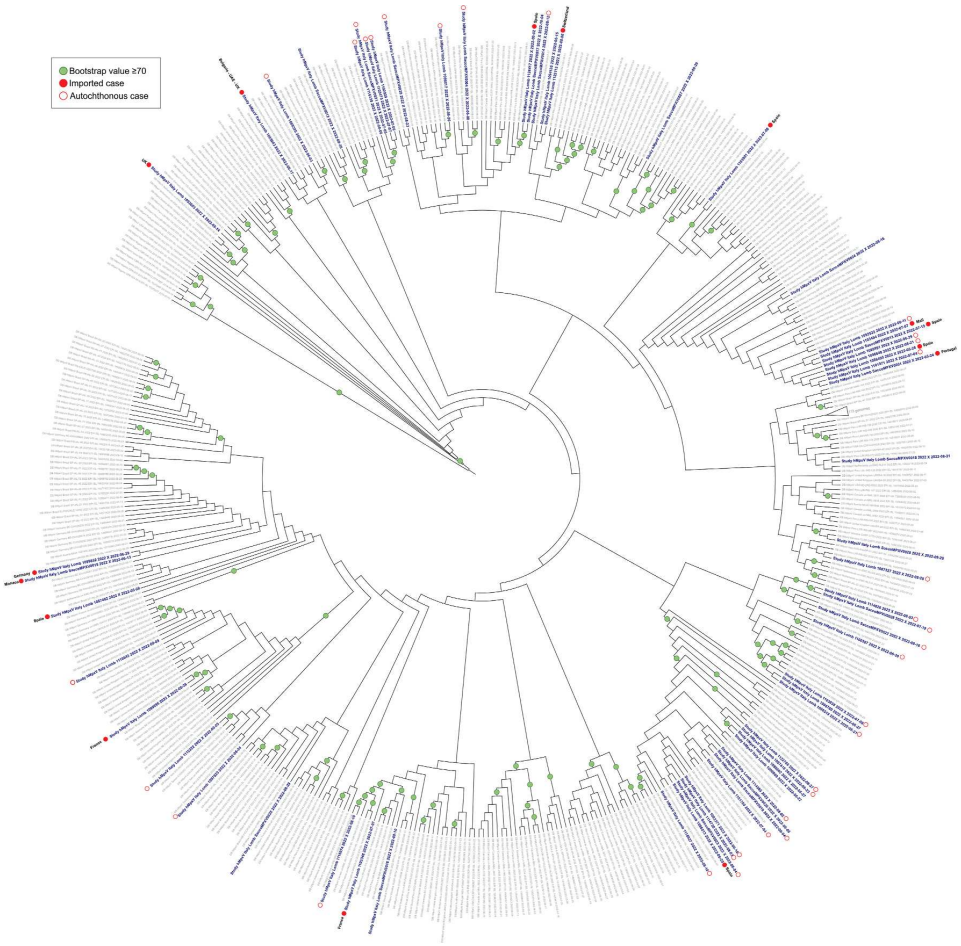

Fig. 4. Phylogenetic three of complete coding sequences of MPXV strains originated in this study (n = 61) and references (n = 523). Nodes of tree with a bootstrap value ≥ 70 were highlighted with a green dot. In order to differentiate the MPXV cases, autochthonous cases are highlighted with a white circle (with red border) while imported cases are highlighted with a red circle. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

described in this study are distributed in 37 local subclusters of the outbreak clade. Two clusters contain genomes relative to both imported infections and to autochthonous ones. E.g., sample 1096013 is associated with a patient that was infected in Spain and clusters with three other MPXV genomes associated with local infections. Both the tree topology and the sampling date suggest that the virus was imported by the patient who had travelled, and it was spread to the other three subjects.

After running association tests, 44 genic mutations were found linked to the 2022 outbreak clade (Table S1) of which 24/44 were non-synonymous. Interestingly, 42 characterizing mutations were also identified by Isidro et al. and by Wang et al., 40 of which were common to both studies [8,23]. Moreover, the two aforementioned works also identified intergenic mutations, which were not the focus of the present study. Notably, the vast majority of the single nucleotide mutations detected (617/757; 81.5%) followed the G > A or the C > T mutation pattern, which is known to be associated to the APOBEC enzymes' function. The prevalence of these substitutions is even higher in these outbreak-associated mutations (43/44; 97.7%).

4. Discussion

The recent MPXV global outbreak represented a major public health concern, requiring a strong effort in terms of individuals' management, spread containment, and diagnostic resources. However, it also gave the opportunity to elucidate the clinical and virological aspects of a neglected tropical disease. Our results confirm that viral DNA detected by qPCR on swabs from lesions is the most appropriate standard tool to make a prompt MPXV laboratory diagnosis [24]. Taken alongside data from previous studies [4,15–17,25–27], swabs from skin lesions were PCR positive at the time of diagnosis in almost all samples, showed the highest viral load (low cq value), the longer viral shedding (median 16 days after symptoms onset) and an isolation rate of nearly 40%. Suner et al. reported a median time of 25 days for DNA detection by qPCR in skin lesions but a shorter period of DNA detection for other body fluids as also observed in our study [26]. On the contrary, the isolation rate from the oropharyngeal swabs (n = 117), which proved to be an optimal biological sample at diagnosis (median shedding 14 days), was below 10%. This finding suggests the persistence of MPXV DNA in the upper respiratory tract that was not associated with a viable virus. Our results,

are slightly different from those reported by Hernaez in a Spanish cross-sectional study involving respiratory samples from 44 patients describing viable virus in 66% of qPCR-positive saliva samples [25]. The reduced infectivity in aerosol samples in our study may reflect the efficiency of virus replication our culture system used, but conversely, our results on other body fluids (e.g. skin lesion, anogenital) are in keeping with other reports [16]. Higher isolation rates (more than 50%) were observed in anogenital and urethral samples with higher isolation rate in the first 14 days after symptoms onset, supporting the possibility of a sexual transmission of the disease. Conversely, MPXV was not isolated from any of the 25 positive plasma samples. The associated high Cq values (low viral load) assessed in these samples likely justifies the lack of viral isolation.

In our study, vaccinated subjects showed milder symptoms (no skin lesions), faster viral clearance and lower Cq values in multiple biological specimens. Indeed, most of the recruited patients were below 50 years of age and therefore unvaccinated. However, based on our data no major conclusions could be draw on the implication of MPXv vaccine discontinuation in the origin and spread of 2022 MPXV outbreak. Moreover, independently from vaccination, none of the individuals included in this study showed severe complications and hospitalization was necessary only in 3.1% of cases, mostly at the beginning of the outbreak due to a worrying symptomatology, pain management and the need for antiviral treatment. Indeed, our hospitalization rate was slightly lower than those observed in a recent systemic review reporting however a high level of heterogeneity worldwide [28].

Since the beginning of this outbreak, most cases in Europe have been registered among MSM [2], and among those who have multiple sex partners [29]. This epidemiologic feature was also observed in the population included in our study. Indeed, sexual activity entails close physical contacts, favouring the chance of transmission, irrespective of sexual orientation and route of infection, as furtherly supported by the three MPXV-positive females, who reported sexual intercourse with a confirmed positive partner. Yet, whether MPXV is transmitted through sexual secretions and/or oral, genital, or anal mucosa to date remains under investigation. So far, viral DNA in seminal fluid has been detected in four and 22 patients in Italy [30,31], two cases in Germany [32], and in 29/32 (91%) patients belonging to a larger case series [3]. In addition, viable virus has been recently documented few other reports [33,34] also summarized in a recent systematic review by Reda et al. [35]. In our series, replication-competent virus was isolated in 100% (3/3) seminal specimens supporting the evidence of infectiousness of MPXV in semen. Virus isolation confirms semen as a potential source of infection, but additional analyses are required to assess whether the virus could be associated with seminal cell infection, whether it stems from passive diffusion from urethra, or genital lesions, and whether viral replication occurs in the genital tract.

How the infective virus is conveyed from the entry site to the several infected anatomical sites remains to be elucidated. Based on this observation, it emerged that the first two weeks after symptoms onset likely represent the most important phase in terms of virus infectiousness. Moreover, our data suggest that, on average, during the fourth week from symptoms onset the infectious potential might be considered drastically reduced.

The whole sequencing data consistently detected the IIb clade (previously named "West African"), mirroring the other reports on the 2022 outbreak. Genomic diversity among the outbreak samples is low and to date there is no evidence of emerging variants of concern suggestive of immune escape [8]. Phylogeny of previously published and novel genomes highlighted the scarce genomic variability of the outbreak samples, while allowing the identification of small high-confidence local epidemiological clusters (Fig. 4). Single mutation analysis led us to identify 24 non-synonymous mutations that characterize the 2022 outbreak clade (Table S1). Of note, three of these mutations (L108F in the DNA polymerase, S30L and D88 N in the Late transcription factor VLTF-1) were indicated among the putative causes of the 2022 outbreak by Kannan et al. [36]. In this study, the authors observe that the mutations contributing to the enhanced viral spread are related to the replication process. Indeed, a large part of the non-synonymous mutations that we have identified as outbreak-related affect proteins that regulate the genome replication (e.g. gene_124 DNA Helicale, gene_50 DNA Polymerase). Moreover, two other mutations affect proteins that regulate the interaction with the host immune system: S105L in chemokine binding protein [37] and S54F in Crm-B TNF-alpha-receptor-like protein [38] (both are in the inverted repeat regions and are thus listed twice in Table S1). The two aminoacidic changes might have improved the protein affinity with the human TNF and chemokines. The vast majority of all the mutations detected in our dataset follow the substitution pattern associated with the APOBEC deaminases. As pointed out by Isidro et al. [8] and Gigante et al. [39] as well such enzymes can be considered the evolutionary driving force of MPXV, which led to the development of the 2022 epidemic. In fact, 43 of the 44 outbreak-associated mutations follow these patterns.

Our study has some limitations. First, follow-up samples could not be collected from all patients and not for all clinical samples, thus viral shedding duration has been assessed only in subset of patients. The rapid evolving of outbreak has reduced the capacity to collected information regarding on skin healing (e.g. fresh pustules or desquamation of crust), thus the onset of symptoms was used as starting point to calculate the starting point of infection.

5. Conclusions

Our study describes clinical and epidemiological overview of the 2022 MPXV outbreak, including a clinical track record to real-time PCR performed on specimens from different anatomical sites (oropharyngeal, anal, urethral, and skin lesion swabs, together with plasma, urine and sperm) as well as MPXV isolation in an "*in vitro*" model. Our study will contribute to a better understanding of the MPXV dissemination, with a particular focus on the different route of transmissions. The provided insight on MPXV 2022 outbreak has highlighted the need to refine the clinical management and diagnosis, and for defining appropriate public health guidelines and preventive strategies, suited to the most affected communities.

Funding statement

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Data sharing

All sequenxing data included in this study are available under BioProject ID: PRJNA909472 (http://www.ncbi.nlm.nih.gov/bioproject/909472). De-identified participant data collected for the study, including individual participant data will be made available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Antonio Piralla: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Davide Mileto:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Alberto Rizzo:** Methodology, Formal analysis, Data curation. **Guglielmo Ferrari:** Methodology, Formal analysis, Data curation. **Federica Giardina:** Methodology, Formal analysis, Data curation. **Stefano Gaiarsa:** Writing – original draft, Visualization, Software, Data curation. **Greta Petazzoni:** Writing – original draft,

Visualization, Software, Data curation. **Micol Bianchi:** Investigation, Formal analysis, Data curation. **Federica Salari:** Methodology, Formal analysis, Data curation. **Fiorenza Bracchitta:** Methodology, Formal analysis, Data curation. **Josè Camilla Sammartino:** Methodology, Formal analysis, Data curation. **Alessandro Ferrari:** Methodology, Formal analysis, Data curation. **Gloria Gagliardi:** Methodology, Formal analysis, Data curation. **Alessandro Mancon:** Methodology, Formal analysis, Data curation. **Claudio Fenizia:** Methodology, Formal analysis, Data curation. **Mara Biasin:** Methodology, Formal analysis, Data curation. **Francesca Rovida:** Formal analysis, Data curation. **Stefania Paolucci:** Formal analysis, Data curation. **Elena Percivalle:** Formal analysis, Data curation. **Alessandra Lombardi:** Formal analysis, Data curation. **Valeria Micheli:** Formal analysis, Data curation. **Silvia Nozza:** Data curation. **Antonella Castagna:** Data curation. **Davide Moschese:** Data curation. **Spinello Antinori:** Data curation. **Andrea Gori:** Data curation. **Paolo Bonfanti:** Data curation. **Roberto Rossotti:** Data curation. **Antonella D'Arminio Monforte:** Data curation. **Federica Attanasi:** Data curation. **Marcello Tirani:** Data curation. **Danilo Cereda:** Data curation. **Fausto Baldanti:** Writing – review & editing, Supervision, Funding acquisition. **Maria Rita Gismondo:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tmaid.2024.102698.

References

[1] CDC. Monkeypox in the U.S. Centers for Disease Control and Prevention; 2022. published online Aug 31, https://www.cdc.gov/poxvirus/monkeypox/response/2022/world-map.html. [Accessed 12 April 2023].
[2] Monkeypox situation update, as of 1 March 2023. European Centre for Disease Prevention and Control; 2023. published online Mar 01, https://monkeypoxreport.ecdc.europa.eu/. [Accessed 12 March 2023].
[3] Thornhill JP, Barkati S, Walmsley S, Rockstroh J, Antinori A, Harrison LB, et al. Monkeypox virus infection in humans across 16 countries - april-june 2022. N Engl J Med 2022;387:679–91. https://doi.org/10.1056/NEJMoa2207323.
[4] Adler H, Gould S, Hine P, Snell LB, Wong W, Houlihan CF, et al. Clinical features and management of human monkeypox: a retrospective observational study in the UK. Lancet Infect Dis 2022;22:1153–62. https://doi.org/10.1016/S1473-3099(22)00228-6.
[5] Eisenstadt R, Liszewski WJ, Nguyen CV. Recognizing minimal cutaneous involvement or systemic symptoms in monkeypox. JAMA Dermatol 2022;158:1457–8. https://doi.org/10.1001/jamadermatol.2022.4652.
[6] Likos AM, Sammons SA, Olson VA, Frace AM, Li Y, Olsen-Rasmussen M, et al. A tale of two clades: monkeypox viruses. J Gen Virol 2005;86:2661–72. https://doi.org/10.1099/vir.0.81215-0.
[7] Català A, Clavo-Escribano P, Riera-Monroig J, Martín-Ezquerra G, Fernandez-Gonzalez P, Revelles-Peñas, et al. Monkeypox outbreak in Spain: clinical and epidemiological findings in a prospective cross-sectional study of 185 cases. Br J Dermatol 2022;187:765–72. https://doi.org/10.1111/bjd.21790.
[8] Isidro J, Borges V, Pinto M, Sobral D, Santos JD, Nunes A, et al. Phylogenomic characterization and signs of microevolution in the 2022 multi-country outbreak of monkeypox virus. Nat Med 2022;28:1569–72. https://doi.org/10.1038/s41591-022-01907-y.
[9] Mileto D, Riva A, Cutrera M, Moschese D, Mancon A, Meroni L, et al. New challenges in human monkeypox outside Africa: a review and case report from Italy. Trav Med Infect Dis 2022;49:102386. https://doi.org/10.1016/j.tmaid.2022.102386.
[10] Vaughan A, Aarons E, Astbury J, Brooks T, Chand M, Flegg P, et al. Human-to-Human transmission of monkeypox virus, United Kingdom, october 2018. Emerg Infect Dis 2020;26:782–5. https://doi.org/10.3201/eid2604.191164.
[11] Altindis M, Puca E, Shapo L. Diagnosis of monkeypox virus - an overview. Trav Med Infect Dis 2022 Nov-Dec;50:102459. https://doi.org/10.1016/j.tmaid.2022.102459. Epub 2022 Sep 13. PMID: 36109000; PMCID: PMC9534096.
[12] WHO. Monkeypox outbreak: global trends. https://worldhealthorg.shinyapps.io/mpx_global/. [Accessed 12 April 2023].
[13] Billioux BJ, Mbaya OT, Sejvar J, Nath A. Potential complications of monkeypox. Lancet Neurol 2022;21:872.
[14] Huhn GD, Bauer AM, Yorita K, Graham MB, Sejvar J, Likos A, et al. Clinical characteristics of human monkeypox, and risk factors for severe disease. Clin Infect Dis 2005;41:1742–51. https://doi.org/10.1086/498115.
[15] Peiró-Mestres A, Fuertes I, Camprubí-Ferrer D, Marcos MÁ, Vilella A, Navarro M, et al. Frequent detection of monkeypox virus DNA in saliva, semen, and other clinical samples from 12 patients, Barcelona, Spain, May to June 2022. Euro Surveill 2022;27:2200503. https://doi.org/10.2807/1560-7917.ES.2022.27.28.2200503.
[16] Lim CK, McKenzie C, Deerain J, Chow EPF, Towns J, Chen MY, et al. Correlation between monkeypox viral load and infectious virus in clinical specimens. J Clin Virol 2023;161:105421. https://doi.org/10.1016/j.jcv.2023.105421.
[17] Palich R, Burrel S, Monsel G, Nouchi A, Bleibtreu A, Seang S, et al. Viral loads in clinical samples of men with monkeypox virus infection: a French case series. Lancet Infect Dis 2023;23:74–80. https://doi.org/10.1016/S1473-3099(22)00586-2.
[18] Number of confirmed monkeypox cases in Italy as of March 17. by region, https://www.statista.com/statistics/1323947/monkeypox-confirmed-cases-by-region-italy/. [Accessed 12 April 2023].
[19] Li Y, Zhao H, Wilkins K, Hughes C, Damon IK. Real-time PCR assays for the specific detection of monkeypox virus West African and Congo Basin strain DNA. J Virol Methods 2010;169:223–7. https://doi.org/10.1016/j.jviromet.2010.07.012.
[20] Maksyutov RA, Gavrilova EV, Shchelkunov SN. Species-specific differentiation of variola, monkeypox, and varicella-zoster viruses by multiplex real-time PCR assay. J Virol Methods 2016;236:215–20. https://doi.org/10.1016/j.jviromet.2016.07.024.
[21] Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 2012;19:455–77. https://doi.org/10.1089/cmb.2012.0021.
[22] Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol 2015;32:268–74. https://doi.org/10.1093/molbev/msu300.
[23] Wang L, Shang J, Weng S, Aliyari SR, Ji C, Cheng G, et al. Genomic annotation and molecular evolution of monkeypox virus outbreak in 2022. J Med Virol 2023;95:e28036. https://doi.org/10.1002/jmv.28036.
[24] WHO. Monkeypox posted on 19 may 2022. https://www.who.int/news-room/fact-sheets/detail/monkeypox. [Accessed 13 April 2023].
[25] Hernaez B, Muñoz-Gómez A, Sanchiz A, Orviz E, Valls-Carbo A, Sagastagoitia I, et al. Monitoring monkeypox virus in saliva and air samples in Spain: a cross-sectional study. Lancet Microbe 2023;4:e21–8. https://doi.org/10.1016/S2666-5247(22)00291-9.
[26] Suñer C, Ubals M, Tarín-Vicente EJ, Mendoza A, Alemany A, Hernández-Rodríguez Á, et al. Viral dynamics in patients with monkeypox infection: a prospective cohort study in Spain. Lancet Infect Dis 2023;23:445–53. https://doi.org/10.1016/S1473-3099(22)00794-0.
[27] Tarín-Vicente EJ, Alemany A, Agud-Dios M, Ubals M, Suñer C, Antón A, et al. Clinical presentation and virological assessment of confirmed human monkeypox virus cases in Spain: a prospective observational cohort study. Lancet 2022;400:661–9. https://doi.org/10.1016/S0140-6736(22)01436-2.
[28] DeWitt ME, Polk C, Williamson J, Shetty AK, Passaretti CL, McNeil CJ, et al. Global monkeypox case hospitalisation rates: a rapid systematic review and meta-analysis. EClinicalMedicine 2022;54:101710. https://doi.org/10.1016/j.eclinm.2022.101710.
[29] Brown K, Leggat PA. Human monkeypox: current state of knowledge and implications for the future. Trav Med Infect Dis 2016;1:8. https://doi.org/10.3390/tropicalmed1010008.
[30] Antinori A, Mazzotta V, Vita S, Carletti F, Tacconi D, Lapini LE, et al. Epidemiological, clinical and virological characteristics of four cases of monkeypox support transmission through sexual contact, Italy, May 2022. Euro Surveill 2022;27:2200421. https://doi.org/10.2807/1560-7917.ES.2022.27.22.2200421.
[31] Raccagni AR, Candela C, Mileto D, Canetti D, Bruzzesi E, Rizzo A, et al. Monkeypox infection among men who have sex with men: PCR testing on seminal fluids. J Infect 2022;85:573–607. https://doi.org/10.1016/j.jinf.2022.07.022.
[32] Noe S, Zange S, Seilmaier M, Antwerpen MH, Fenzl T, Schneider J, et al. Clinical and virological features of first human monkeypox cases in Germany. Infection 2023;51:265–70. https://doi.org/10.1007/s15010-022-01874-z.
[33] Lapa D, Carletti F, Mazzotta V, Matusali G, Pinnetti C, Meschi S, et al. Monkeypox virus isolation from a semen sample collected in the early phase of infection in a patient with prolonged seminal viral shedding. Lancet Infect Dis 2022;22:1267–9. https://doi.org/10.1016/S1473-3099(22)00513-8.
[34] Colavita F, Mazzotta V, Rozera G, Abbate I, Carletti F, Pinnetti C, et al. Kinetics of viral DNA in body fluids and antibody response in patients with acute Monkeypox virus infection. iScience 2023;26:106102. https://doi.org/10.1016/j.isci.2023.106102.
[35] Reda A, Abdelaal A, Brakat AM, Lashin BI, Abouelkheir M, Abdelazeem B, et al. Monkeypox viral detection in semen specimens of confirmed cases: a systematic

review and meta-analysis. J Med Virol 2023;95:e28250. https://doi.org/10.1002/jmv.28250.
[36] Kannan SR, Sachdev S, Reddy AS, Kandasamy SL, Byrareddy SN, Lorson CL, et al. Mutations in the monkeypox virus replication complex: potential contributing factors to the 2022 outbreak. J Autoimmun 2022;133:102928. https://doi.org/10.1016/j.jaut.2022.102928.
[37] Smith CA, Smith TD, Smolak PJ, Friend D, Hagen H, Gerhart M, et al. Poxvirus genomes encode a secreted, soluble protein that preferentially inhibits beta chemokine activity yet lacks sequence homology to known chemokine receptors. Virology 1997;236:316–27. https://doi.org/10.1006/viro.1997.8730.
[38] Cunnion KM. Tumor necrosis factor receptors encoded by poxviruses. Mol Genet Metabol 1999;67:278–82. https://doi.org/10.1006/mgme.1999.2878.
[39] Gigante CM, Korber B, Seabolt MH, Wilkins K, Davidson W, Rao AK, et al. Multiple lineages of monkeypox virus detected in the United States, 2021-2022. Science 2022;378:560–5. https://doi.org/10.1126/science.add4153.

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Short Communication

Increased circulation of echovirus 11 in the general population and hospital patients as elicited by the non-polio enterovirus laboratory-based sentinel surveillance in northern Italy, 2023

Laura Pellegrinelli[1], Cristina Galli[1], Federica Giardina[2], Guglielmo Ferrari[3], Sara Colonia Uceda Renteria[4], Ferruccio Ceriotti[4], Arlinda Seiti[1], Sandro Binda[1], Antonino Maria Guglielmo Pitrolo[3], Roberta Schiavo[5], Sergio Maria Ivano Malandrin[6], Annalisa Cavallero[6], Marco Arosio[7], Claudio Farina[7], Massimo Oggioni[8], Pierluigi Congedo[8], Danilo Cereda[9], Francesca Rovida[2,3], Antonio Piralla[3,#], Elena Pariani[1,#,*], Fausto Baldanti[2,3,#], Non-polio community-based surveillance group[†], Non-polio hospital-based surveillance group[‡]

[1] *Department of Biomedical Sciences for Health, University of Milan, Milan, Italy*
[2] *Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy*
[3] *Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy*
[4] *Virology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy*
[5] *Microbiology Unit, Hospital Guglielmo da Saliceto, Piacenza, Italy*
[6] *Microbiology Unit, Fondazione IRCCS San Gerardo dei Tintori, Monza, Italy*
[7] *Microbiology and Virology Laboratory, ASST "Papa Giovanni XXIII", Bergamo, Italy*
[8] *S.S.D. Microbiologia, Dipartimento dei Servizi Diagnostici, ASST della Brianza, Vimercate, Italy*
[9] *Direzione Generale Welfare Regione Lombardia, Milano, Italy*

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ABSTRACT

Objectives: Following the alert of echovirus 11 (E-11) infection in neonates in EU/EEA Member States, we conducted an investigation of E-11 circulation by gathering data from community and hospital surveillance of enterovirus (EV) in northern Italy from 01 August 2021 to 30 June 2023.
Methods: Virological results of EVs were obtained from the regional sentinel surveillance database for influenza-like illness (ILI) in outpatients, and from the laboratory database of ten hospitals for inpatients with either respiratory or neurological symptoms. Molecular characterization of EVs was performed by sequence analysis of the VP1 gene.
Results: In our ILI series, the rate of EV-positive specimens showed an upward trend from the end of May 2023, culminating at the end of June, coinciding with an increase in EV-positive hospital cases. The E-11 identified belonged to the D5 genogroup and the majority (83%) were closely associated with the

* Corresponding author: Elena Pariani, Department of Biomedical Sciences for Health, University of Milan, Via Carlo Pascal 36, 20133, Milan, Italy.
E-mail address: elena.pariani@unimi.it (E. Pariani).

# These authors contributed equally to the work and share the last authorship.

† **Non-polio community-based surveillance group**: Sentinel physicians of RespiVirNet in Lombardy; Federica Attanasi, Marcello Tirani, Michela Viscardi (Direzione Generale Welfare Regione Lombardia, Milano).

‡ **Non-polio hospital-based surveillance group**: Stefania Paolucci (Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy), Alessandro Borghesi, Stefano Ghirardello, Amelia Di Comite (Neonatal Intensive Care Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy), Thomas Fojadelli (Pediatric Clinic, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy), Gian Luigi Marseglia (Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy, Pediatric Clinic, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy), Giuliana Lo Cascio (Microbiology Unit, Hospital Guglielmo da Saliceto, Piacenza, Italy); Davide Oggioni (S.S.D. Microbiologia, Dipartimento dei Servizi Diagnostici, ASST della Brianza, Vimercate, Italy); Valentina Sottili (Maternal and Child Department, Pediatrics Unit, ASST della Brianza, Vimercate, Italy); Giovanna Mangili, (Neonatal Intensive Care Unit, ASST "Papa Giovanni XXIII", Bergamo); Maria Teresa Sinelli (Neonatal Intensive Care Unit, Fondazione IRCCS San Gerardo dei Tintori); Fabio Sagradi (Laboratorio di Microbiologia e Virologia, ASST Cremona, Italy), Diana Fanti, Alice Nava (S.C. Microbiologia Clinica- ASST Grande Ospedale Metropolitano Niguarda, Milan, Italy), Federica Novazzi, Nicasio Mancini (Department of Medicine and Surgery, University of Insubria, Varese, Italy, Laboratory of Microbiology, ASST Sette Laghi, Varese, Italy), Annapaola Callegaro (Laboratory Medicine Department, Asst Bergamo Est, Bergamo Italy).

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novel E-11 variant, first identified in severe neonatal infections in France since 2022. E-11 was identified sporadically in community cases until February 2023, when it was also found in hospitalized cases with a range of clinical manifestations. All E-11 cases were children, with 14 out of 24 cases identified through hospital surveillance. Of these cases, 60% were neonates, and 71% had severe clinical manifestations.
Conclusion: Baseline epidemiological data collected since 2021 through EV laboratory-based surveillance have rapidly tracked the E-11 variant since November 2022, alongside its transmission during the late spring of 2023.

1. Introduction

Since the initial report of enterovirus (EV), echovirus 11 (E-11) infection in neonates in France in May 2023 [1], further cases have been reported in Italy and other European countries. The World Health Organization (WHO), having evaluated the limited data available, considers the public health risk to be low although countries are advised to investigate cases (https://www.who.int/emergencies/disease-outbreak-news/item/2023-DON474). Given that non-polio EV infections are not considered notifiable infectious diseases, this investigation aims to examine the circulation of E-11 in Lombardy (northern Italy). Non-polio EV infections spread by the respiratory and oral-faecal routes and usually present with a prodrome of influenza-like symptoms or fever, which can range from mild and self-limited disease to severe and life-threatening manifestations, including myocarditis, sepsis, hepatitis, meningitis, encephalitis and acute flaccid myelitis/paralysis [2]. They play a significant role in respiratory infections, causing a range of respiratory symptoms such as cough, cold and difficulty breathing, and can also lead to respiratory complications, particularly in infants and young children [3]. Understanding their role is crucial for effective diagnosis, management and public health interventions, highlighting the importance of on-going surveillance and research to understand their impact on public health and to develop targeted prevention strategies.

The aim of this study was to conduct event-based surveillance of non-polio EV by examining the regional virological database for influenza-like illness (ILI) surveillance (community-based surveillance) and reviewing laboratory data from 10 hospitals, including inpatients with respiratory or systemic/neurological symptoms (hospital-based surveillance), from 01 August 2021 to 30 June 2023.

2. Materials and methods

To evaluate EV circulation within the community, EV detection results were obtained from the virological ILI database of Lombardy's regional reference laboratory, which is part of the Italian respiratory virus surveillance network (https://www.iss.it/en/respivirnet). This network relies on the voluntary participation of sentinel physicians who are tasked with collecting respiratory specimens to monitor respiratory viruses (https://www.iss.it/en/respivirnet), including the identification of EVs through a real-time RT-PCR assay [4].

Additionally, a hospital-based laboratory surveillance of EVs has been established in Lombardy since October 2021 to evaluate the molecular characteristics of EVs in inpatients hospitalized with respiratory or neurological symptoms. Samples from individuals who tested positive for EVs and were either admitted to the emergency department or hospitalized with respiratory or neurological symptoms in 10 hospitals in northern Italy were analysed.

Each sample that tested positive for EV-RNA was further tested by real-time RT-PCR to detect EV-D68 genome [5,6]. EV-RNA positive samples with a viral load of Ct $<$ 33 underwent molecular characterization through sequencing of the VP1 gene [7], followed by nucleotide sequence similarity analysis using the Basic Local Alignment Search Tool BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the RIVM enterovirus genotyping tool (https://www.rivm.nl/mpf/typingtool/enterovirus/).

3. Results

During the study period, 3781 respiratory specimens from ILI cases were examined, of which 8.2% (n = 309) were positive for EV-RNA. EVs were detected every week from week 2021-40, when the study started, showing a pattern of circulation that resulted in two epidemic waves (Figure 1). The first wave started in week 2022-19 (weekly EV-positivity rate: 20%), peaked in week 2022-28 (50%) and subsided in week 2022-37 (10%). The second wave started in week 2023-21 (12.5%) and peaked in week 2023-25 with a positivity rate of 38.6%. During the hospital-based surveillance, 341 EV-positive patients were identified. The temporal distribution of EV-positive cases identified during hospital- and community-based surveillance overlapped (Figure 1). In total, 22 EV-positive individuals (22/341; 6.5%) were newborns, all of whom were identified in the hospital setting.

All respiratory specimens collected during ILI surveillance were tested routinely for other respiratory viruses, namely, SARS-CoV-2, influenza virus A/B, respiratory syncytial virus (RSV), metapneumovirus (MPV), rhinovirus, adenovirus (AdV) and parechovirus (PeV). The presence of viral co-infection was observed in 45% of EV-positive samples; in detail, rhinovirus and RSV were identified in 48.9% and 23% of EV-positive samples, respectively, while SARS-CoV-2 and influenza virus A were detected in 4.3% and 5.8% of EV-positive respiratory specimens. AdV was identified in 14.4% of EV-positive samples and MPV in 3.6%; no EV/PeV coinfection was identified. For 87.7% (n = 299) specimens collected during hospital-based surveillance, data of test for other respiratory viruses (namely, SARS-CoV-2, influenza virus A/B, RSV, MPV, rhinovirus, AdV) were available; in detail, rhinovirus and RSV were identified in 47.3% and 11.5% of EV-positive samples, respectively, while SARS-CoV-2 and influenza virus A were detected in 3.4% and 6.1% of EV-positive respiratory specimens. AdV was identified in 24.4% of EV-positive samples and MPV in 7.4%.

Considering E-11 cases, no other viruses were detected in clinical samples collected in the hospital setting, but AdV and rhinovirus were detected in two ILI cases of E-11 (Table 1).

Analysis of EV-D68 specific assay and EV sequencing results revealed that 294 (45,2%) strains were classified as an EV type, while 356 (54.8%) EVs remained untyped due to low viral load. Twenty distinct EV types belonging to all the four EV groups (A-D) were identified at different frequencies including: EV-D68 (n = 146),

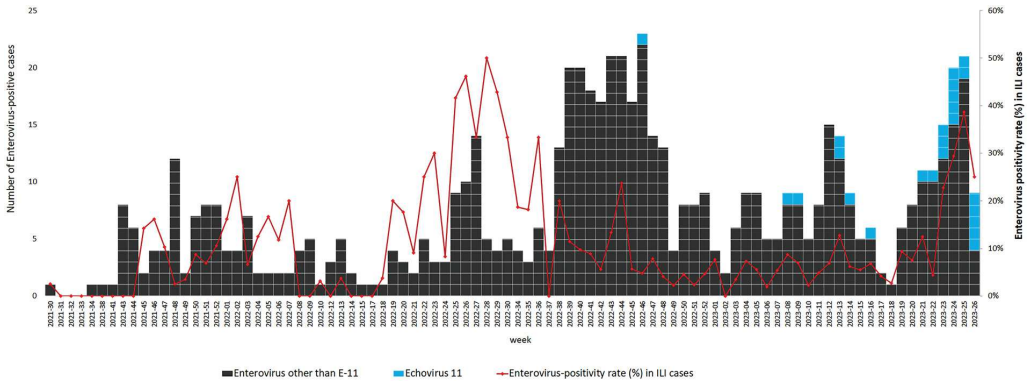

Figure 1. Weekly positivity rate of EV in community surveillance (ILI series) and number of EV-positive cases and E-11 positive cases in Lombardy, northern Italy from week 2021-30 to week 2023-26.

Table 1
Demographic, clinical and molecular characterization of E-11 cases identified in Lombardy, northern Italy from week 2021-30 to week 2023-26. The following abbreviations are used: NPS (nasal-pharyngeal swab), NPA (nasal-pharyngeal aspirate), CSF (cerebral spinal fluid), and BAL (brocho-alveolar lavage).

E-11 cases	Surveillance setting	Clinical manifestation	Type of specimen	Age	Week of sample collection	Weekly EV-positivity rate	E-11 genogroup	Detection of other viruses in clinical sample
1	Community	ILI	NPS	8 months	2022-46	0.2%	D5	None
2	Community	ILI	NPS	2 years	2023-08	8.7%	D5	None
3	Community	ILI	NPS	2 years	2023-09	7.0%	D5	None
4	Community	ILI	NPS	3 years	2023-13	12.8%	D5	None
5	Community	ILI	NPS	2 years	2023-13	12.8%	D5	Rhinovirus
6	Hospital	Fever	NPS	10 months	2023-14	6.1%	D5	None
7	Hospital	Fulminant hepatitis [2]	Blood	4 days	2023-16	6.9%	D5	None
8	Hospital	Fulminant hepatitis [2]	Blood	6 days	2023-16	6.9%	D5	None
9	Hospital	Fever, hyporeactivity, mild hypotonia	NPS	7 days	2023-21	12.5%	D5	None
10	Hospital	Acute otitis	NPA	5 years	2023-22	4.3%	D5	None
11	Community	ILI	NPA	10 years	2023-23	22.7%	D5	Adenovirus
12	Hospital	Encephalitis	CSF and faeces	6 years	2023-23	22.7%	D5	None
13	Hospital	Acute hepatitis	Blood	18 days	2023-23	22.7%	D5	None
14	Community	ILI	NPA	4 years	2023-24	29.4%	D5	None
15	Hospital	Fever and gastroenteritis	NPS	45 days	2023-24	29.4%	D5	None
16	Hospital	Fever and gastroenteritis	NPS	22 days	2023-24	29.4%	D5	None
17	Hospital	Respiratory distress	BAL	4 months	2023-24	29.4%	D5	None
18	Hospital	Asymptomatic	Faeces	6 months	2023-24	29.4%	D5	None
19	Hospital	Hyporeactivity, mild hypotonia, mild apnoea	NPS	3 days	2023-25	25.0%	D5	None
20	Hospital	Fever	NPS	10 days	2023-25	25.0%	D5	None
21	Community	ILI	NPS	3 years	2023-26	38.6%	D5	None
22	Community	ILI	NPS	9 years	2023-26	38.6%	D5	None
23	Community	ILI	NPS	4 years	2023-26	38.6%	D5	None
24	Hospital	Meningitis	NPS and CSF	10 years	2023-26	38.6%	D5	None

coxsackievirus (CV) A6 ($n = 27$), echovirus (E) 11 ($n = 24$), CV-A4 ($n = 15$), CV-B5 ($n = 15$), CV-B4 ($n = 12$), CV-A16 ($n = 10$), CV-B2 ($n = 7$), E-3 ($n = 7$), E-18 ($n = 7$), CV-A5 ($n = 5$), EV-C105 ($n = 4$), CV-B3 ($n = 4$), CV-A9 ($n = 3$), CV-A2 ($n = 2$), E-25 ($n = 2$), CV-A10 ($n = 1$), EV-A71 ($n = 1$), CV-A21 ($n = 1$), EV-C109 ($n = 1$).

Twenty-four cases of E-11 were identified (24/294: 8.2%). All E-11 cases were children aged between 3 days and 10 years. Out of these, 10 cases were identified within community surveillance and 14 within hospital surveillance. Overall, 14 (58.3%) E-11 cases had mild infection (ILI, fever, or acute otitis) and all were children aged between 22 days and 10 years. Ten cases (41.7%) had severe clinical manifestations, of which six were in neonates. Overall, of the 22 EV-positive neonates, 6 (27.3%) were E-11 cases, 4 (18.2%) were CV-B cases, 1 was CV-A16 (4.5%) and 1 was E-9 (4.5%).

As shown in Table 1, aside from the two cases of fulminant hepatitis [8], a wide range of symptoms were recorded, making the clinical diagnosis of E-11 infection highly unpredictable based on the observation of symptoms alone.

E-11 was sporadically detected in November 2022 (one case) and February 2023 (two cases), with an additional three cases detected in April 2023. Subsequently, the number of E-11 cases increased, with 16 cases recorded in May/June 2023.

Our analysis of the VP1 gene from 24 E-11 sequences identified that all belonged to genogroup D, lineage D5, similarly to other E-11 strains recently reported in France [1]. The phylogenetic tree (Figure S1) included 23 out of 24 E-11 sequences (one was excluded due to its limited lenght) and showed that 19 study E-11 sequences (83%) segregated together (mean nucleotide identity: 98.5%). These sequences clustered with recent French E-11 strains ($N = 9$) [1], sharing a mean nucleotide identity of 98.6%. The other four E-11 study strains showed separate segregation. Four sequences clustered with E-11 strains identified in China in 2017 and 2019.

4. Discussion and conclusion

In light of the WHO advisement and risk assessment of severe E-11 infection in Europe and given the lack of on-going surveillance of EV infection in Italy, we performed event-based surveillance to assess the spread of E-11 in northern Italy. From the virological data gathered through community-based surveillance of ILI, we observed a low level of EV circulation from August 2021 to May 2022, followed by two epidemics. The first occurred from May to July 2022 and was caused by EV-D68. The second wave occurred from end of May to July 2023. E-11 was detected both in individuals with mild symptoms and in hospitalized patients with self-limited severe manifestations, ranging in age from 9 days to 10 years. It is noteworthy that E-11 was also sporadically identified from November 2022 to April 2023, but only in individuals with ILI. Furthermore, phylogenetic analysis of the VP1 gene showed that 83% of the E-11 strains grouped together and clustered with the French E-11 strains [1]. As previously noted, recombination of E-11 with other EV types may have led to the emergence and spread of novel variants with chimaeric genome structures [9]. This could explain the severe illness and epidemic nature of the strains observed in Italy in 2013 [10] and in France in 2022-2023 [1]. In contrast, data from the US national enterovirus surveillance system has reported a significantly higher mortality from E-11 infection in neonates compared to infants older than 1 month [11], similar to what has been recently observed in a multicentre retrospective cohort study [12]. Consistent with these findings, our E-11 cases were either children >1 year of age or neonates with no underlying medical conditions, likely resulting in a mild self-limited clinical manifestation.

There are a few drawbacks to this study. First, it was not possible to molecularly characterize all EV samples with respect to their viral load. In addition, the routinely used typing techniques based on the VP1 fragment provide only partial information on viral evolution and no information on recombination events. Finally, although it would be useful to have information on possible co-infections, it was not possible to obtain this information for all cases included in this study: a viral panel was evaluated in all respiratory samples from ILI cases, but different pathogen panels were used in the hospital setting.

In conclusion, our community-based sentinel laboratory surveillance has detected an increase in the incidence of E-11 in recent months. In addition, hospital-based surveillance has captured the clinical features and severity of E-11 in neonates during the outbreak caused by a new variant of E-11 initially identified in France.

Declarations of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical statement

Ethical approval was not required for this study because the study was part of the routine activities of the Italian respiratory virus surveillance network (RespiVirNet) and the routine management and treatment of patients.

Author contributions

LP, AS, CG conducted virological investigation of ILI cases; SCUR, FC, RS, SMIM, AC, MA, CF, MO, PC conducted virological investigation of hospitalized cases; CG, FG, GF, AMGP carried out phylogenetic analyses, EP, DC, FR, SB retrieved epidemiological data, LP, EP, AP, FB wrote the first draft of the manuscript. AP, EP, FB revised the manuscript and supervise. All authors were involved in the study group on surveillance of enterovirus infections, contributed to the manuscript and approved the final version.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2024.106998.

References

[1] Grapin M, Mirand A, Pinquier D, Basset A, Bendavid M, Bisseux M, et al. Severe and fatal neonatal infections linked to a new variant of echovirus 11, France, July 2022 to April 2023. *Euro Surveill* 2023;**28**:2300253.
[2] Fischer TK, Simmonds P, Harvala H. The importance of enterovirus surveillance in a post-polio world. *Lancet Infect Dis* 2022;**22**(1):e35–40. doi:10.1016/S1473-3099(20)30852-5.
[3] Imamura T, Oshitani H. Global reemergence of enterovirus D68 as an important pathogen for acute respiratory infections. *Rev Med Virol* 2015;**25**(2):102–14. doi:10.1002/rmv.1820.
[4] Galli C, Pellegrinelli L, Bubba L, Primache V, Anselmi G, Delbue S, et al. When the COVID-19 pandemic surges during influenza season: lessons learnt from the sentinel laboratory-based surveillance of influenza-like illness in Lombardy during the 2019-2020 season. *Viruses* 2021;**13**:695.
[5] Piralla A, Girello A, Premoli M, Baldanti F. A new real-time reverse transcription-PCR assay for detection of human enterovirus 68 in respiratory samples. *J Clin Microbiol* 2015;**53**:1725–6.
[6] Poelman R, Schuffenecker I, Van Leer-Buter C, Josset L, Niesters HG, Lina B, et al. European surveillance for enterovirus D68 during the emerging North-American outbreak in 2014. *J Clin Virol* 2015;**71**:1–9.
[7] Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. *J Clin Microbiol* 2006;**44**:2698–704.
[8] Piralla A, Mojoli F, Pellegrinelli L, Ceriotti F, Valzano A, Grasselli G, et al. Impact of SARS-CoV-2 omicron and delta variants in patients requiring intensive care unit (ICU) admission for COVID-19, Northern Italy, December 2021 to January 2022. *Respir Med Res* 2023;**83**:100990.
[9] Chu PY, Tyan YC, Chen YS, Chen HL, Lu PL, Chen YH, et al. Transmission and demographic dynamics of coxsackievirus B1. *PLoS One* 2015;**10**:e0129272.
[10] Rueca M, Lanini S, Giombini E, Messina F, Castilletti C, Ippolito G, et al. Detection of recombinant breakpoint in the genome of human enterovirus E11 strain associated with a fatal nosocomial outbreak. *Virol J* 2022;**19**:97.
[11] Khetsuriani N, Lamonte-Fowlkes A, Oberst S, Pallansch MACenters for Disease Control and Prevention. Enterovirus surveillance—United States, 1970-2005. *MMWR Surveill Summ* 2006;**55**:1–20.
[12] Wang P, Xu Y, Liu M, Li H, Wang H, Liu Y, et al. Risk factors and early markers for echovirus type 11 associated haemorrhage-hepatitis syndrome in neonates, a retrospective cohort study. *Front Pediatr* 2023;**11**:1063558.

The Journal of Infectious Diseases

MAJOR ARTICLE

Epidemiological and Clinical Insights into the Enterovirus D68 Upsurge in Europe 2021–2022 and Emergence of Novel B3-Derived Lineages, ENPEN Multicentre Study

Margarida Pires Simoes,[1,2] Emma B. Hodcroft,[3,4,5] Peter Simmonds,[6] Jan Albert,[7,8] Enagnon K. Alidjinou,[9] Katia Ambert-Balay,[10] Cristina Andrés,[11,12] Andrés Antón,[11,12] Christelle Auvray,[10] Jean-Luc Bailly,[13] Fausto Baldanti,[14,15] Capser Bastings,[16] Stuart Beard,[17] Carla Berengua,[18] Natasa Berginc,[19] Mandy Bloemen,[20] Soile Blomqvist,[21] Froukje Bosma,[22] Sindy Böttcher,[23] Laura Bubba,[24] Stafan Buderus,[25] Maria Cabrerizo,[26] Cristina Calvo,[27] Cristina Celma,[17] Ferruccio Ceriotti,[28] Gemma Clark,[29] Inës Costa,[30] Marianne Coste-Burel,[31] Karen Couderé,[32] Jeroen Cremer,[1] Margarita del Cuerpo Casas,[18] Theo Daehne,[33] Jessica de Beer,[22] Maria de Ceano-Vivas,[27] Cillian De Gascun,[34] Alexis de Rougemont,[10] Jonathan Dean,[34] Jennifer L. Dembinski,[35] Sabine Diedrich,[23] Javier Diez-Domingo,[36] Lena Dillner,[37] Dagny H. Dorenberg,[35] Alexandra Ducancelle,[38] Susanne Dudman,[39,40] Robert Dyrdak,[7,8] Anna-Maria Eis-Huebinger,[41] Iker Falces-Romero,[12] Agnes Farkas,[42] Susan Feeney,[43] Maria D. Fernandez-Garcia,[26] Jacky Flipse,[44] Kristina T. Franck,[45] Cristina Galli,[46] Isabelle Garrigue,[47] Felix Geeraedts,[22] Irina Georgieva,[48] Federica Giardina,[15] Raquel Guiomar,[30] Elenor Hauzenberger,[37] Esther Heikens,[49] Cécille Henquell,[13,50] Didier Hober,[9] Mario Hönemann,[51] Hannah Howson-Wells,[29] Željka Hruškar,[52] Niina Ikonen,[21] Berthemarie Imbert,[31] Arjan R. Jansz,[16] Marion Jeannoël,[53] Helena Jiřincová,[54] Laurence Josset,[53] Kathrin Keeren,[55] Naomie Kramer-Lindhout,[56] Sidsel Krokstad,[57] Mouna Lazrek,[9] Hélène Le Guillou-Guillemette,[38] Caroline Lefeuvre,[38] Andreas Lind,[39] Maja M. Lunar,[58] Melanie Maier,[51] Stéphanie Marque-Juillet,[59] C. Patrick McClure,[60] James McKenna,[43] Adam Meijer,[1] Ana Menasalvas Ruiz,[61] Beatriz Mengual-Chuliá,[36] Sofie Midgley,[45] Audrey Mirand,[13,50] Richard Molenkamp,[62] Milagrosa Montes,[63] Antonio Moreno-Docón,[64] Ursula Morley,[34] Jean-Luc Murk,[32] Ana Navascués-Ortega,[65] Roel Nijhuis,[66] Lubomira Nikolaeva-Glomb,[48] Svein A. Nordbø,[67] Sanela Numanovic,[35] Massimo Oggioni,[68] Eider Oñate Vergara,[63] Jordi Pacaud,[47] Marie L. Pacreau,[59] Marcus Panning,[33] Elena Pariani,[46] Lili Pekova,[69] Laura Pellegrinelli,[46] Miroslav Petrovec,[70] Corinna Pietsch,[51] Léa Pilorge,[71] Luis Piñeiro,[63] Antonio Piralla,[14] Mario Poljak,[58] Birgit Prochazka,[72] Nuria Rabella,[18] Janette C. Rahamat-Langendoen,[62] Petra Rainetova,[54] Marijke Reynders,[73] Annelies Riezebos-Brilman,[22] Lieuwe Roorda,[74] Carita Savolainen-Kopra,[21] Isabelle Schuffenecker,[53] Leo C. Smeets,[75] Asya Stoyanova,[48] Karl Stefic,[76] Caroline Swanink,[44] Irena Tabain,[52] Jeroen Tjhie,[16,32] Luc Thouault,[71] Camille Tumiotto,[47] Sara Uceda Renteria,[28] Tina Uršič,[70] Sophie Vallet,[71] Marc Van Ranst,[20] Peter Van Wunnik,[75] Jaco J. Verweij,[32] Jorgina Vila,[77] Bas Wintermans,[56] Elke Wollants,[20] Katja C. Wolthers,[78] F. Xavier López-Labrador,[36] Thea Kolsen Fischer,[79,80] Heli Harvala,[81,82] and Kimberley S. M. Benschop[1]

[1]Centre for Infectious Disease Control, Dutch National Public Health Institute, Bilthoven, The Netherlands; [2]European Program for Public Health Microbiology Training, European Centre for Disease Prevention and Control, Stockholm, Sweden; [3]Geneva Center of Emerging Viral Diseases, Geneva University Hospital and University of Geneva, Geneva, Switzerland; [4]Swiss Institute of Bioinformatics, Lausanne, Switzerland; [5]Institute of Social and Preventive Medicine, University of Bern, Bern, Switzerland; [6]Nuffield Department for Medicine, University of Oxford, Oxford, United Kingdom; [7]Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden; [8]Department of Microbiology, Tumor, and Cell Biology, Karolinska Institutet, Stockholm, Sweden; [9]Laboratoire de Virologie ULR, Univ Lille, Centre Hospitalier Universitaire de Lille, Lille, France; [10]National Reference Centre for Gastroenteritis Viruses, Laboratory of Virology-Serology, University Hospital of Dijon Bourgogne, Dijon, France; [11]Respiratory Viruses Unit, Microbiology Department, Vall d'Hebron Hospital Universitari, Vall d'Hebron Institut of Research, Vall d'Hebron Barcelona Hospital Campus, Barcelona, Spain; [12]Microbiology Department, Hospital Unviersitario La Paz, Centro de Investigación Biomédica en Red de Enfermedades Infecciosas, Instituto de Salud Carlos III, Madrid, Spain; [13]Labaratoire Microorganismes: Génome Environnement-Epidemiology and Physiopathology of Enterovirus Diseases LMGE-EPIE Team, Université Clermont Auvergne, CNRS, Clermont-Ferrand, France; [14]Microbiology and Virology Department, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, Pavia, Italy; [15]Department of Clinical Surgical Diagnostic and Pediatric Sciences, Università Degli Studi di Pavia, Pavia, Italy; [16]Laboratory for Medical Microbiology, Eurofins-PAMM, Veldhoven, The Netherlands; [17]Enteric Virus Unit, UK Health Security Agency, London, United Kingdom; [18]Microbiology Department, Hospital Universitari de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain; [19]National Laboratory of Health, Environment, and Food, Laboratory for Public Health Virology, Ljubljana, Slovenia; [20]Clinical and Epidemiological Virology, Rega Institute, Katholieke Universiteit Leuven, Leuven, Belgium; [21]Department of Health Security, Expert Microbiology Unit, Finnish Institute for Health and Welfare, Helsinki, Finland; [22]Laboratory for Medical Microbiology and Public Health, Hengelo, The Netherlands; [23]National Reference Laboratory for Poliomyelitis and Enteroviruses, Robert Koch Institute, Berlin, Germany; [24]European Non-Polio Enterovirus Network; [25]GFO Kliniken Bonn, Betriebsstätte St Marien, Bonn, Germany; [26]Enterovirus and Viral Gastroenteritis Lab, National Centre for Microbiology, Instituto de Salud Carlos III and the Spanish Research Networks Consortium of Epidemiology and Public Health, Madrid, Spain; [27]Pediatric and Infectious Diseases Department, Hospital Universtiario La Paz, Fundación IdiPaz, Centro de Investigación Biomédica en Red de Enfermedades Infecciosas, Instituto de Salud Carlos III, Madrid, Spain; [28]Virology Unit, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [29]Clinical Microbiology, Nottingham University Hospitals National Health Service Trust, Nottingham, United Kingdom; [30]National Reference Laboratory for Influenza and Other Respiratory Viruses, National Institute of Health Dr Ricardo Jorge, Lisbon, Portugal; [31]Virology Department, Centre Hospitalier Universitaire Hôtel Dieu, University Hospital, Nantes, France; [32]Microvida, Laboratory of Medical Microbiology and Immunology, Elisabeth Tweesteden Hospital, Tilburg, The Netherlands; [33]Institute of Virology, Medical Center University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany; [34]National Virus Reference Laboratory, University College Dublin, Dublin, Ireland; [35]Department of Virology, Norwegian Institute of Public Health, Oslo, Norway; [36] Center for Public Health Research (Foundation for the Promotion of Health and Biomedical Research in the Valencian Community), Generalitat

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Correspondence: Kimberley S.M. Benschop, PhD, Centre for infectious Disease Control, Centre for Infectious Disease Research, Diagnostics and Laboratory Surveillance, Dutch National Institute for Public Health and the Environment, PO Box 1, 3720 BA Bilthoven, The Netherlands (kim.benschop@rivm.nl).

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Valenciana, Valencia, Spain, and the Spanish Research Networks Consortium of Epidemiology and Public Health, Instituto de Salud Carlos III, Madrid Spain; [37]Department of Microbiology, Public Health Agency of Sweden, Solna, Sweden; [38]Laboratoire de Virologie, Département de Biologie des Agents Infectieux, Centre Hospitalier Universitaire Angers, Angers, France; [39]Department of Microbiology, Oslo University Hospital, Oslo, Norway; [40]Insititute of Clinical Medicine, University of Oslo, Oslo, Norway; [41]Insitute of Virology, Medical Faculty, University Bonn, Bonn, Germany; [42]National Public Health Center, Budapest, Hungary; [43]Regional Virus Laboratory, Belfast Health and Social Care Trust, Royal Victoria Hospital, Belfast, United Kingdom; [44]Laboratory for Medical Microbiology and Immunology, Rijnstate, Velp, The Netherlands; [45]Danish World Health Organization National Reference Laboratory for Poliovirus, Statens Serum Institut, Copenhagen, Denmark; [46]Department of Biomedical Sciences for Health, University of Milan, Milan, Italy; [47]Virology Department, University Hospital of Bordeaux, Bordeaux, France; [48]National Reference Laboratory for Enteroviruses, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria; [49]Department of Medical Microbiology, St Jansdal Hospital, Harderwijk, The Netherlands; [50]National Reference Centre for Enteroviruses and Parechoviruses-Associated Laboratory, Centre Hospitalier Universitaire Clermont-Ferrand, Clermont-Ferrand, France; [51]Institute of Medical Microbiology and Virology, University of Leipzig, Leipzig, Germany; [52]Department of Virology, Croatian Institute of Public Health, Zagreb, Croatia; [53]National Reference Center for Enteroviruses and Parechoviruses, Institut des Agents Infectieux, Hospices Civils de Lyon, Lyon, France; [54]National Reference Laboratory for Enteroviruses, National Institute of Public Health, Prague, Czech Republic; [55]Commission for Polio Eradication in Germany, Robert Koch Institute, Berlin, Germany; [56]Laboratory Medical Microbiology and Immunology, Admiraal de Ruijter Hospital, Goes, The Netherlands; [57]Department of Medical Microbiology, St Olavs Hospital, Trondheim University Hospital, Trondheim, Norway; [58]Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia; [59]Service de Biologie, Centre Hospitalier de Versailles Le Chesnay, France; [60]Wolfson Centre for Global Virus Research, School of Life Sciences, University of Nottingham, Nottingham, United Kingdom; [61]Pediatric Infectious Diseases Unit, Hospital Universitario Virgen de la Arrixaca, Murcia, Spain; [62]Department of Viroscience, Erasmus Medical Center, Rotterdam, The Netherlands; [63]Microbiology Department, Donostia University Hospital and Biogipuzkoa Health Research Institute, San Sebastián, Spain; [64]Microbiology Department, Hospital Clínico Universitario Virgen de la Arrixaca, Instituto Murciano De Investigación Biosanitaria Arrixaca, Murcia University, Murcia, Spain; [65]Microbiology Department, Complejo Hospitalario de Navarra, Navarra, Spain; [66]Department of Medical Microbiology and Immunology, Meander Medical Center, Amersfoort, The Netherlands; [67]Department of Clinical and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway; [68]Microbiology and Virology Unit, Department of Diagnostic Services, Azienda Socio Sanitaria Territoriale della Brianza, Vimercate, Italy; [69]Clinic of Infectious Diseases, University Hospital Prof Dr Stoyan Kirkovich AD, Stara Zagora, Bulgaria; [70]Institute of Microbiology and Immunology, Laboratory for the Diagnosis of Viral Infections, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia; [71]Unité de Virologie, Département de Bactériologie-Virologie-Parasitologie-Mycologie-Hygiène, Pôle de Biologie-Pathologie, Centre Hospitalier Régional et Universitaire de Brest, Brest Cedex, France; [72]Austrian Agency for Health and Food Safety, National Reference Laboratory for Poliomyelitis, Vienna, Austria; [73]Laboratory Medicine, Molecular Microbiology, AZ St Jan Brugge-Oostende AV, Bruges, Belgium; [74]Department of Medical Microbiology, Maasstad Hospital, Rotterdam, The Netherlands; [75]Department of Medical Microbiology, Reinier Haga Medical Diagnostic Center, Delft, The Netherlands; [76]Laboratoire de Virologie INSERM U1259, Centre Hospitalier Régional, Universitaire de Tours, Tours, France; [77]Paediatric Hospital Medicine, Department of Paediatrics, Hospital Universitari Vall d'Hebron, Barcelona, Spain; [78]Department of Medical Microbiology, OrganoVIR Labs, Amsterdam University Medical Centers, Amsterdam, The Netherlands; [79]Department of Clinical Research, Nordsjællands Hospital, Hilleroed, Denmark; [80]Department of Public Health, University of Copenhagen, Copenhagen, Denmark; [81]Nuffield Division of Clinical Laboratory Sciences, Radcliffe Department of Medicine, Oxford, United Kingdom; and [82]National Health Service Blood and Transplant, Microbiology Services, Colindale, United Kingdom

Enterovirus D68 (EV-D68) infections are associated with severe respiratory disease and acute flaccid myelitis (AFM). The European Non-Polio Enterovirus Network (ENPEN) aimed to investigate the epidemiological and genetic characteristics of EV-D68 infections and its clinical impact during the fall-winter season of 2021–2022. From 19 European countries, 58 institutes reported 10 481 (6.8%) EV-positive samples of which 1004 (9.6%) were identified as EV-D68 (including 852 respiratory samples). Clinical data were reported for 969 cases; 78.9% of infections were reported in children (0–5 years); and 37.9% of cases were hospitalized. Acute respiratory distress was commonly noted (93.1%) followed by fever (49.4%). Neurological problems were observed in 6.4% of cases including 6 diagnosed with AFM. Phylodynamic/Nextstrain and phylogenetic analyses based on 694 sequences showed the emergence of 2 novel B3-derived lineages, with no regional clustering. In conclusion, we describe a large-scale European EV-D68 upsurge with severe clinical impact and the emergence of B3-derived lineages.

Keywords. enterovirus D68 (EV-D68); respiratory infection; nonpolio enterovirus (NPEV); European Non-Polio Enterovirus Network (ENPEN); acute flaccid myelitis (AFM).

Enterovirus D68 (EV-D68) primarily infects the human upper respiratory tract and is mainly associated with mild to moderately severe upper respiratory symptoms, including sore throat, cough, congestion, and fever. However, infections can also be associated with lower respiratory tract infections and severe neurological conditions such as meningitis, encephalitis, or acute flaccid myelitis (AFM) [1–3]. Children up to 5 years of age are most commonly affected and are at greatest risk of developing severe disease [4]. Nevertheless, severe forms of EV-D68 have also been observed in adults, especially in elderly, immunosuppressed, or those with other underlying clinical conditions [2, 5].

EV-D68 is a member of the species *Enterovirus D* in the genus *Enterovirus,* family Picornaviridae [6]. The genus *Enterovirus* comprises 9 species and more than 200 types. Molecular detection is the gold standard to diagnose EV-D68 infections, either by an EV-D68 specific reverse transcription polymerase chain reaction (RT-PCR) or by an EV-generic assay targeting the conserved 5′ untranslated region followed by genotyping of the genes encoding capsid proteins VP1 or VP4–VP2 [7].

Phylogenetic analysis of VP1 sequences enables the differentiation of EV-D68 strains into genotypes A through D. The B genotype is further divided into subgenotypes/clades B1, B2, and B3, while the A subgenotype/clade is divided into A1 and A2, whereas A2 has been further divided into D1 and D2 (also referred to as A2/D1 and A2/D2, respectively). The most common EV-D68 subgenotype/clade circulating worldwide is B3, followed by A2/D2 [8].

The first EV-D68 reported outbreaks of acute respiratory disease in Europe occurred between 2008 and 2010 in the Netherlands and Italy [9–11]. Prior to that, clinical EV-D68 cases were rarely reported [12]. Since 2008, the epidemiology of EV-D68 in Europe and North America has shown a biennial epidemic cycle [13], with infections occurring predominantly in early fall and winter [14–17]. From 2014 onwards, the biennial EV-D68 outbreaks have been accompanied by reports of AFM cases testing positive for EV-D68 in the United States where AFM is subject to enhanced surveillance (reviewed in [16, 18]). AFM cases associated with EV-D68 were first reported in Europe in 2014 [19–21]. In 2019, a disruption of the biennial cycle was noted when an upsurge of EV-D68 infections with 93 cases was reported in 5 European countries [22]. The study also identified 5 EV-D68–infected children with severe

neurological disease and the circulation of B3-derived clusters, designated US18, EU18, and EU19.

During 2020, the first year of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, no EV-D68 cases were reported in Europe. However, during the fall of 2021, when countries eased nonpharmaceutical coronavirus disease 2019 (COVID-19) interventions, a substantial rise in EV infections was detected, in part due to increased EV-D68 circulation in some countries [4, 23]. The aim of this study was to examine the epidemiological, clinical, and molecular characteristics of this EV-D68 upsurge in Europe.

METHODS

Data Collection and Analysis

An invitation to participate in the study was sent to members of the European Non-Poliovirus Enterovirus Network (ENPEN). ENPEN brings together specialists from different fields including clinical virology, neurological and pediatric infectious diseases, academic/molecular virology, epidemiology, and public health [12, 24].

Institutes provided information on the source of the samples, sample types screened, and detection and typing methodology (Supplementary Table 1). Epidemiological data on the total number of samples tested and the total number of EV and EV-D68–positive samples were collected and analyzed per month from January through December 2021. All participating institutes were coded using a 2-digit country code, followed by a sequential number (eg, XX99; Supplementary Table 1).

Demographic and clinical data were reported in aggregated format or pseudonymized manner and included cases from 2021 and 2022 (until February; Table 1 and Table 2). The data collected is summarized in Figure 1.

EV-D68 Phylodynamic and Phylogenetic Analysis

Sequences were processed for phylodynamic analysis with the Nextstrain augur pipeline to show the time-related divergence [15, 22, 25]. Briefly, the pipeline combines EV-D68 sequences from this study and those publicly available EV-D68 sequences from National Center for Biotechnology Information GenBank (via ViPR). The lattter were randomly subsampled down to 240 sequences per country per year in order to reduce disparity in representation between countries with different levels of sequencing. Sequences were then aligned using IQTree, and a time-resolved phylogeny was produced with Treetime, along which ancestral sequences were reconstructed also using Treetime (both programs as implemented in Nextstrain). EV-D68 subgenotypes/clades were assigned using mutational markers on the phylogeny. Sequence analysis included metadata on country, sample type, date of collection, and age groups. Three analyses were performed based on the length of sequence available: (1) >300 bp, which included the partial VP1 sequences >300 bp, the near-complete VP1 sequences >700 bp, and the complete genomes (n = 692); (2) >700 bp, which included the near-complete VP1 sequences >700 bp and the complete genomes (n = 210); and (3) only complete genomes (n = 82) (Figure 1). Near-complete VP1 sequences and complete genome sequences were used to achieve a higher phylogenetic resolution. In the VP1 analyses, we included all available VP1 study sequences longer than 300 bp or 700 bp and sequences >700 bp extracted from GenBank on 21 September 2022 (n = 3740) (subsampled as described earlier) (Figure 1). In the full-genome analysis we included all available study sequences longer than 6 kbp and all available sequences longer than 6 kb from GenBank on 3 September 2022 (n = 976) (Figure 1). The code used to run the analysis is available at https://github.com/emmahodcroft/ev_d68_enpen2022.

To highlight the evolutionary divergence shown in Nextstrain, neighbor-joining trees (Jukes-Cantor corrected) using MEGA 7 software were constructed [26]. Mean pairwise (uncorrected *p* distances) distances between sequence groups were calculated using SSE software [27]. VP1 study sequences that were >80% complete between nucleotide positions 2501–2842 (numbering based on the Fermon prototype sequence, KU844179) were analyzed together with 3300 publicly available sequences between these positions (>90% complete) extracted from GenBank on 31 October 2022 (Figure 1). Complete genome sequences were analyzed together with 1025 publicly available complete genomes with known dates extracted from GenBank on 31 October 2022 (Figure 1).

GenBank Accession Numbers

Sequences were deposited in GenBank under the following accession numbers: OM811651-OM811652, OM831155-OM831207, ON006421-ON006422, OP267493-OP267535, OQ120627-OQ120631, OQ126930-OQ127239, OQ139546-OQ139558, OQ139565-OQ139630, OQ148174-OQ148361, OQ586762-OQ586804, OQ589870 and PP069743.

Ethical Statement and Privacy

Patients' privacy and confidentiality issues, according to General Data Protection Regulation, were managed in compliance with national/European legislation. Approval from an ethics committee and informed consent for virus screening was attained in accordance to participating institutes' regulations.

RESULTS

Detection Frequencies of EV and EV-D68

A total of 58 institutions from 19 European countries participated in this study (Table 1 and Supplementary Table 1). Tested samples ranged from respiratory (852 of 969 [88%]) to feces and cerebral spinal fluid, depending on institute/country and diagnostic/surveillance system. EV-D68 laboratory

Table 1. Enterovirus Testing and EV-D68 Detection in Participating European Countries From January 2021 and February 2022

			Sampling and Testing Data					Clinical Records		
			EV 2021			EV-D68 2021		EV-D68 Total, 2021–2022		
Country	Country Code	Institute (n)	Samples Tested (n)	Positive Samples (n)	EV Positive Detection, %	Positive Samples (n)	EV-D68 % of EV	Clinical Cases (n)	AFM Cases/ Acute Myelitis (n)	Sequences (n)
Austria	AT	1	381	2	0.5	1	50.0	1	0	0
Belgium	BE	2	11 275	1038	9.2	100	9.6	98	0	18
Bulgaria	BG	1	399	13	3.3	0	NA	0	0	0
Croatia	HR	1	106	14	13.2	0	NA	0	0	0
Czechia	CZ	1	465	120	25.8	0	NA	0	0	0
Denmark	DK	1	NR	431	NA[c]	17	3.9	12[d]	0	11
Finland	FI	1	176[b]	NA[b]	NA[b]	0	NA[b]	0	0	0
France	FR	10	28 237	1921	6.8	156	8.2	153[d,e]	1	132
Germany	DE	4[a]	6064	149	2.5	5	3.4	5	1	5
Hungary	HU	1	360	6	1.7	0	NA	0	0	0
Ireland	IE	1	10 075	1964	19.5	14	0.7	16[d]	0	16
Italy	IT	3	5296	415	7.8	20	4.8	24[d]	0	8
Netherlands	NL	11	18 258	1004	5.5	106	10.6	105	2	97
Norway	NO	3	8289	205	2.5	32	11.7	15[d]	0	1
Portugal	PT	1	1040	5	0.5	0	NA	3[d,f]	0	2
Slovenia	SI	3	5629	163	2.9	0	NA	0	0	0
Spain	ES	8[a]	39 738	1194	3.0	248	20.5	253[d]	1	249
Sweden	SE	2	6122	84	1.4	11	13.1	11	1	1
United Kingdom	UK	3	11 533	1753	15.2	294	16.8	273[d]	0	204
Total		58	153 443	10 481	6.8	1004	9.5	969	6	744

Abbreviations: AFM, acute flaccid myelitis; EV, enterovirus; NA, not applicable; NR, not reported.

[a]Contributor reported for other institutes.

[b]Finland only reported on tested samples for EV-D68.

[c]Denmark did not report on the total samples tested.

[d]Countries reporting EV-D68 cases in 2022.

[e]French institute reporting EV-D68 cases only in 2022.

[f]Portuguese institute only reporting EV-D68 cases in 2022.

confirmation was mostly based on respiratory samples. Of the institutes that reported the sample type information (n = 49), respiratory samples comprised 66% of the samples tested (Supplementary Table 1). Testing more than 150 000 samples revealed a total of 10 481 EV-positive samples (6.8%) from 1 January through 31 December 2021. Of the EV-positive samples, 1004 were confirmed as EV-D68–positive (9.6%). Large differences in the number of samples tested and proportions of EV and EV-D68–positive samples were observed among countries/reporting institutes (Table 1 and Supplementary Table 1, respectively). However, data could not be compared due to different catchment population and testing strategies. Most institutes tested multiple sample types, and higher proportions of respiratory samples tested did not reflect a higher proportion in EV-D68–positive detections. Notably, the 2 institutes that only performed testing on cerebrospinal fluid (CSF) samples did not report any EV-D68 cases.

Seasonality of EV and EV-D68

The number of samples tested for EV remained similar throughout the first 8 months of 2021 (average 10 000 tests/month) during the period when an increasing number and proportion of EV-positive samples were observed (Figure 2). The first EV-D68–positive sample was detected in June 2021 and EV-D68–positive samples were sporadically detected from June through August 2021. From September 2021 onwards, the number of samples tested for EV increased accompanying a higher number of EV-positive samples. During this time, the number of EV-D68–positive samples increased exponentially and reached a peak in October 2021 (405 of 1004, 40%).

Clinical Characteristics of EV-D68 Cases

Clinical data were reported by 41 institutes (13 countries) on 969 EV-D68 cases (Figure 1, Table 1, and Supplementary Table 1). Most EV-D68 cases were identified by testing a respiratory sample (n = 852, sample type known for 870 cases; 98%) whereas fecal (n = 16), vesicle (unknown origin; n = 1), or plasma (n = 1) samples were positive in the remaining cases. Most

Table 2. Demographic and Clinical Characteristics of EV-D68 Cases Reported by 14 European Countries From January 2021 Through February 2022 (n = 968)

Characteristic	EV-D68 Cases, No. (%)
Demographic characteristics	
Age group	
0–2 mo	79 (8.2)
3–23 mo	288 (29.7)
3–12 mo	215 (22.2)
13–23 mo	73 (7.5)
2–5 y	398 (41.1)
6–15 y	101 (10.4)
16–25 y	14 (1.4)
26–45 y	34 (3.5)
46–65 y	24 (2.5)
> 65 y	17 (1.8)
Unknown	14 (1.4)
Total	969
Sex	
Male	524 (54.08)
Female	361 (37.25)
Unknown	84 (8.67)
Clinical information	
Symptoms, data reported for	
Any symptom reported	668 (68.9)
Respiratory	622 (93.1)
Fever	330 (49.4)
Enteric	99 (14.8)
Neurological[a]	43 (6.4)
Rash	28 (4.2)
Coinfections	
Any coinfection reported	241 (24.9)
Rhinovirus	115 (47.7)
Adenovirus	45 (18.7)
RSV	33 (13.7)
CoV (OC43, 229E, and SARS-CoV-2)	12 (5.0)
Clinical history and hospital information	
Preexisting condition[b]	249[c] (25.7)
Hospitalized	369 (38.1)
Hospital stay, average days	2.8
Intensive care unit admission	61 (16.5)

Abbreviations: CoV, coronavirus; RSV, respiratory syncytial virus.

[a]Reported neurological symptoms included headache, dizziness and agitation, seizures, encephalitis, meningitis, acute myelitis, and acute flaccid myelitis/acute flaccid paralysis.

[b]Reported preexisting conditions were asthma, congenital malformations, epilepsy, prematurity, and cancer.

[c]There were 235 patients with comorbidities displaying respiratory signs.

infections were reported in children between 2 and 5 years of age (41.1%) followed by children between 3 and 12 months of age (22.2%) (Table 2). In total, 79% (n = 765) of EV-D68 cases were in the age group 0–5 years (median age of 2.9 years, range from new-borns to 93 years). More than half of the cases were male (54%). Detailed clinical information was available for 668 EV-D68 cases showing respiratory distress as the predominant symptom (93.1%). The second most common clinical sign was fever, being reported in approximately half of these cases.

As shown in Table 2, coinfections were reported for 241 of 969 (24.9%) of EV-D68 cases, of which almost half were also infected with human rhinovirus. More than 2 coinfecting viruses were reported in 69 cases (28.6%).

Of 969 EV-D68 cases, 369 (38.1%) were hospitalized between 0.5 and 136 days (interquartile range, 0.5–3 days). A total of 249 individuals with EV-D68 infection (25.7%) had known underlying medical conditions, for example prematurity, congenital malformations, asthma, and different cancer types (Table 2).

Neurological conditions were identified in 43 patients (6.4%), most of which were in the age group 0–5 years (n = 34; 79%). The neurological problems ranged from headache, dizziness, and agitation to seizures. One case was reported with encephalitis (8 years of age with comorbidities) and 4 cases were diagnosed with meningitis (up to 5 months of age). AFM was reported in 6 children: 5 cases up to 5 years of age and 1 in an older child (6–15 years of age). Patients with neurological disorders typically showed respiratory distress (32 of 43; 74.4%), fever (26 of 43; 60.5%), enteric symptoms (12 of 43; 27.9%), or rash (7 of 43, 16.3%); and 37.2% (16 of 43) had an underlying medical condition. Twenty-eight of 43 patients had severe neurological disorders requiring intensive care unit (ICU) admission, 82.1% (23 of 28) being between 0 and 5 years of age and 39.3% (11/28) with known comorbidities.

Phylodynamics and Divergence of EV-D68 Strains

To investigate the circulation of EV-D68 strains, in this study we included a maximum of 694 sequences of the received 744 sequences for analysis (Figure 1). A majority of the strains were reported by submitting institutes as B3, and 8 strains as A2/D2. The B3 strains were detected throughout the study period. The A2/D2 strains were detected from October through December 2021 (data not shown). Figure 3 shows a screenshot of the Nextstrain build of the 300-bp VP1 EV-D68 sequences over time, accessible at https://nextstrain.org/community/enterovirus-phylo/evd68-2022/vp1-300. All of the Nextstrain runs are available at https://github.com/enterovirus-phylo/evd68-2022. The phylogenetic tree showed a temporal ladder-like evolution and the emergence of 2 novel B3-derived lineages, designated lineage 1 and lineage 2 for this study. These patterns are visible in both of the VP1 analyses and the full-genome analysis.

The divergence between these lineages was estimated to be 4.2% based on the complete genome (5.2% based on the partial VP1). Lineage 1 descended from B3 strains reported in 2019 (previously designated US18 with the 2019–2020 upsurge) and was detected across Europe (Figure 4) predominantly in the United Kingdom, Netherlands, France, and Spain from August 2021 throughout January 2022. For the 4 AFM cases with available sequence data available, all fell into the B3-derived lineage 1.

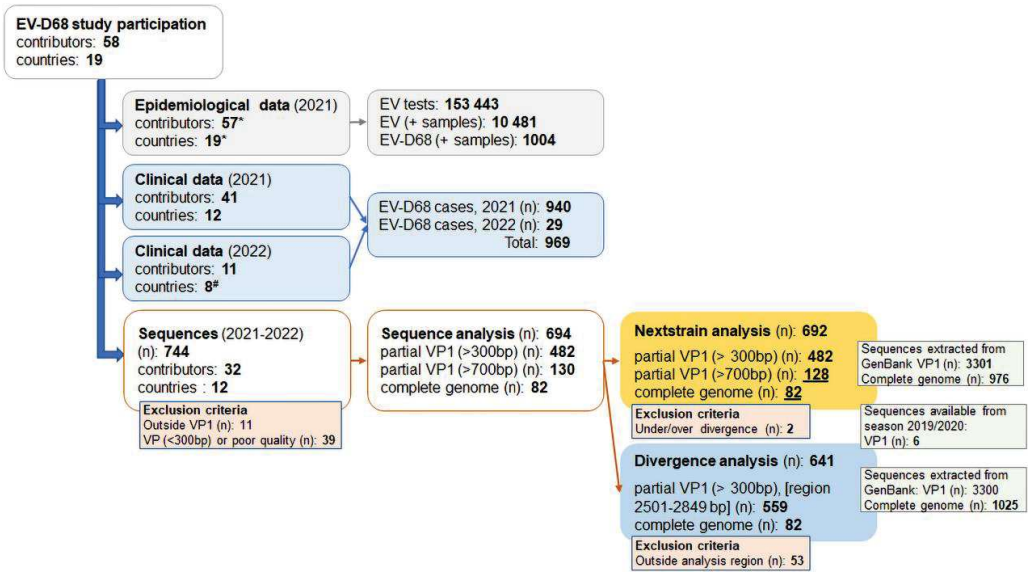

Figure 1. Diagram of the information collected: epidemiological data, clinical records, and sequence data with associated clinical metadata used for the analysis of the EV-D68 upsurge in Europe, 2021–2022. Sequence information is also represented separately for phylogenetic reconstruction (Nextstrain analysis) and divergence analysis. Publicly accessible sequences, extracted from Gen Bank, used for epidemiologic analysis are depicted in boxes alongside exclusion criteria. *Monthly testing data were received from 55 institutes, 18 countries; and monthly EV-positive data received from 57 institutes, 19 countries. #Two institutes only reported EV-D68 cases for 2022 (1 also representing the country cases).

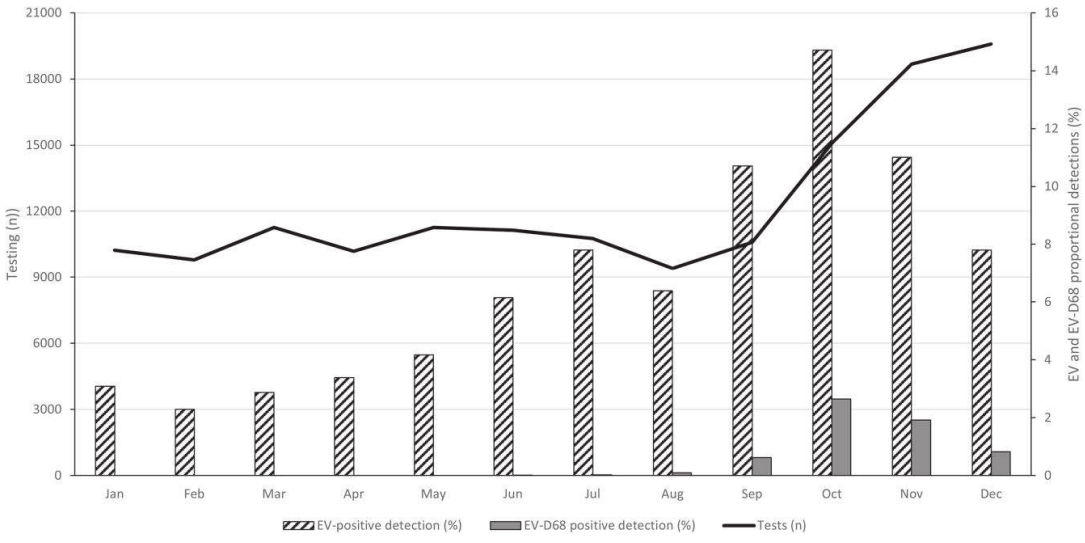

Figure 2. Proportion of samples found positive for EV (lined bars, % of EV-positive samples/number of samples tested) and EV-D68 (grey bars, % of EV-D68–positive samples/number of EV positive samples), and monthly totals of EV tests (line) reveal an increasing trend from September 2021 onwards. The highest numbers of EV and EV-D68 detections were observed in October 2021.

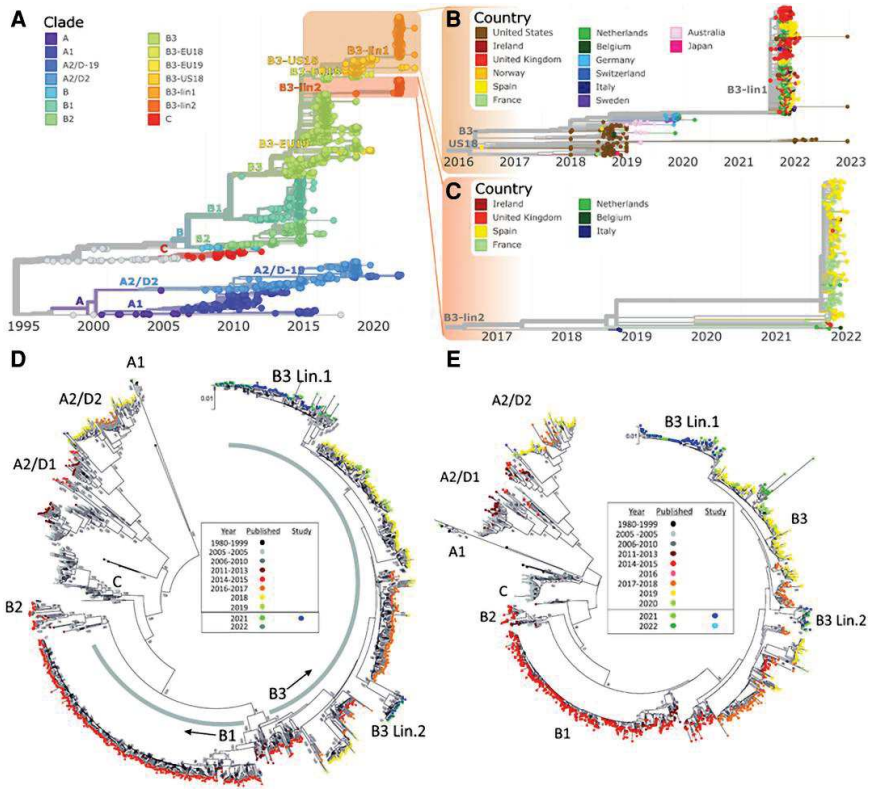

Figure 3. *A–C*, Phylodynamic analysis of EV-D68 with Nextstrain using 692 partial study VP1 sequences > 300 bp and 3307 publicly available VP1 sequences. *B* and *C*, Zoomed views of areas of the tree containing the 2 novel lineages. *D*, Neighbor-joining phylogenetic tree of complete genome from the study samples (n = 82) and 1025 sequences with data annotations from GenBank. *E*, Neighbor-joining phylogenetic tree of VP1 region sequences (positions 2501–2842, numbered using the Fermon prototype sequence, KU844179) from the study samples (n = 559) and 3306 sequences with data annotations from GenBank.

Lineage 2 was distantly related to a B3 sample from the 2016 outbreak in Europe and predominantly detected in southwestern European countries, such as Spain and France (Figure 4) from June 2021 through January 2022, with similar kinetics to lineage 1 variants (data not shown). Notably, lineage 2 showed a deletion at VP1:S143 not widely seen elsewhere on the phylogeny.

DISCUSSION

In this study, we describe valuable information on the demographic and clinical features of nearly a thousand EV-D68 cases mostly reported in the fall and winter of 2021–2022 by 13 of 19 European countries participating in the study, and the emergence of novel B3-derived lineages. For the first time, the collection of denominator data, that is, the number of samples tested for EV and EV-D68 across Europe, allowed for a depiction of the proportion of both EV and EV-D68 infections found within different institutes in 2021. However, it should be noted that catchment population and testing strategies varied, and data could not be compared directly between institutes nor among countries. Nevertheless, the study revealed the importance of sharing data across Europe, which aimed to improve diagnostic awareness based on the increased circulation of EV-D68. It also enforced our understanding of the epidemiology and evolution of EV-D68. The study also revealed gaps in data comparability and the need for better harmonized medical and diagnostic practices.

Based on the biennial circulation pattern of EV-D68 previously recorded in Europe, an EV-D68 upsurge was expected in summer/fall of 2020. However, this 2-year cycle had already been disrupted by the previous EV-D68 upsurge in Europe in 2019 [22] and was disturbed further by COVID-19 nonpharmaceutical interventions. For EV-D68 (and other viral pathogens) the disruption in their circulation is also hypothesized to have led to a much larger EV-immune–naive cohort

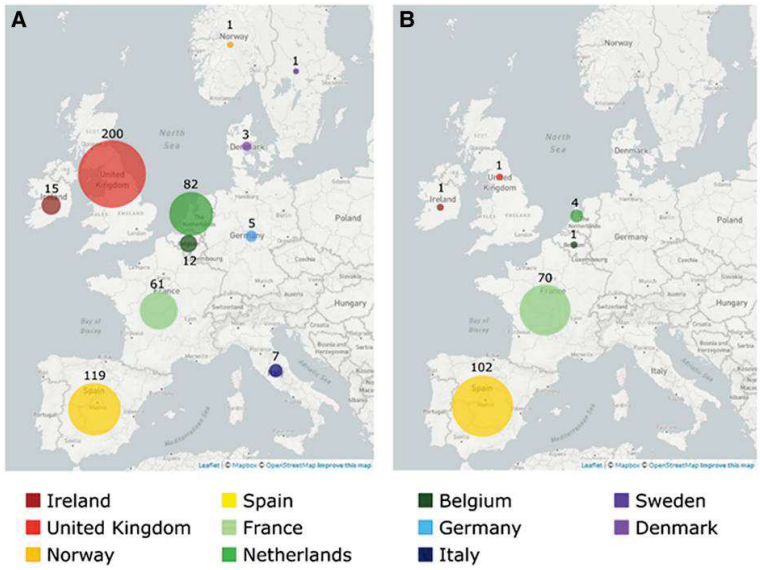

Figure 4. Maps showing the geographic distribution of the 2 novel EV-D68 B3-derived lineages, lineage 1 (*A*) and lineage 2 (*B*).

compared to the ones found in previous incidence cycles [15, 23, 28]. As a result, the easing of COVID-19 nonpharmaceutical interventions may have spurred new upsurges far greater than in previous cycles in Europe and beyond [4, 29–31]. Similarly, increased detections of other pathogens has been noted due to resumption of community circulation [32–34]. An additional contributory factor to the greater number of samples tested for EV and EV-D68 detections may have been the increased number of respiratory samples that were collected for syndromic testing on respiratory viruses, including SARS-CoV-2.

EV-D68 infections were predominantly associated with respiratory symptoms (93.1%), and nearly a quarter of EV-D68 infections were in individuals with underlying medical conditions, which may have played a role in the severity of EV-D68 infections [5, 16, 35, 36]. Similar proportions of underlying medical conditions among EV-D68 infections related to severity were also reported in previous studies, albeit the populations studied were different [16]. Most cases were detected by diagnostic testing of respiratory samples, highlighting the importance of including respiratory samples in EV surveillance as EV-D68 RNA is rarely detected in fecal and CSF samples, even in patients with neurological disease [7, 37]. It is noteworthy that rhinovirus was the predominant coinfection, which was also seen in other studies [36, 38]. It should be considered which underlying medical conditions and coinfections are to be included in the data collection to best promote standardization and further implementation in future EV-D68 studies [7, 12].

A total of 43 EV-D68 patients (6.4%) displayed neurological disorders, and half of them were diagnosed with seizures, encephalitis, meningitis, or AFM, providing further evidence for a potentially neurovirulent property of this virus [39]. Although data are reported on only 6 AFM cases with confirmed EV-D68 infection, it was noted that for several other patients with similar paralytic clinical presentations laboratory testing for EV-D68 was not performed or failed due to the delayed onset of neurological symptoms (unpublished data; [40]). This could have resulted from inappropriate sampling for EV-D68 testing or clinical presentations that were not identified as AFM due to lack of clinical awareness. Furthermore, AFM initially starts with a respiratory prodromal phase and samples may not have been collected at an appropriate time before the onset of AFM [41]. Inappropriate sampling and diagnosis can lead to underdiagnosing and underreporting [42, 43] and thus, clear guidelines on how to diagnose AFM are required.

Of the reported clinical data, over 38% EV-D68 cases required hospitalization and 17% of them at ICU level, revealing a substantial utilization of health care resources from EV-D68 infections in our study population, especially in young children. These results are consistent with previous studies [36]. The high proportions of hospitalization can be the result of a sampling bias due to testing strategy. Thus, standardized surveillance that includes the general asymptomatic population is essential to estimate the true burden of disease. The EV-D68 hospitalization rate is concordant with that associated

with influenza virus infections (34%) [31], and ICU admission rates due to Respiratory Syncytial Virus infections resemble those of EV-D68 (both 17%) [44].

By comparing the 2021–2022 sequence data to previous EV-D68 strains, we were able to detect a similar stepwise diversification of EV-D68 as observed with other viruses [45–47]. In this study, the majority of the sequences encompassed partial VP1, as most institutes use the assay developed by Nix and colleagues [48].

Despite the interruption in EV-D68 circulation during the COVID-19 pandemic, EV-D68 evolution continued, resulting in the emergence of 2 novel postpandemic B3-derived lineages. Lineage 1 clearly originated from the prepandemic strains designated as B3-US18 by Midgley et al [22]. In that study, 2 other clusters/lineages were observed and designated as B3-EU18 and B3-EU19 [15, 22]. These were not observed in 2021–2022, and may no longer be circulating, or circulating only at very low levels.

In contrast, another B3-derived lineage (lineage 2) was identified in 2021 and is less clearly linked to recent outbreaks, with a common ancestor in 2016. This highlights the need for more comprehensive surveillance of EV-D68 to better understand where such lineages may have circulated before becoming widespread. To track whether these novel lineages persist in the coming years or are replaced by other novel variants, vigilant monitoring and rapid molecular characterization shared among institutes is required.

This study has a number of limitations, particularly related to differences in surveillance systems without a uniform case definition and sampling strategy, and differences in screening and typing methods across institutes. In addition, not all institutes were able to provide detailed monthly testing data. Clinical records were incomplete in some cases due to different reasons such as General Data Protection Regulation (GDPR) or constraints of the reporting systems used by the institutes. The varied and incomplete reporting may have led to biased data, therefore we propose the standardization of data collection with comprehensive reporting to better determine the disease burden of EV-D68 infections. Finally, EV-D68 samples are generally only collected from symptomatic and hospitalized individuals, which most likely do not fully reflect the demographics or overall geographic distribution. Despite these limitations, the epidemiological and clinical data show the considerable disease burden of this infection, especially in younger children, as well its reemergence and continued evolution during and after the alleviation of nonpharmaceutical interventions in the wake of the SARS-CoV-2 pandemic. With globalization and human connectedness, pathogenic agents are also constantly on the move and continue to evolve. EV-D68 should be considered in the differential diagnosis, especially when attending children with respiratory and/or neurological symptoms. It is our understanding that timely diagnosis could improve medical handling, in particular when neurological signs are present. Concurrently, standardizing sampling, clinical and viral diagnosis, and typing requirements would all provide better data, which can then contribute towards the improved understanding of the clinical and public health impact of EV-D68 and other enteroviruses [7, 12].

CONCLUSION

This study substantiates and extends the previous description of an upsurge in EV-D68 infections in September 2021 [23], which raised awareness of EV-D68–associated respiratory and neurological infections in many countries and led to enhanced vigilance. The data shown in this study underline the clinical and public health impact of EV-D68. The observation of rapid genetic diversification of EV-D68 into novel B3-derived lineages emphasizes the value of continued phylogenetic monitoring of EV-D68 and calls for further genomic analyses to investigate potential strain-associated differences in neuropathogenicity suspected in previous outbreaks [1, 2]. Overall, we highlight the need for the implementation of a mandatory and harmonized pan-European EV surveillance system.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Author contributions. K. B., H. H., and T. K. F. initiated the study. K. B. and M. S. collected and analyzed the data, wrote the first draft, prepared the tables and figures, and edited the final manuscript. E. H., K. B., and P. S. analyzed the sequence data. E. H., H. H., P. S., and T. K. F. helped with drafting the manuscript. All other authors were responsible for diagnostics, testing, and data collection at partner sites, provided and checked data, critically reviewed and edited the manuscript, and have accepted the final version of the manuscript.

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References

1. Hixon AM, Frost J, Rudy MJ, Messacar K, Clarke P, Tyler KL. Understanding enterovirus D68-induced neurologic disease: a basic science review. Viruses **2019**; 11:821.
2. Sooksawasdi Na Ayudhya S, Laksono BM, van Riel D. The pathogenesis and virulence of enterovirus-D68 infection. Virulence **2021**; 12:2060–72.
3. Elrick MJ, Pekosz A, Duggal P. Enterovirus D68 molecular and cellular biology and pathogenesis. J Biol Chem **2021**; 296:100317.
4. Zhang W, Luan R. Estimating the impact of non-pharmaceutical interventions against COVID-19 on mumps incidence in Sichuan, China. BMC Infect Dis **2021**; 21:886.
5. Larsson SB, Vracar D, Karlsson M, Ringlander J, Norder H. Epidemiology and clinical manifestations of different enterovirus and rhinovirus types show that EV-D68 may still have an impact on severity of respiratory infections. J Med Virol **2022**; 94:3829–39.
6. Zell R, Delwart E, Gorbalenya AE, et al. ICTV virus taxonomy profile: Picornaviridae. J Gen Virol **2017**; 98:2421–2.
7. Harvala H, Broberg E, Benschop K, et al. Recommendations for enterovirus diagnostics and characterisation within and beyond Europe. J Clin Virol **2018**; 101:11–7.
8. Andres C, Vila J, Creus-Costa A, et al. Enterovirus D68 in hospitalized children, Barcelona, Spain, 2014–2021. Emerg Infect Dis **2022**; 28:1327–31.
9. Meijer A, van der Sanden S, Snijders BE, et al. Emergence and epidemic occurrence of enterovirus 68 respiratory infections in The Netherlands in 2010. Virology **2012**; 423: 49–57.
10. Piralla A, Lilleri D, Sarasini A, et al. Human rhinovirus and human respiratory enterovirus (EV68 and EV104) infections in hospitalized patients in Italy, 2008–2009. Diagn Microbiol Infect Dis **2012**; 73:162–7.
11. Poelman R, Schuffenecker I, Van Leer-Buter C, et al. European surveillance for enterovirus D68 during the emerging North-American outbreak in 2014. J Clin Virol **2015**; 71:1–9.
12. Fischer TK, Simmonds P, Harvala H. The importance of enterovirus surveillance in a post-polio world. Lancet Infect Dis **2022**; 22:e35–40.
13. Kramer R, Sabatier M, Wirth T, et al. Molecular diversity and biennial circulation of enterovirus D68: a systematic screening study in Lyon, France, 2010 to 2016. Euro Surveill **2018**; 23:1700711.
14. Cottrell S, Moore C, Perry M, Hilvers E, Williams C, Shankar AG. Prospective enterovirus D68 (EV-D68) surveillance from September 2015 to November 2018 indicates a current wave of activity in Wales. Euro Surveill **2018**; 23:1800578.
15. Hodcroft EB, Dyrdak R, Andres C, et al. Evolution, geographic spreading, and demographic distribution of enterovirus D68. PLoS Pathog **2022**; 18:e1010515.

16. Holm-Hansen CC, Midgley SE, Fischer TK. Global emergence of enterovirus D68: a systematic review. Lancet Infect Dis **2016**; 16:e64–75.
17. Xiang Z, Wang J. Enterovirus D68 and human respiratory infections. Semin Respir Crit Care Med **2016**; 37:578–85.
18. Messacar K, Tyler KL. Enterovirus D68-associated acute flaccid myelitis: rising to the clinical and research challenges. JAMA **2019**; 321:831–2.
19. Helfferich J, de Lange MM, Benschop KS, et al. Epidemiology of acute flaccid myelitis in children in The Netherlands, 2014 to 2019. Euro Surveill **2022**; 27:2200157.
20. Lang M, Mirand A, Savy N, et al. Acute flaccid paralysis following enterovirus D68 associated pneumonia, France, 2014. Euro Surveill **2014**; 19:20952.
21. Pfeiffer HC, Bragstad K, Skram MK, et al. Two cases of acute severe flaccid myelitis associated with enterovirus D68 infection in children, Norway, autumn 2014. Euro Surveill **2015**; 20:21062.
22. Midgley SE, Benschop K, Dyrdak R, et al. Co-circulation of multiple enterovirus D68 subclades, including a novel B3 cluster, across Europe in a season of expected low prevalence, 2019/20. Euro Surveill **2020**; 25:1900749.
23. Benschop KS, Albert J, Anton A, et al. Re-emergence of enterovirus D68 in Europe after easing the COVID-19 lockdown, September 2021. Euro Surveill **2021**; 26:2100998.
24. Benschop KSM, Broberg EK, Hodcroft E, et al. Molecular epidemiology and evolutionary trajectory of emerging echovirus 30, Europe. Emerg Infect Dis **2021**; 27:1616–26.
25. Huddleston J, Hadfield J, Sibley TR, et al. Augur: a bioinformatics toolkit for phylogenetic analyses of human pathogens. J Open Source Softw **2021**; 6:2906.
26. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol **2016**; 33:1870–4.
27. Simmonds P. SSE: a nucleotide and amino acid sequence analysis platform. BMC Res Notes **2012**; 5:50.
28. Tedcastle A, Wilton T, Pegg E, et al. Detection of enterovirus D68 in wastewater samples from the UK between July and November 2021. Viruses **2022**; 14:143.
29. Park SW, Pons-Salort M, Messacar K, et al. Epidemiological dynamics of enterovirus D68 in the United States and implications for acute flaccid myelitis. Sci Transl Med **2021**; 13:eabd2400.
30. van Summeren J, Meijer A, Aspelund G, et al. Low levels of respiratory syncytial virus activity in Europe during the 2020/21 season: what can we expect in the coming summer and autumn/winter? Euro Surveill **2021**; 26:2100639.
31. Wang LP, Han JY, Zhou SX, et al. The changing pattern of enteric pathogen infections in China during the COVID-19 pandemic: a nation-wide observational study. Lancet Reg Health West Pac **2021**; 16:100268.
32. Liu P, Xu M, Lu L, et al. The changing pattern of common respiratory and enteric viruses among outpatient children in Shanghai, China: two years of the COVID-19 pandemic. J Med Virol **2022**; 94:4696–703.
33. Olsen SJ, Winn AK, Budd AP, et al. Changes in influenza and other respiratory virus activity during the COVID-19 pandemic-United States, 2020–2021. Am J Transplant **2021**; 21:3481–6.
34. Honemann M, Thiem S, Bergs S, et al. In-depth analysis of the re-emergence of respiratory syncytial virus at a tertiary care hospital in Germany in the summer of 2021 after the alleviation of non-pharmaceutical interventions due to the SARS-CoV-2 pandemic. Viruses **2023**; 15:877.
35. Cassidy H, Poelman R, Knoester M, Van Leer-Buter CC, Niesters HGM. Enterovirus D68—the new polio? Front Microbiol **2018**; 9:2677.
36. Penela-Sanchez D, Gonzalez-de-Audicana J, Armero G, et al. Lower respiratory tract infection and genus enterovirus in children requiring intensive care: clinical manifestations and impact of viral co-infections. Viruses **2021**; 13: 2059.
37. Freeman MC, Wells AI, Ciomperlik-Patton J, et al. Respiratory and intestinal epithelial cells exhibit differential susceptibility and innate immune responses to contemporary EV-D68 isolates. Elife **2021**; 10:e66687.
38. Giardina FAM, Piralla A, Ferrari G, Zavaglio F, Cassaniti I, Baldanti F. Molecular epidemiology of rhinovirus/enterovirus and their role on cause severe and prolonged infection in hospitalized patients. Microorganisms **2022**; 10: 755.
39. Sinclair W, Omar M. Enterovirus. StatPearls. Treasure Island, FL: StatPearls Publishing, **2022**.
40. Christy A, Messacar K. Acute flaccid myelitis associated with enterovirus D68: a review. J Child Neurol **2019**; 34: 511–6.
41. Knoester M, Helfferich J, Poelman R, et al. Twenty-nine cases of enterovirus-D68-associated acute flaccid myelitis in Europe 2016: a case series and epidemiologic overview. Pediatr Infect Dis J **2019**; 38:16–21.
42. Helfferich J, Knoester M, Van Leer-Buter CC, et al. Acute flaccid myelitis and enterovirus D68: lessons from the past and present. Eur J Pediatr **2019**; 178:1305–15.
43. Murphy OC, Messacar K, Benson L, et al. Acute flaccid myelitis: cause, diagnosis, and management. Lancet **2021**; 397: 334–46.
44. Rha B, Curns AT, Lively JY, et al. Respiratory syncytial virus-associated hospitalizations among young children: 2015–2016. Pediatrics **2020**; 146:e20193611.
45. Chen Z, Schiffman M, Herrero R, et al. Classification and evolution of human papillomavirus genome variants: alpha-5 (HPV26, 51, 69, 82), alpha-6 (HPV30, 53, 56,

66), alpha-11 (HPV34, 73), alpha-13 (HPV54) and alpha-3 (HPV61). Virology **2018**; 516:86–101.
46. Graf T, Bello G, Venas TMM, et al. Identification of a novel SARS-CoV-2 P.1 sub-lineage in Brazil provides new insights about the mechanisms of emergence of variants of concern. Virus Evol **2021**; 7:veab091.
47. Huang C, Yu J, Hause BM, et al. Emergence of new phylogenetic lineage of influenza D virus with broad antigenicity in California, United States. Emerg Microbes Infect **2021**; 10:739–42.
48. Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol **2006**; 44:2698–704.