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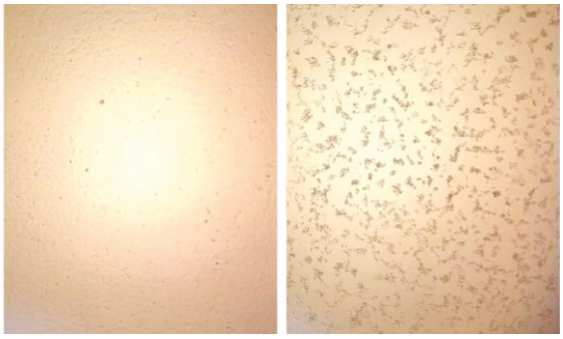
SARS-CoV-2 emergence: dissecting mechanisms of viral infection and immunological response



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Ciclo XXXIV – A.A. 2018-2021

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Cover image: cytopathic effect of SARS-CoV-2 on VERO E6 cells

A mia madre e mio padre

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Abstract

The new Coronavirus Disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in late 2019 and rapidly became a global health problem. In few months, the world scientific committee and health organizations had been involved in one of the most dramatic pandemic of the modern world. In less than one year, international scientific efforts contributed to the rapid introduction of therapies (e.g. monoclonal antibodies) and vaccines. More recently, the emergence of SARS-CoV-2 variants of concern (VOC) has posed new issues in terms of vaccine immunogenicity and immune protection against reinfection in convalescent subjects. At the same time, those subjects with immunological lesions might be exposed to increased risk of disease, even in presence of a complete vaccination schedule. Thus, one of the most crucial topics is related to the understanding of immune response against SARS-CoV-2 infection and vaccination, including both humoral and cell-mediated immune response.

Overall, this work provides a comprehensive evaluation of immunological response of SARS-CoV-2 infection and vaccination, helping to define future vaccination strategies (e.g. administration of booster doses in frail patients). In detail, during my PhD work, we designed and applied a microneutralization assay for quantification of SARS-CoV-2 neutralizing antibodies in different epidemiological and clinical settings, including the screening population for selection of hyperimmune plasma donors. Later, we investigated the T-cell mediated immunity against SARS-CoV-2 by the assessment and development of a in-house SARS-CoV-2 ELISpot assay. After the introduction of vaccination, the methods were optimized and used in different cohorts of vaccinated subjects, including healthcare workers and frail patients. Furthermore, the impact of SARS-CoV-2 variants on immune response elicited by vaccination was explored. Finally, mechanisms of SARS-CoV-2 infection and transmission were analysed using in vitro models in order to demonstrate the potential role of macrophages and monocytes in viral spread.

List of abbreviations

SARS-CoV-2	Severe acute respiratory syndrome virus 2
COVID-19	Coronavirus disease 2019
hCoVs	Human Coronaviruses
ACE2	Angiotensin converting enzyme 2
TMPRSS2	Transmembrane Serine Protease 2
RBD	Receptor binding domain
MAbs	Monoclonal antibodies
NT Abs	Neutralizing antibodies
IFNγ	Interferon gamma
ELISpot	Enzyme-linked immunospot assay
VOC	Variant of concern
ICI	Immune checkpoint inhibitor
MOI	Multiplicity of infection
TCID50	Median tissue culture infectious dose
PFU	Plaque-forming unit
PBMC	Peripheral blood mononuclear cells

1. Introduction

Novel coronavirus-related disease, named Coronavirus Disease 2019 (COVID-19) was firstly discovered in Wuhan (Hubei province, China) in late 2019, since local health authorities reported clusters of subjects with pneumonia of unknown aetiology linked to a local seafood and wet animal market (Wuhan Municipal Health Commission, 2019). Diagnostic procedures performed in four respiratory samples obtained from patients with unknown pneumonia led to the identification of a new beta coronavirus (Zhu *et al.,* 2019). Later, the virus was named as severe acute respiratory syndrome coronavirus- 2 (SARS-CoV-2) (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020) and spread rapidly all over the world, being responsible of the first coronavirus-related pandemic after the SARS and MERS epidemics in 2003 (Drosten *et al*., 2003) and 2012 (Corman *et al*., 2012). Only on March 11th, 2020, the World Health Organization (WHO) declared global emergency status. Italy has been one of the most affected European countries, reporting a dramatic number of hospitalizations and deaths. On 29th January 2020, two Chinese tourists reporting fever and other respiratory symptoms were hospitalized at the National Institute for Infectious Diseases "L. Spallanzani" in Rome and with hindsight a diagnosis of SARS-CoV-2 infection was made (Capobianchi *et al*., 2020). In Lombardy, the first case of documented SARS-CoV-2 infection was reported in a young man in Castiglione d'Adda (Lodi province, south of Milan) on 20th February, 2020 and in few hours other 36 cases, reporting no contact with the first patient or with other documented cases, were diagnosed (Livingston *et al.,* 2020). The exponential increase of SARS-CoV-2 cases and related diseases led to the definition of the first lockdown area of the Lombardy region. Diagnostic procedures were rapidly intensified as well as therapeutic and prophylactic social strategies that led to a national lockdown on 9th March 2020, with a subsequent decline of SARS-CoV-2 active cases during summer months. However, in September 2020 a new increase in cases and deaths was reported in our country and in other European and non-European countries. Despite the great efforts of scientific committees and organizations, more than 200 million cases and 4,5 million deaths were counted worldwide, by August 2021. On the other hand, the international scientific efforts contributed to the rapid introduction of several vaccines against SARS-CoV-2, allowing the most impressive vaccination campaign ever. One of the major concerns in vaccine immunogenicity was related to the emergence of SARS-CoV-2 variants that may be associated with a decreased effectiveness of vaccination. At the same time, those subjects with immunological defects due to their clinical conditions, including transplanted subjects or oncologic patients, might still be exposed to increased risk of SARS-CoV-2 disease, even though they complete the vaccination schedule.

1.1. Coronaviridae

Human Coronaviruses (HCoVs) (Figure 1), belonging to the Coronaviridae family (order Nidovirales), are enveloped single positive-stranded non-fragmented RNA viruses, firstly identified in1965 from a patient with common cold (Tyrrell *et al.*, 1965). They are characterized by a typical "crown" structure, due to the presence of trimeric Spike glycoprotein at the envelope level (Lai *et al.,* 1997).

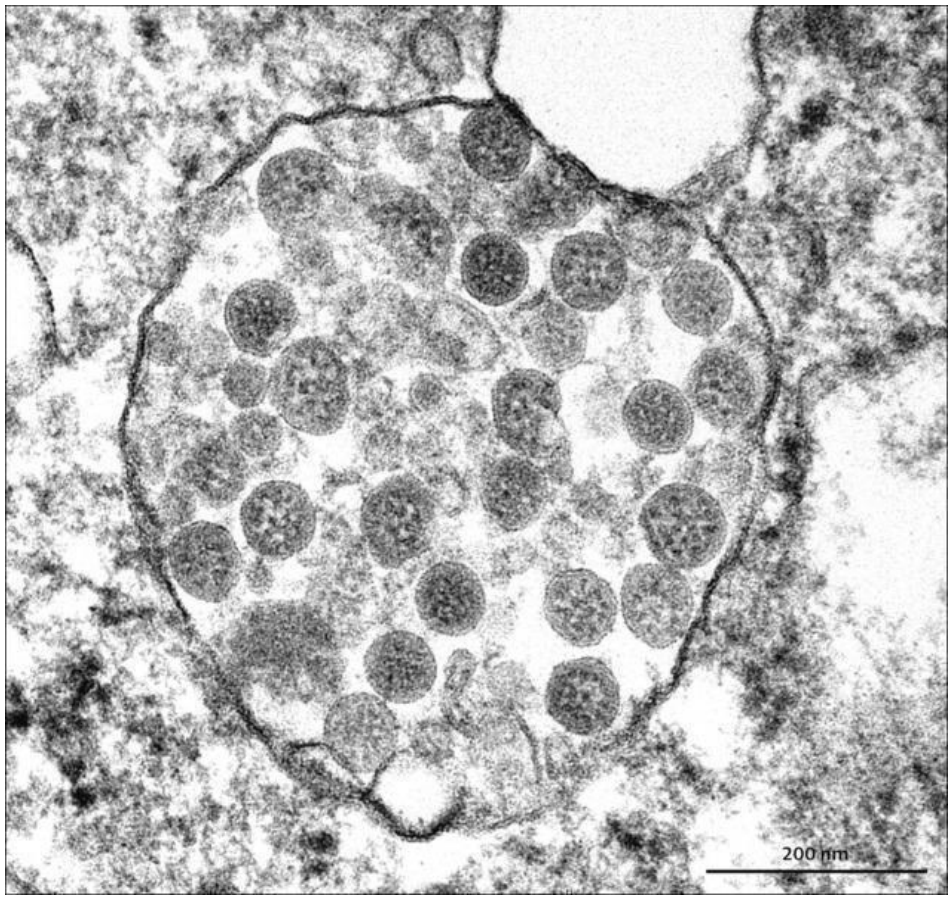

Figure 1. SARS-CoV-2 isolate in cell culture at electron microscopy (Goldsmith *et al.*, 2020).

HCoVs belong to subfamily Coronavirinae and are classified in four genera (Alpha, Beta, Gamma and Delta) based on phylogenesis and genome structure. In Figure 2 phylogenetic relation between animal and human Coronaviruses before the emergence of SARS-CoV-2 are shown. In general,

alphacoronaviruses and betacoronaviruses are involved in mammal infections while gammacoronaviruses and deltacoronaviruses are mainly related to bird infections. Respiratory syndromes in humans are related to alphacoronaviruses and betacoronaviruses. So far, four HCoVs are endemic in human population (229E, NL63, HKU-1 and OC-43) which cause common cold and mild self-limiting respiratory syndromes or infection at gastrointestinal level. However in some cases, especially in children, elderly subjects and immunosuppressed hosts (Su *et al.*, 2016) severe respiratory syndrome are observed (Forni *et al.*,2017; Wevers *et al.*, 2009).

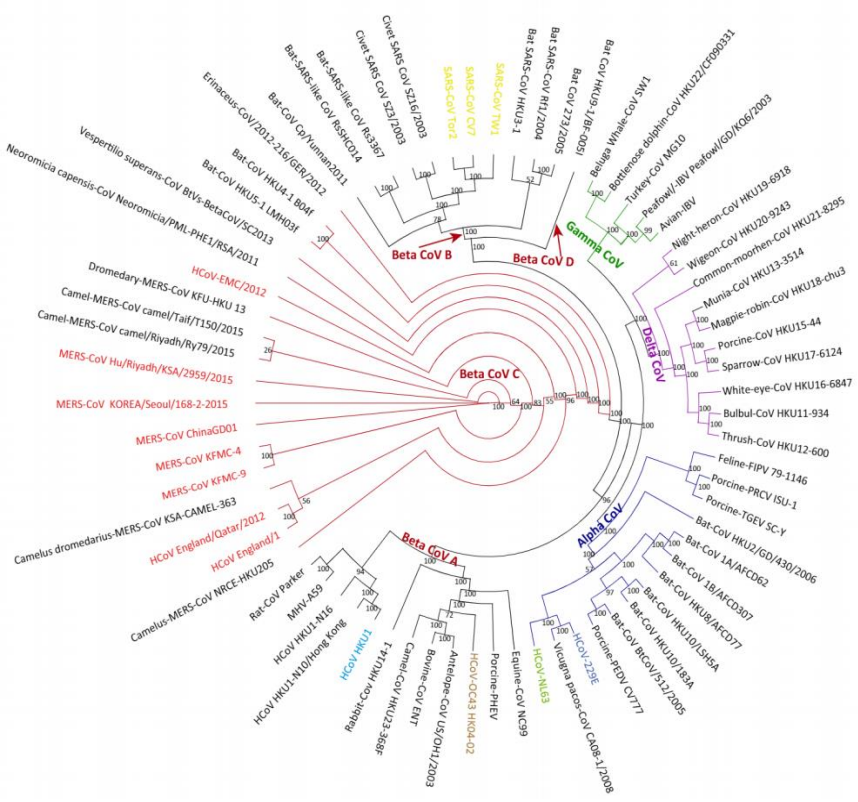

Figure 2. Phylogenetic relations of the known animal and human coronaviruses before the emergence of the new SARS-CoV-2 subfamily members (Su *et al.*, 2016).

The HCoV genome is considered one of the largest genomes known in RNA viruses (Su *et al.*, 2016), counting approximatively 26-32 kilobases (kb); the large majority of RNA (about two thirds) from 5' terminal extremity codifies for

non structural proteins while the remaining one third codifies for structural proteins Spike (S), Envelope (E), Membrane (M) and Nucleocapsid (N) (Cui *et al.*, 2019; Figure 3).The first step in the virus lifecycle is the binding of S protein to a cellular receptor. For the large majority of HCoVs the receptor is angiotensin-converting enzyme 2 (ACE-2) but the aminopeptidase N and dipeptidyl-peptidase 4 are used by HCoV-229E and MERS-CoV, respectively. Receptor binding is generally accompanied by the cleavage of S protein by proteases, such as cathepsin or transmembrane serine protease 2 (TMPRS2) that occurs in the S2 part of the S protein, which is essential for the separation of receptor binding domain (RBD) and the fusion domain of the protein (Belouzard *et al.*, 2009). A second cleavage is necessary for the exposure of the fusion peptide (Fehr *et al.*, 2015).

After the cleavage of the S2 portion of the S protein, the exposed fusion peptide inserts into the host cell membrane and, through the formation of a six-helix bundle, the fusion between viral and host membranes occurs. Then, the viral RNA is released into the cell cytoplasm. The RNA genome is flanked by two untranslated regions (5' and 3') containing cis-acting RNA structures crucial for the normal RNA synthesis. The first translated region is the replicase gene (ORF-1a and 1b) that encodes for 16 non-structural proteins (nsp 1-11 and 1-16, respectively), 15 of them involved in the synthesis of viral replication and transcription complexes (Gorbalenya *et al.*, 2006). In detail, nsp12 codifies for the RNA dependent RNA polymerase (RdRp), while nsp7 and nsp8 are two RdRp cofactors. Nsp14 codifies for a 3'-5' exonuclease activity that is crucial for a correct RNA synthesis due to its proofreading function. Viral synthesis leads to the production of both genomic and subgenomic RNAs, both produced through a negative strand intermediate. Sub-genomics RNAs are mRNA that lead to structural and accessory protein synthesis. These sub-genomic RNAs form a set of so-called "nested" RNA since they are 3′ co-terminal with the full-length genome; this characteristic is typical of the Nidovirales order (Fehr *et al.*, 2015). After the phase of replication and the synthesis of sub-genomic RNAs, the synthesis of structural proteins occurs. These proteins are released in the endoplasmic reticulum and migrate to endoplasmic reticulum-Golgi intermediate compartment (ERGIC) where the formation of mature virions occurs. Looking at the other structural proteins, N protein is responsible for the viral genome encapsidation into the endoplasmic reticulum, while M protein has been associated with many protein-to-protein interactions. However, despite its function, laboratory evidence reported that M protein alone is not sufficient for the virus-like particle (VLP) formation, but E protein is also required (Bos *et al.*, 1996). After the complete assembly, virions are transported to cell surface by vesicles and released through exocytosis (Fehr *et al.*, 2015).

Focusing on the origin of the current SARS-CoV-2, it has been observed that 96% of the sequences is shared with bat coronavirus BatCoV RaTG13 virus (Zhang *et al.*, 2020), supporting the possible origin from bats' coronaviruses, as previously reported for SARS and MERS (Li *et al.*, 2005). The discovery that pangolin coronavirus genomes have 85.5% to 92.4% sequence similarity to SARS-CoV-2 (Lam *et al.*, 2020) suggests that pangolins might be considered as possible hosts in the emergence of the new human coronavirus.

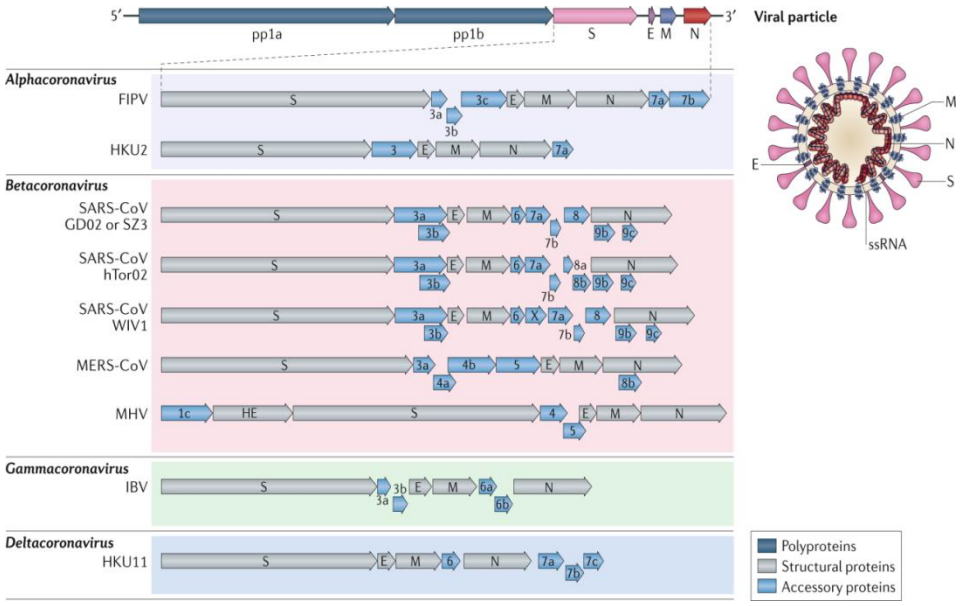

Figure 3. Structure and classification of Coronaviruses (Cui *et al.*, 2019).

1.2. SARS-CoV-2 emergence: origin, genome and transmission

The first cases of pneumonia of unknown aetiology were reported in China in December 2019, in association with Wuhan Seafood market. Based on the surveillance strategy for "pneumonia of unknown aetiology" (Xiang *et al.*, 2013), the first four cases were identified as associated with a novel coronavirus, firstly named 2019-nCoV (Li *et al.*, 2020). As reported by Zhu and colleagues, the suspected cases were identified using both molecular and cellular approaches, including genome sequencing and electron microscopy (Zhu *et al.*, 2020; Figure 4). Moreover, other cases were reported in Wuhan and only a few cases were reported in association to direct contact with the live-market context, thus supporting a human-to-human transmission of the virus (Chan *et al.*, 2020).

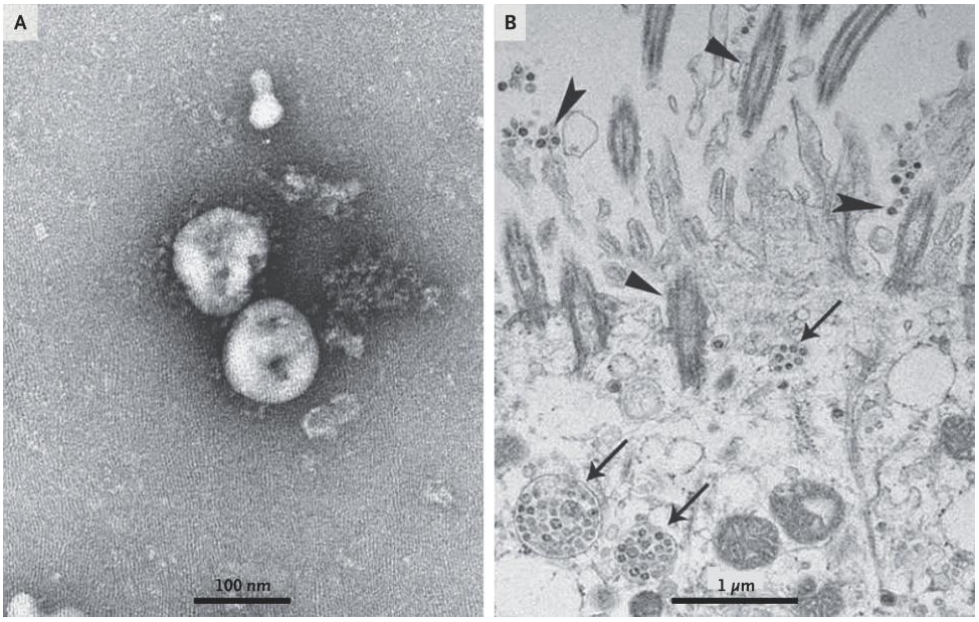

Figure 4. 2019-nCoV particles with "crow" structure (A) and 2019-nCoV particles in the human airway epithelial cell (B) (Zhu *et al.*, 2020).

Overall, SARS-CoV-2 shares 80% and 50% genome sequence identity with SARS-CoV and MERS-CoV, respectively (Lu *et al*., 2020). Of note, even if SARS-CoV-2 shares 90% amino acid identity for the structural genes with SARS-CoV, the sequence of S protein of SARS-CoV-2 diverges from that of SARS-CoV (Zhou *et al*., 2020) and the sequence similarity of RBD of the two viruses is 73% (Hu *et al.*, 2020). On the other side, an identity of 85% was found between SARS-CoV-2 and SARS-CoV in terms of non-structural proteins that are mainly involved in transcription and virus replication (Chan *et al.*, 2020). Similarly to SARS-CoV, the receptor for viral entry of SARS-CoV-2 is the ACE2 (Hoffmann *et al*., 2020) which is recognized and bound by S protein (Figure 5). ACE2 is expressed on the surface of cells of the respiratory tract including pulmonary cells, but also on extrapulmonary cells, including renal, cardiac, gastrointestinal and endothelial cells (Albini *et al.,* 2020).

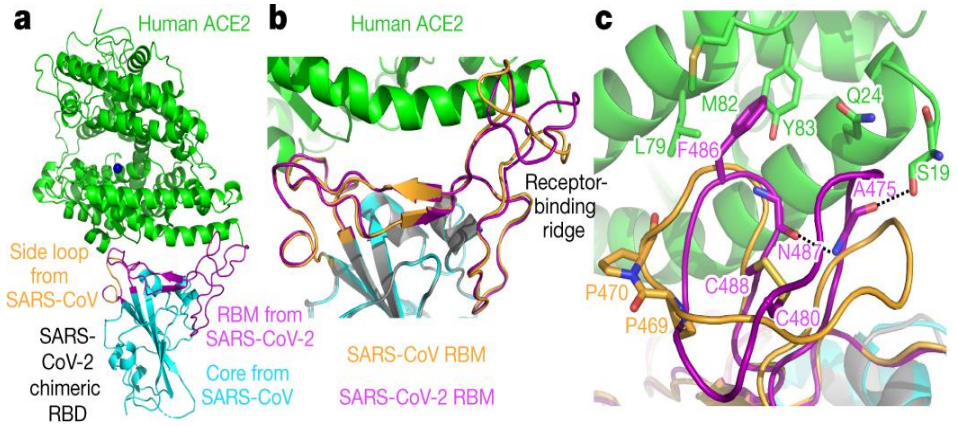

Figure 5. Crystal structure of the SARS-CoV-2 chimeric RBD complexed with ACE2 (Shang *et al*., 2020).

In detail, the subunit S1 of the S protein contains a region of 211 amino acids at C-terminal domain that is recognized as receptor binding domain (RBD) and represents the key target of neutralizing antibody activity. ACE2 binding is necessary but not sufficient for allowing a complete virus entry, since the cleavage of S protein by host protease is needed for activating the SARS-CoV-2 entry. Of note, SARS-CoV-2 seems to have a greater affinity for ACE2 than SARS-CoV (Shang *et al*., 2020). Moreover, TMPRSS2, cathepsin L and furin contribute to viral entry (Ou et al., 2020; Shang et al., PNAS 2020; Sungnak et al., 2020). The furin cleavage seems to facilitate the binding of a higher proportion of the S protein to the human ACE2 (Wrobel *et al.*, 2020), thus helping in the emergence and spread of SARS-CoV-2 in humans. So far, the loss of furin cleavage site seems to reduce the viral infectivity (Johnson *et al*., 2021). TMPRSS2 receptor facilitates the entry of virus and cathepsin L has a role in viral entry also in those cells that lack TMPRSS2 (Hoffmann *et al*., 2020). After the viral entry in the upper respiratory tract, replication occurs in alveolar epithelial cells, vascular endothelial cells and alveolar macrophages, as observed for SARS-CoV (Hamming *et al*., 2004; Jia *et al.,* 2005; Kuba *et al.*, 2005).

SARS-CoV-2 replication is similar to that described for other HCoVs: the genome is released into the cell cytoplasm cytosol and viral replication mechanism starts (Lu *et al*., 2020). As a first step the two ORFs, 1a and 1b, are translated into viral replicase proteins, then cleaved in individual non structural proteins by both host and viral proteases. After the RNA-dependent RNA polymerase synthesis, virus-induced double membrane vesicles (DMVs) at endoplasmic reticulum level are generated and viral replication of genomic and subgenomics RNAs (sgRNAs) occurs. Translation of sgRNAs

results in structural and accessory proteins that are inserted into the endoplasmic reticulum and Golgi compartment for subsequent virion assembly. At the end, RNA genomes are included into virions and released at plasma membrane level are secreted from the plasma membrane (Figure 6).

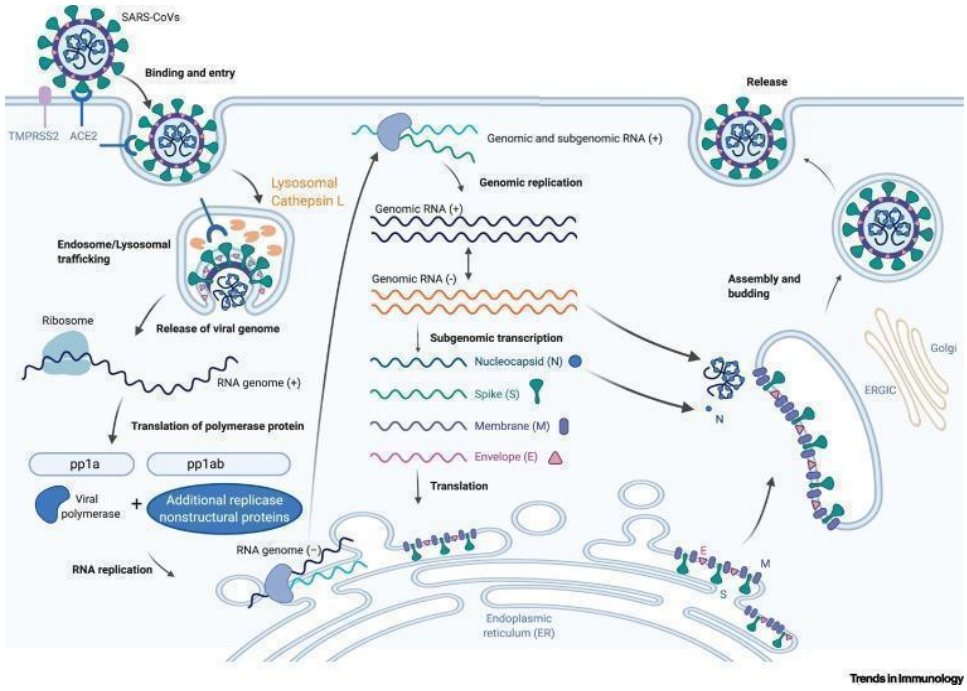

Figure 6. SARS-CoV-2 replication and life cycle (Harrison *et al.*, 2020).

Like the other HCoVs, SARS-CoV-2 is mainly transmitted by respiratory droplets, aerosol and human-to-human contact with SARS-CoV-2 infected subjects (Figure 7). Moreover, contamination of inanimate surfaces might contribute to viral spread (Liu *et al*., 2020; Ong *et al*., 2020). However, it has to be pointed out that the presence of detectable SARS-CoV-2 RNA on an inanimate surface may not correlate with the presence of infectious viral particles (Colaneri *et al*., 2020). Furthermore, the evidence of asymptomatic and pre-symptomatic infected patients that can potentially transmit the infection, differently from what observed for SARS-CoV (Arons *et al*., M 2020; Harrison *et al*., 2020), represents a crucial concern in terms of viral spread and infection control. Indeed, these subjects hardly produce droplets, whch supports the assumption that aerosols play and important role in viral transmission. Finally, faecal–oral transmission has also been proposed as a possible transmission route (Harrison *et al*., 2020). However, in this setting

the real contribute of fecal-oral transmission in viral spread remain to be elucidated.

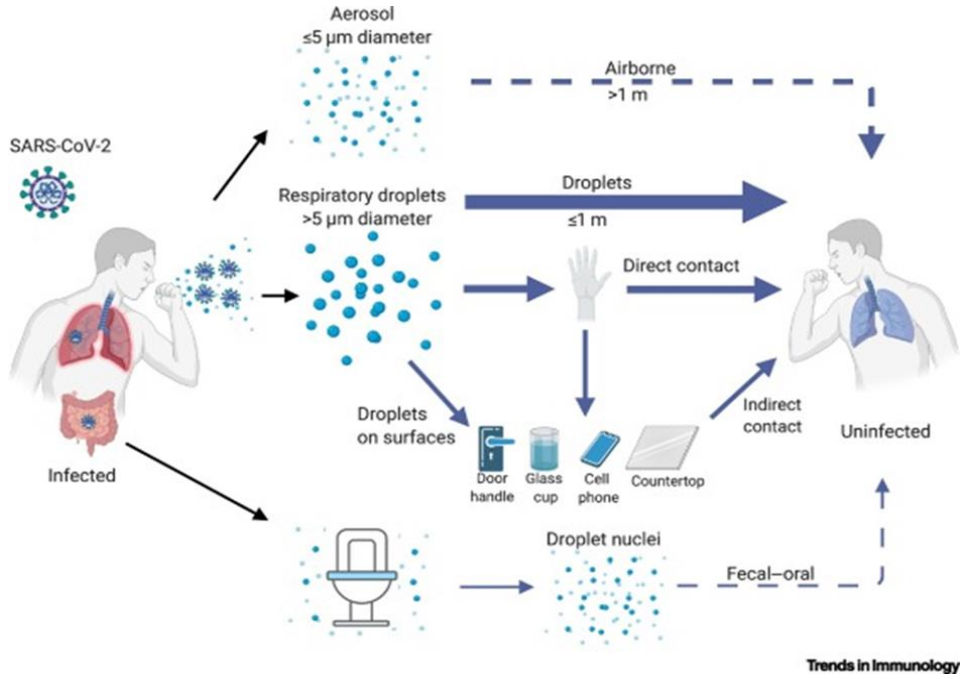

Figure 7. Transmission's routes of SARS-CoV-2 infection (Harrison *et al.*, 2020)

1.3. Clinical manifestations of SARS-CoV-2 infection

1.3.1. SARS-CoV-2 respiratory syndrome: from mild to severe condition

The incubation period of SARS-CoV-2 ranges from 4 to 6 days but it can reach 14 days (Li *et al.*, 2020). Mild symptoms with clinical manifestation restricted mainly at the upper respiratory level are reported in 80% of infected patients; in these subjects, symptoms are normally resolved in 6-10 days. However, severe COVID-19 may occur in 20% of subjects with lower respiratory tract involvement and extrapulmonary symptoms (Figure 8). Death was reported in about 3% of the cases worldwide at the beginning and declined to less than 1% in the last period of pandemic.

In this setting, the role of immune response seems to be crucial (Perico *et al.*, 2021). COVID-19 morbidity and mortality have been linked to male gender, elderly age and comorbidities, leading to a poorer outcome of the viral infection for frail patients and resulting in increased risk of hospitalization,

intensive care unit admittance and invasive tracheal intubation (Bersanelli *et al.,* 2021). Among such individuals, cancer patients represent a large subgroup at high risk of developing COVID-19 and its severe complications. A recent report from Italy showed that nearly 20% of the deceased patients infected with SARS-CoV-2 had a history of active cancer in the past 5 years (Onder *et al.,* 2020).

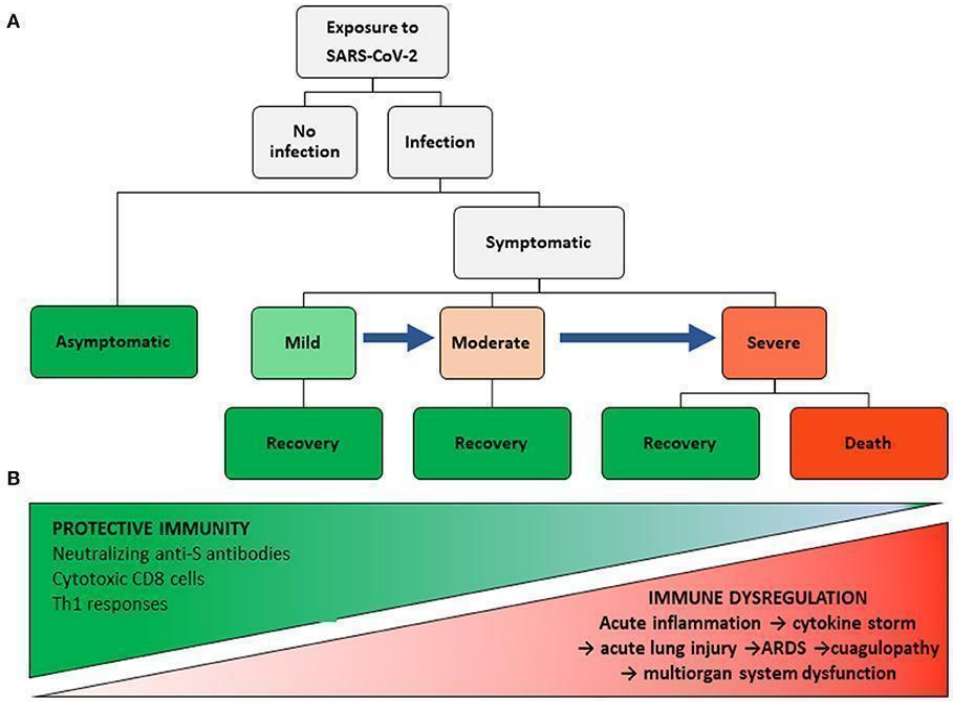

Figure 8. Spectrum of clinical manifestation of SARS-CoV-2 infection (Perico *et al*., 2021).

From a biological point of view, after SARS-CoV-2 infection at epithelial cell levels, apoptosis occurs as part of the normal viral cycle, leading to a first wave of inflammation with recruitment of cells of the innate immune system, mainly macrophages and neutrophils, elicit a specific innate immune response to eradicate infected cells (Cao, 2020). The innate immune response, in turn, leads to an increase in pro-inflammatory cytokines able to recruit cells of adaptive immune response (T and B cells). At this level, T helper cells are mainly involved in mediate antibody production by B cells and enhance cytotoxic CD8+ T-cell and NK responses, leading to eradication of infected cells (Figure 9).

On the other side, mainly in subjects with comorbidities or other risk factors, an abnormal immune response, characterized by lymphopenia and suppression of CD4+ T cells, might be associated with severe COVID-19 and poor prognosis. In the absence of a sustained T helper response, B cells might be not efficient enough in antibody production (especially neutralizing antibodies). A decreased proliferation and effector activity of cytotoxic T cells is normally related to an increase in those cells reporting a higher expression of programmed cell death protein 1 (PD1). At the same time, an increased expression of CD94/NK group 2 member A (NKG2A) receptor linked to functional exhaustion of NK and CD8+ T cells has been reported (Antonioli *et al.*, 2020).

This immune impairment is responsible for an uncontrolled viral replication that, in turn, leads to a persistent viral shedding with a consequent abnormal amplification of innate immune response activity with consequent abnormal cytokine production (Figure 9). This clinical condition may lead to pulmonary disease linked to SARS-CoV-2 infection but also to extrapulmonary involvement and systemic disease (Perico *et al.*, 2021).

Based on SARS-CoV-2 immuno-pathogenesis, COVID-19 clinical manifestations may range from asymptomatic disease to severe respiratory syndrome, including acute respiratory distress syndrome (ARDS), that may require hospitalization in subintensive or intensive care units (SICU or ICU). ARDS has been described as severe pulmonary complication with oxygen refractory hypoxia (Guan *et al.,* 2020) in about one third of SARS-CoV-2 positive patients admitted to hospital (Rodriguez-Morales *et al.,* 2020).

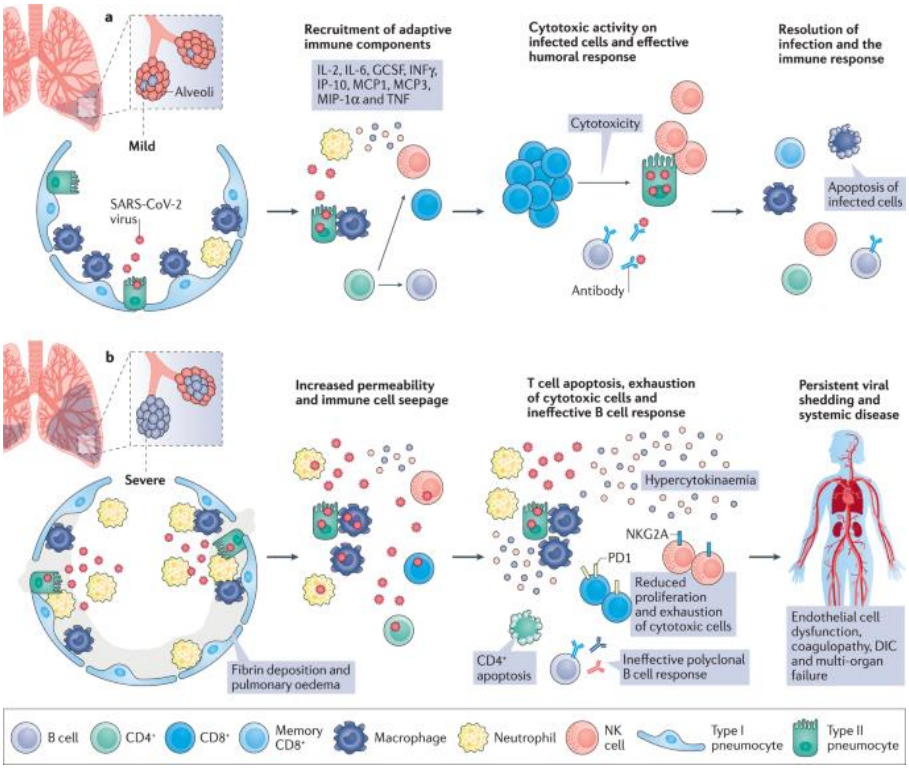

Figure 9. Immunopathology in mild (a) and severe (b) COVID-19 (Garcia, 2020)

Fever and cough are normally the most frequent initial symptoms observed (Chen *et al.*, 2020; Colaneri *et al.*, 2020) while diarrhoea and haemoptysis are less frequently reported (Huang *et al.*, 2020). In hospitalized subjects, abnormal computer tomography (CT) was reported in the large majority of patients (Figure 10) while pulmonary fibrosis was observed in about 45% of patients (Sun *et al.*, 2020), mainly characterized by linear opacities, crazy-paving pattern, and bronchial wall thickening especially in severe and critical patients as well as the so called “ground glass opacity” (Li *et al.*, 2020).

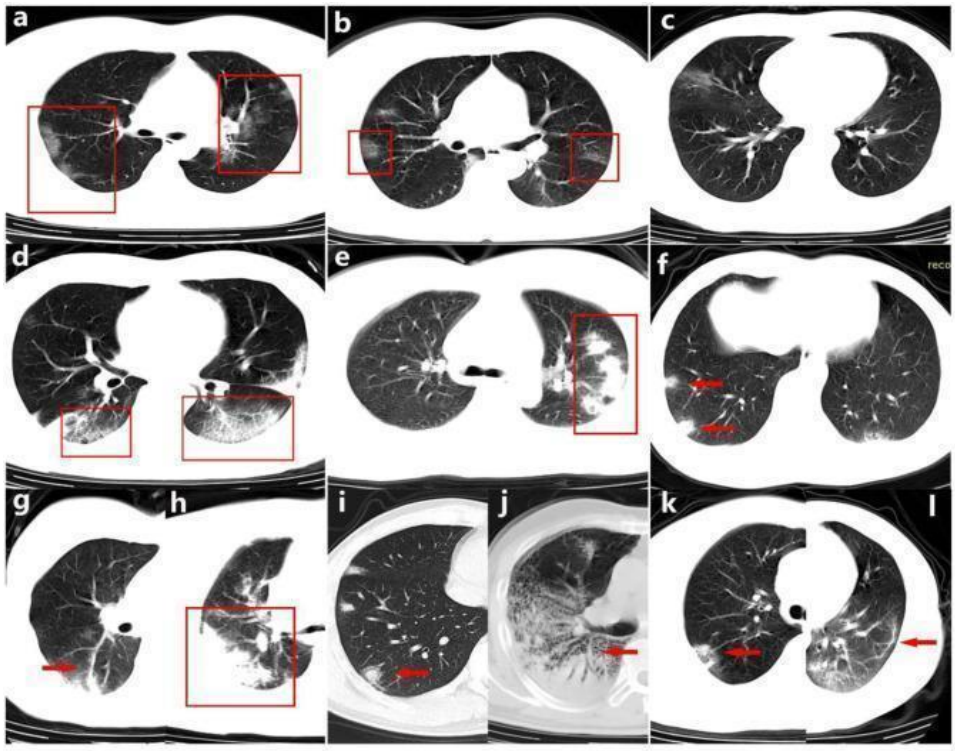

Figure 10. Chest CT scan features of COVID-19. a-b. GGO. c. GGN. d. Crazy Paving Pattern. e. Long Axis Parallelism. f. Patchy Ground-glass Opacities. g. Increased Vascular Margins. h. Consolidation. i. Nodule with Halo Sign. j. Air Bronchogram. k. Reversed Halo Sign. l. Parenchymal Bands (Luo *et al.*, 2020).

Cytokines' levels, blood cell counts and other biochemical parameters have been widely investigated in order to define prognostic markers of severe disease. So far, C-reactive protein, ferritin and D-dimers as well as several inflammatory cytokines have been associated with COVID-19 severity and increased risk of death (Wu *et al.*, 2020; Zhou *et al.*, 2020; Qin *et al.*, 2020). From a laboratory point of view, the reduction of lymphocytes count in severe cases of COVID-19 has been widely reported (Chen *et al.*, 2020; Qin *et al.*, 2020; Tan *et al.*, 2020; Huang *et al.*, 2020; Paolucci *et al.*, 2020).

1.3.2. SARS-CoV-2 extrapulmonary manifestations

According to previous observation, extrapulmonary diseases may be reported in SARS-CoV-2 positive patients (Figure 11). For example, neurological involvement, including central nervous system manifestations, peripheral

nervous system (PNS) manifestations and skeletal muscular injury manifestations have been reported (Mao *et al.*, 2020). In particular, total or partial loss of smell (anosmia) and taste (ageusia) has been widely associated with COVID-19, accounting for 33.9-68% of subjects, mainly females (Meng *et al*., 2020). Even if neurologic symptoms are frequent in COVID-19 it is still unclear if these symptoms are a direct consequence of the replication of the virus in neural cells or are due to post-infectious immune-mediated disease (Ellul *et al.,* 2020; Iadecola *et al*., 2020). Conflicting data have been reported on SARS-CoV-2 neurotropism and expression of ACE2 in neural progenitor cells and mature neurons (Zhang *et al.*, 2020; Ramani *et al.*, 2020; Pellegrini *et al*., 2020). Interestingly, the brain represents a potential high-replicative site for SARS-CoV-2 causing a significant neuronal damage, as demonstrated by using human brain organoids (Song *et al.*, 2021).

Gastrointestinal disorders have been also reported, even before the respiratory disease. Vomiting, diarrhoea, nausea and abdominal pain have been described as the most frequent manifestations. Interestingly, it seems that subjects with gastrointestinal disease show higher frequency of severe COVID-19, leading to ARDS and ICU admission, than those without gastrointestinal symptoms (Jin *et al*., 2020). A possible route of fecal-oral transmission has been proposed as a mechanism involved in gastrointestinal disease (Wong *et al*.,2020). Similarly to SARS in 2003 (Wu *et al*., 2004), hepatic involvement has been also reported in COVID-19 patients, with increase in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and decrease of albumin level (Chen *et al.*, 2020; Saini *et al*., 2020). Although data on hepatic disorders are still scarce, two possible mechanisms underlying hepatic manifestations have been proposed. Firstly, the liver damage may be caused by hyper activation of pro-inflammatory response, including IL-6; additionally, a possible direct damage could be hypothesized due to the presence of ACE2 receptor on hepatic endothelial cells. In this regards, a recent study reported SARS-CoV-2 tropism for kidneys and liver but also for heart and brain, suggesting that this organotropism might be involved in the evolution of SARS-CoV-2 related disease (Puelles *et al*., 2020). Another evidence of direct mechanisms involved in hepatic disease is the presence of viral particles observed by electron microscopy in the liver of two deceased COVID-19 patients (Wang *et al*., 2020).

Myocarditis, pericarditis, acute coronary syndrome (ACS) and heart failure are some of the most important cardiovascular manifestations that may be observed in COVID-19 (Johnson *et al*., 2020). Guo and colleagues reported that mortality in hospitalized COVID-19 patients was mainly associated to myocardial injury; in the same way, it is largely known that diseases such as hypertension and other cardiovascular diseases are important risk factors for severe COVID-19, hospitalization, admission at ICU and death (Guo *et al*.,

2020; Zhou *et al*., 2020). Of note, experimental data proved that SARS-CoV-2 is able to infect human cardiomyocytes in culture and SARS-CoV-2 was also isolated in heart slices obtained by a dead COVID-19 patient (Bojkova *et al*., 2020). Finally, ocular and also dermatological manifestations have been reported in a number of patients, even if these types of diseases are less known or might be underestimated (Johnson *et al*., 2020).

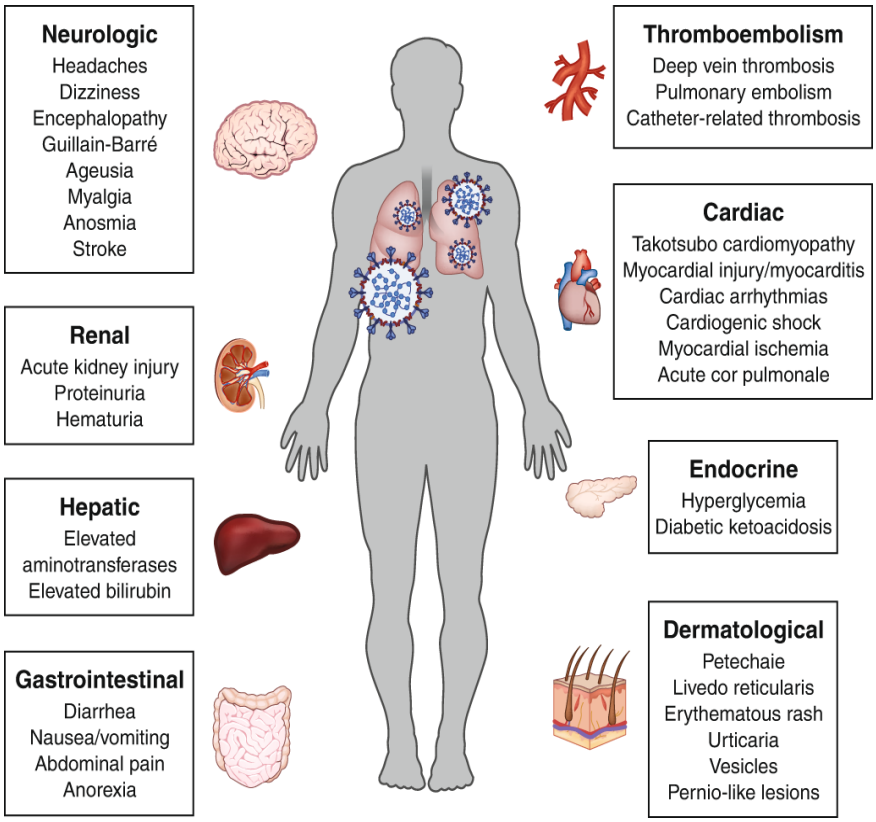

Figure 11. Extrapulmonary manifestation of SARS-CoV-2 infection (Gupta, 2020).

1.3.3. SARS-CoV-2 clinical manifestation in children

During the initial phase of the pandemic, children were the group of population less affected by SARS-CoV-2 infection. Preliminary results obtained by Chinese studies (Wu *et al.,* 2020) reported that milder infections occurred in children and symptoms were mainly fever, cough and nasal congestion; cases of asymptomatic SARS-CoV-2 positive children were also described (Jiehao *et al*., 2020; Pan *et al*., 2020; Castagnoli *et al*., 2020). In Italy, the

same trend was reported; with less than 1% of infected subjects reporting age lower than 18 years old in Lombardy region up to 15 March 2020 (Rovida *et al.*, 2020). Similarly, by May 8, 2020, less than 2% of cases in all the country were reported in subjects under 18 years of age and only 3 deaths were counted (Task force COVID-19 ISS, 2020).

Otherwise, cases of SARS-CoV-2 positive children reporting extrapulmonary manifestation were described in literature. In particular, SARS-CoV-2 has been related to Kawasaki-like hyper inflammatory syndrome, called MIS-C, probably linked to a delayed response against the virus. Licciardi and colleagues reported two young patients affected by SARS-CoV-2 infection reporting persistent fever, diarrhoea, abdominal pain. Moreover, biochemical and haematological parameters revealed high levels of procalcitonin and C-reactive protein and low lymphocyte count. For the similarities with Kawasaki disease the authors proposed the name of SARS-CoV-2–induced Kawasaki-like hyper inflammatory syndrome for describing these peculiar clinical features, mainly related to post-infection immune response (Licciardi *et al.*, 2020).

A peculiar systemic COVID-19 related manifestation observed in both children and adult is called thrombotic microangiopathy (TMA) linked to endothelial cell damage at blood vessels level and leading to haemolytic anaemia, thrombocytopenia and, eventually, organ damage (Oxley *et al.*, 2020). The dysregulation of C5b9 and other complement factors is a clear marker of TMA in COVID-19 patients (Diorio *et al.,* 2020). Even if systemic response is involved in endothelial dysfunction and thrombotic microangiopathy, it has also been described that SARS-CoV-2 is able to directly infect human blood vessels and kidney organoids (Monteil *et al.*, 2020).

1.4. Diagnosis of SARS-CoV-2 infection

The rapid emergence of SARS-CoV-2 pandemic required a huge effort in terms of diagnosis and consequent isolation of infected people. Looking at Figure 12, after the infection with SARS-CoV-2 a period of viral replication in absence of symptoms occurs and, during this period, the RNA detection or viral isolation is unlikely. At symptoms' onset the role of diagnostic assays become of paramount importance for the detection of viral RNA or for viral culture. After 7-10 days from symptoms' onset the detection of SARS-CoV-2 specific IgM and IgG might help in the definition of acute or past infection. After three or four weeks from the symptoms' onset the molecular approaches are not useful, since the decay of viral RNA occurs; otherwise, the only diagnostic approach is the antibody detection. Procedures for SARS-CoV-2

diagnosis, including virological, molecular and immunological approaches are described here below.

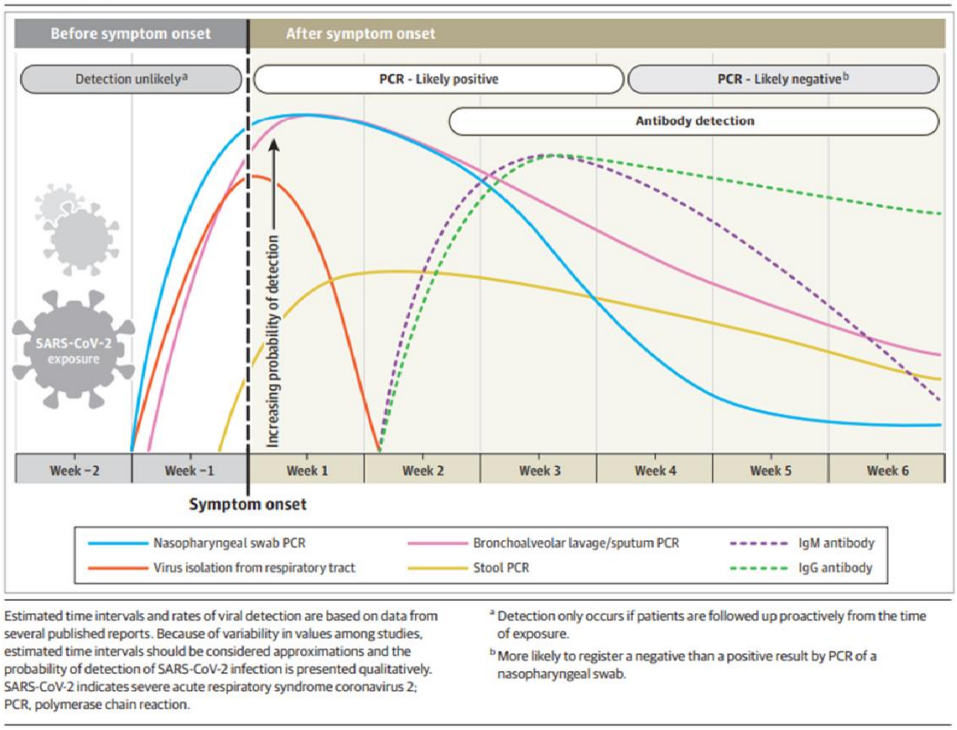

Figure 12. Estimated time intervals and rates of viral detection according to time after SARS-CoV-2 exposure (Sethuraman *et al*., 2020).

1.4.1. Viral isolation

Before the introduction of molecular assays, virus cultures and isolation were considered gold standards for viral diagnosis (Leland and Ginocchio, 2007), despite the time-consuming procedures and the need of highly specialized personnel. With the SARS-CoV-2 emergence, in relation to the need to study the new virus many research groups decided to approach viral culture for diagnostic and research purposes. In particular, one of the most important fields requiring cellular virology approaches is the quantification of neutralizing antibodies (NT Abs) in convalescent subjects (Percivalle *et al*., 2020; Cao *et al.,* 2020; Wajnberg *et al*., 2020; Manenti *et al*., 2020). Neutralizing antibodies are able to bind specific epitopes of S protein avoiding the binding with ACE2 receptor (Figure 13).

Since the manipulation of SARS-CoV-2 isolates requires biosafety level 3 laboratories, the use of pseudoviral particles instead of viral isolates is widely applied (Nie *et al*., 2020; Robbiani *et al.*, 2020; Hu *et al*., 2020). Pseudovirus is defined as a retrovirus that can integrate the envelope glycoprotein of another virus to form a virus with an exogenous viral envelope, and the genome retains the characteristics of the retrovirus itself. Pseudovirus-based assay have been successfully employed in the study of highly infectious viruses, such as Ebola, MERS, Rabies, Marburg and Lassa (Fan *et al.,* 2018; Nie *et al*., 2017; Liu *et al*., 2017; Li *et al*., 2017)

Even if the two methods have been widely implemented, it has been described that the use of pseudoviruses could give discrepant results with respect to natural strains, likely because the artificial lentiviral particles cannot resemble the complete biology of clinical isolates (Wang *et al*., 2021). The same approach might be used for the evaluation of in vitro efficacy of monoclonal antibodies (Weisblum *et al*., 2020; Chen *et al*., 2021; Liu *et al*., 2020; Gasparo *et al*., 2021). This topic will be further explored in this thesis.

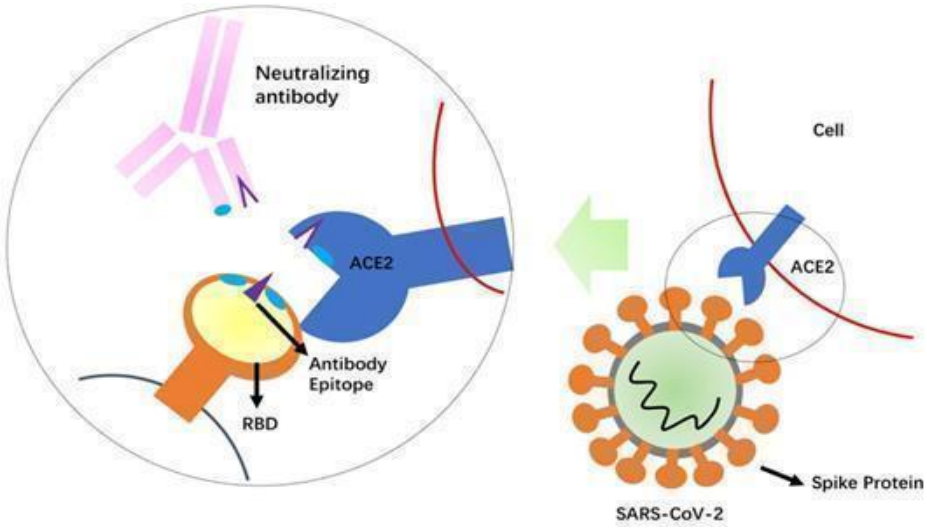

Figure 13. Schematic representation of neutralizing antibodies mechanisms of action (Zhou *et al*., 2020).

Virus isolation and culture might also be useful in those cases of patients with prolonged, low level positive SARS-CoV-2 RNA in order to evidence the presence of active replication (La Scola *et al.*, 2020; Piralla *et al*., 2021). Of note, less than 3-8% of samples with low RNA level (cycle threshold higher than 30-35) are able to infect the cell monolayer (Piralla *et al*., 2021; Singanayagam *et al.*, 2020). Recently, Huang et al. reported that respiratory

samples might be cultured if in presence of less than cycle threshold (Ct) of 30 (Huang *et al.*, 2020). Importantly, the failure to grow virus in culture from convalescent patients still in presence of positive RT-PCR suggests that these patients are unlikely to be contagious (Manzulli *et al.*, 2021).

From a methodological point of view, SARS-CoV-2 is cultivated in VERO CCL-81 or VERO E6 cell lines, since they express a high level of ACE2 receptor. Like other respiratory viruses, SARS-CoV-2 cultures are maintained at 33 °C 5% CO_2 in viral culture medium with 2% fetal bovine serum (FBS). A more efficient viral propagation is obtained using 4-5µg/mL of trypsin (Piralla *et al*., 2021; Caly *et al*., 2020) as observed for other respiratory viruses (Eisfeld *et al*., 2014). Presence of cytopathic effect can be observed after three-five days of incubation (Figure 14).

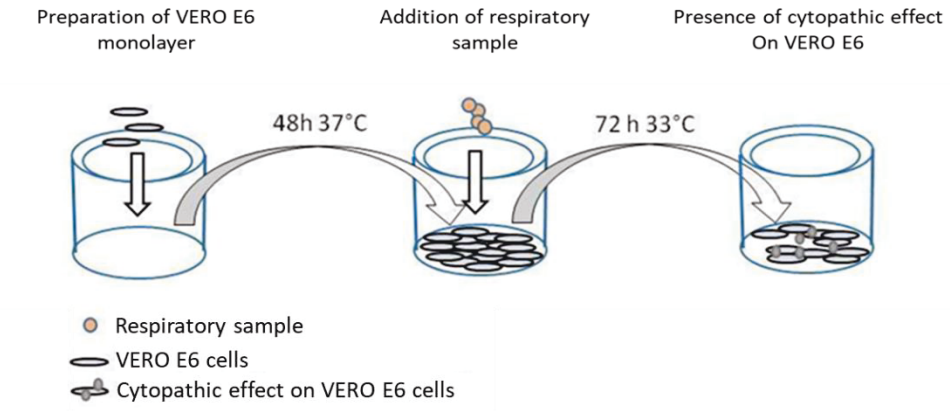

Figure 14. Schematic representation of viral isolation from SARS-CoV-2 positive respiratory sample in VERO E6 cell monolayers.

1.4.2. Molecular assay for SARS-CoV-2 RNA detection and quantification

Immediately after the emergence of the first SARS-CoV-2 cases, a high sensitive real time reverse transcription polymerase chain reaction (RT-PCR) specific for RdRp, E and N genes was assessed for the identification of SARS-CoV-2 in respiratory samples (Corman *et al*., 2020); primers and probes used are shown in Figure 15. Based on the results obtained by this study the limit of detection (LOD) from replicate tests was 3.9 copies per reaction for the E gene assay and 3.6 copies per reaction for the RdRp assay. Later other highly sensitive and rapid molecular assays have been introduced (Bordi *et al*., 2020; Yan *et al*., 2020; Dao Thi *et al.*, 2020) including reverse transcription loop mediated isothermal amplification (RT-LAMP; Ganguli *et al*., 2020).

Primers and probes, real-time RT-PCR for 2019 novel coronavirus

Assay/use	Oligonucleotide	Sequence[a]	Concentration[b]
RdRP gene	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG	Use 600 nM per reaction
	RdRp_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for 2019-nCoV, will not detect SARS-CoV. Use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Pan Sarbeco-Probe will detect 2019-nCoV, SARS-CoV and bat-SARS-related CoVs. Use 100 nM per reaction and mix with P2
	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA	Use 800 nM per reaction
E gene	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Use 400 nm per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	Use 200 nm per reaction
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	Use 400 nm per reaction
N gene	N_Sarbeco_F	CACATTGGCACCCGCAATC	Use 600 nm per reaction
	N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	Use 200 nm per reaction
	N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG	Use 800 nm per reaction

[a] W is A/T; R is G/A; M is A/C; S is G/C. FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

[b] Optimised concentrations are given in nanomol per litre (nM) based on the final reaction mix, e.g. 1.5 µL of a 10 µM primer stock solution per 25 µL total reaction volume yields a final concentration of 600 nM as indicated in the table.

Figure 15. Primer and probes used for assessment of the new RT-PCR for detection of SARS-CoV-2 RNA (Corman *et al*., 2020)

To date, molecular assays performed in respiratory samples (nasopharyngeal swabs and bronchoalveolar lavage) represent the gold standard for SARS-CoV-2 RNA detection and quantification and are extremely useful for diagnostic purposes. A brief and schematic representation of the workflow of RT-PCR based assays is shown in Figure 16. On the other side, since the SARS-CoV-2 infection may be associated with gastrointestinal involvement, the detection of SARS-CoV-2 RNA in faeces and rectal swabs as well as in other body districts might be possible. In a group of Italian subjects with proven SARS-CoV-2 infection, the prevalence of SARS-CoV-2 RNA in rectal swabs was approximatively 10% (Novazzi *et al.*, 2020), which was lower than that observed in other two Chinese studies, 26.7% (Zhang *et al.,* 2020) and 22% (Peng *et al.,* 2020), respectively. Even if the presence of SARS-CoV-2 RNA in fecal samples was demonstrated for about 5 weeks after the corresponding respiratory samples tested negative (Wu *et al*., 2020), the replication of SARS-CoV-2 in gastrointestinal tract remained to be further dissected. In this setting, it has been demonstrated that the expression of TMPRSS2 and TMPRSS4 is able to promote SARS-CoV-2 entry and replication in mature enterocytes expressing ACE receptors in human small intestinal tract (Zang *et al*., 2020). The same observation was made by using human small intestinal organoids and confocal microscopy analysis (Lamers *et al.*, 2020).

Interestingly, few studies reported detection of SARS-CoV-2 RNA also in plasma samples, showing a positive correlation between the detection rate and clinical severity (Veyer *et al.,* 2020; Chen *et al*., 2020) or IL-6 levels (Chen

et al., 2020). In a cohort of 143 hospitalized Italian patients, a prevalence of approximatively 8% of SARS-CoV-2 RNA in plasma was observed (Novazzi *et al.*, 2020), while a rate of 4.6% of SARS-CoV-2 RNA positive plasma was observed in Nigerian cohort (Okwuraiwe *et al.*, 2021). A study from Iran reported that positive blood samples were 24% from a cohort of 100 confirmed COVID-19 cases, with a persistence of median 7 days (Joukar et *al.,* 2021). Whether blood could serve as a source of infection was not shown. On the other side, a large study performed in Hubei province, China, reported no evidence of SARS-CoV-2 RNA in plasma from blood donors (Chang *et al*., 2020).

The presence of SARS-CoV-2 RNA was also documented in other biological materials such as ocular secretion (Colavita *et al.,* 2020). Saliva has been proposed as an attractive biological sample for detection and quantification of SARS-CoV-2 RNA, especially in children, in order to avoid the use of nasopharyngeal specimen collection. A recent meta-analysis provides comparative data on sensitivity and specificity of nucleic acid amplification test (NAAT) in saliva and nasopharyngeal swabs, reporting that diagnostic accuracy of saliva RT-PCR is similar to that obtained with nasopharyngeal swab (Butler-Laporte *et al*., 2021).

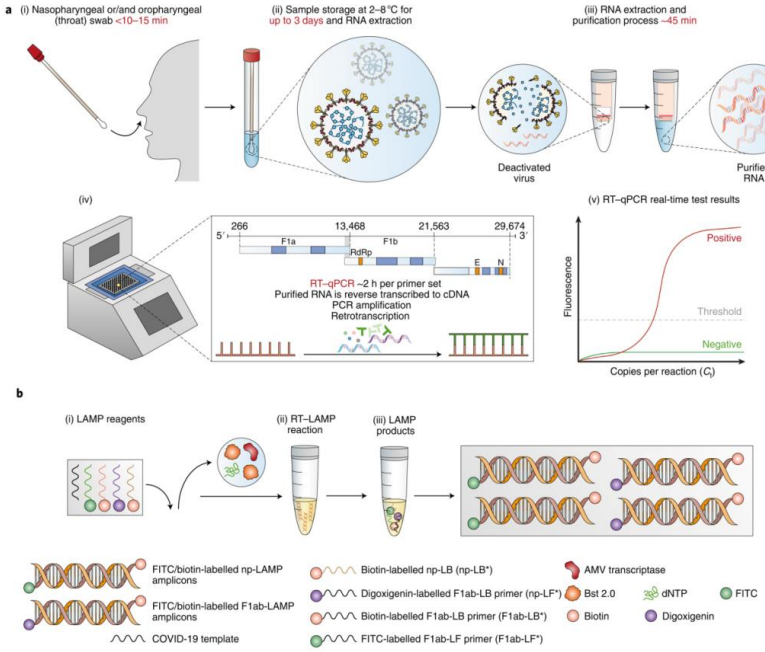

Figure 16. The RT–PCR assay (a) and RT-LAMP reaction (b) (Kevadiya *et al*., 2021).

1.4.3. Antigenic assays

Despite the high sensitivity and accuracy of molecular assays in diagnosis of SARS-CoV-2 infection, the impact of health emergencies as well as the rapid spread of the virus worldwide led to the introduction of rapid tests for the diagnosis of infected subjects. Indeed, molecular assays for the quantification of SARS-CoV-2 RNA require at least 24 hours for obtaining results and need highly qualified technicians and specific laboratory equipment. So far, new assays that rely on direct detection of SARS-CoV-2 antigens (S or N) directly from respiratory samples have been introduced for identification of SARS-CoV-2 positive cases.

This approach is based on lateral flow immunoassay (also called an RDT) able to give a result in less than 30 minutes (WHO, 2020). However, the risk for false negative results is higher compared to molecular assays (Mak *et al.,* 2020). Considering the low sensitivity, it has been suggested that the use of these assays might be reasonable earlier at symptoms' onset when SARS-CoV-2 viral load is higher (Lambert-Niclot *et al.,* 2020; Liotti *et al*., 2021).

1.4.4. Serological assays

Beside the introduction of molecular assays, the concern for the identification of SARS-CoV-2 seropositive subjects required the development and the introduction of serological approaches for the evaluation of total antibodies, IgG, IgM or IgA and for the quantification of neutralizing antibodies. The use of serological assays became of paramount importance for epidemiological studies (Percivalle *et al.,* 2020) also in asymptomatic subjects (Cassaniti *et al*., 2021), in order to define the seroprevalence in specific geographic areas and identify seropositive asymptomatic subjects. Furthermore, this kind of approach might be useful in providing new insights in the field of kinetics of humoral response that will be further explored in the next section of the thesis. Lastly, in the vaccination era, serological approaches seem to be useful also for the definition of immune response after vaccination or for prediction of reinfection (Ohst *et al*., 2018; Gaebler and Nussenzweig, 2020). The large majority of serological assays are solid-phase immunoassays including enzyme-linked immunoassay (ELISA), chemiluminescent immunoassay (CLIA) or enzyme-linked Fluorescent Assay (ELFA) (Galipeau *et al*., 2020). Samples used for antibody quantification are serum and plasma but also saliva for secretory immunoglobulin detection.

The assessment of new serological assay is normally based on comparison with a gold standard, such as microneutralization assay in order to define sensitivity and specificity of the assay, as well as positive and negative

predictive value (Cassaniti *et al*., 2021; Bonelli *et al*., 2020). Furthermore, the application of serology must take in consideration not only the characteristic of a single patient since asymptomatic or mild symptomatic might often show lower levels of SARS-CoV-2 specific antibodies, but also for epidemiologic purposes. So far, a positive or negative result must be followed by an accurate interpretation according to clinical context (Cheng *et al*., 2020) as shown in Figure 17.

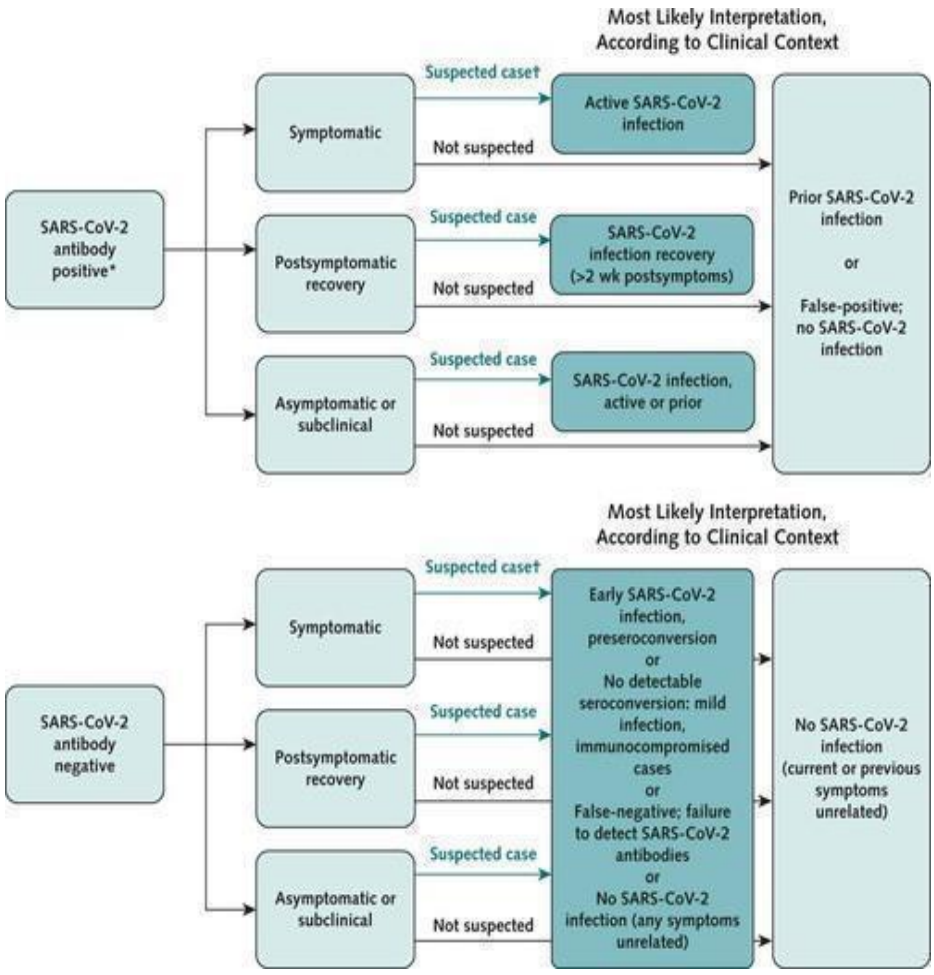

Figure 17. Algorithm for interpretation of serological results according to clinical context (Cheng *et al*., 2020).

1.5. Immune response against SARS-CoV-2 infection

1.5.1. Innate immune response and viral immune evasion in SARS-CoV-2 infection

Innate immune system represents the first immunological barrier for viral infections, including SARS-CoV-2. The balance between innate immune system and viral pathogenesis is influenced by immune escape as well as activation of immunological pathways that are involved in cytokines production and cell recruitment. The binding between S-protein and ACE 2 and the following entry of SARS-CoV-2 is the first step of the activation of detrimental immune responses in COVID-19, that involve both innate and adaptive immune system, including the so called "cytokine storm" (Amor *et al.*, 2020). SARS-CoV-2 has evolved many strategies for immune evasion (Vabret *et al.*, 2020), as also previously reported for SARS-CoV-1 (Figure 18).

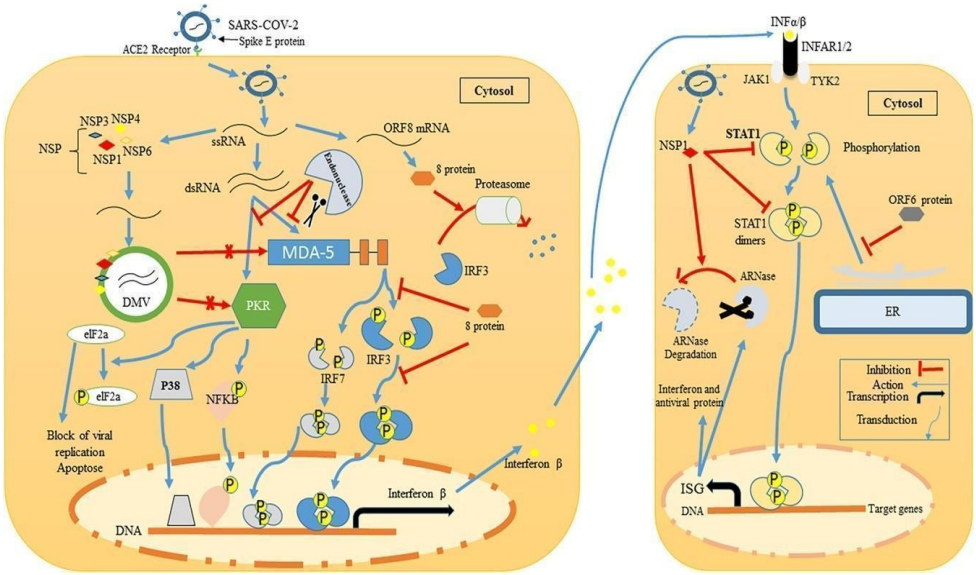

Figure 18. Summary of the immune evasion strategies of SARS-CoV-2 (Bouayad, 2020)

During viral infection the activation of multiple signalling cascades, through the recognition of pathogen-associated molecular patterns (PAMPs), lead to the transcription of type I and type III interferons (IFNs). These IFNs, in turn, lead to the activation of JAK-STAT pathway involved in expression of several genes exerting antiviral activity (Lazear *et al.,* 2019; Yin *et al.,* 2021), including IL-6 and TNF-α. Normally, RNA viruses lead to the targeting of pattern

recognition receptors (PRRs) such as toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors and melanoma differentiation-associated gene 5 (MDA5), that, in turn, is responsible of activation of transcription factors, as nuclear factor-κB (NF-κB) (Matsumiya and Stafforini, 2010), thus leading to the upregulation of antiviral and pro-inflammatory mediators. On the other hand, NSP1 protein of SARS-CoV-2 is able to bind ribosomal subunit 40S leading to suppression of RIG-I activity (Schubert *et al*., 2020).

On the other hand, based on the evidence that IL-6 level is increased in those patients with severe COVID-19, several studies tried to assess the usefulness of treatment and drugs against IL-6. One of them reported that the use of siltuximab in combination with standard ventilator supports significantly reduced 30-day mortality rate than with the use of standard ventilator support only (Gritti *et al*., 2020). Thus, the use of monoclonal antibodies against IL-6 may be considered a valuable tool for reducing the inflammatory environment due to SARS-CoV-2 infection. In parallel, a significant expansion of inflammatory monocytes has been reported in severe COVID-19 cases since the increase of CD14+/CD16+, GM-CSF-CD14+ and IL-6+/CD14+ has been reported (Roschewski *et al.*, 2020).

Finally, the role of macrophages and monocytes in SARS-CoV-2 infection is still controversial. As widely known, macrophages are monocyte-derived cells specialized in phagocytosis but they also have a key role in immune response since they are able to act as antigen-presenting cells, stimulating both T and B cell response. On the other side, a hyperactive immune response seems to be involved in severe SARS-CoV-2 infections (Mehta *et al*., 2020). Indeed, the cytokines profile of patients with a severe COVID-19 is characterized by increased production IL-6, IL-7, TNF-α, CCL2, CCL3 and CXCL10 (Merad *et al*., 2020).

Looking at macrophages, it has been reported that ACE2 is present on macrophages' surfaces. Thus, the virus might be able to directly infect activated macrophages through ACE2 binding (Wang *et al.*, 2020). So far, the ability of macrophages and monocytes to act as a "Trojan Horse" and augment viral spread has been proposed (Park, 2020). According to this hypothesis, macrophages might enable viral anchoring within the pulmonary parenchyma, thus, inducing a prolonged and uncontrolled immune response (Abassi *et al.,* 2020). It has been reported that spleen and lymph nodes ACE2+ macrophages could be infected by SARS-CoV-2 (Xiang *et al*., 2021) suggesting that they could be responsible for viral spread. While infection of SARS-CoV is abortive in monocytes, MERS-CoV can replicate in monocytes, macrophages and dendritic cells (Yilla *et al*., 2005; Zhou *et al.,* 2015). Although SARS-CoV-2 viral particles were detected in monocytes and

macrophages (Boumaza *et al*., 2021), the evidence of productive infection remains to be demonstrated. This topic will be further addressed in this thesis.

1.5.2. Adaptive immune response against SARS-CoV-2 infection

As observed for innate immune response, SARS-CoV-2 infection seems to exert a crucial role in impairing adaptive immune response. Indeed, a common feature observed in COVID-19 patients is the T cell lymphopenia, that involve mainly CD8+ T cells but also NK cells (Paolucci *et al*., 2021). This reduction might be related to the massive recruitment of T cells to inflamed tissues or, eventually, to the use of steroid treatments (Xiang *et al*., 2021; Gu *et al*., 2005). An important feature observed in severe COVID-19 patients was the high level of circulating Epstein Barr virus (EBV) DNA (Paolucci *et al*., 2021) that might be associated with systemic immune suppression.

Adaptive immune response elicited by SARS-CoV-2 infection has been largely studied and, in particular, T-cell response seems to represent a crucial defence system against the infection. Despite the larger number of studies, the durability of SARS-CoV-2 specific T-cell response as well as of humoral response is still unknown. Based on the experience obtained on other common coronaviruses circulation, it is conceivable that humoral response might decline over time even in presence of a sustained long-term cell-mediated response (Callow *et al*., 1990; Guant *et al*., 2010). In terms of humoral response, the role of neutralizing antibodies is of paramount importance, because they prevent the virus from infecting cells. For definition, neutralizing antibodies are those antibodies directed against specific viral antigens, which binding allow the inhibition of receptor binding and viral entry. Thus, the study of kinetics neutralizing antibodies after natural infection as well as after vaccination is crucial.

Severe SARS-CoV-2 infection has been associated with a high level of neutralizing antibody response and, in general, humoral response (Percivalle *et al*., 2020). A significant correlation between neutralizing antibody titers and S-receptor binding domain (S-RBD) specific IgG, but not with anti-nucleoprotein (anti-NP) IgG (Ni *et al*., 2020). Interestingly, T-cell mediated response against SARS-CoV-2 has been detected also in those subjects with no exposure to SARS-CoV-2 (Grifoni *et al*., 2020; Mateus *et al*., 2020; Sette and Crotty, 2020). This finding is important, since it could be assumed that previous exposure to common other HCoVs lead to the generation of a pool of memory T cells able to cross-react with SARS-CoV-2. So far, several epitopes of HCoVs share similarities with those of SARS-CoV-2 and a similarity higher than or equal to 67% has been observed (Mateus *et al*., 2020). Focusing of convalescent patients, SARS-CoV-2 T-cell response has

been detected in almost all subjects affected by SARS-CoV-2 infection (Grifoni *et al*., 2020; Peng *et al*., 2020; Sekine *et al*., 2020), with a higher prevalence of $CD4^+$ mediated T-cell response than respect to $CD8^+$ T cells (Grifoni *et al*., 2020). The breadth and depth of cell-mediated response was highly different in SARS-CoV-2 infected subjects, since those subjects with severe symptoms seems to develop a higher cell-mediated response than subjects with mild symptoms, as observed in the case of neutralizing antibodies (Peng *et al*., 2020). On the other side, even if the frequency of T-cell response was increased in severe COVID-19 cases, it has been also observed that CD4 T cells might have low functional avidity, as well as low TCR clonality (Bacher *et al.,* 2020).

1.6. Therapeutic options for the treatment of COVID-19

After the emergence of COVID-19 pandemic, a strong effort has been made for the rapid development and introduction of effective therapies, acting on different patterns of viral replication (Figure 19). The milestones in SARS-CoV-2 treatment are described.

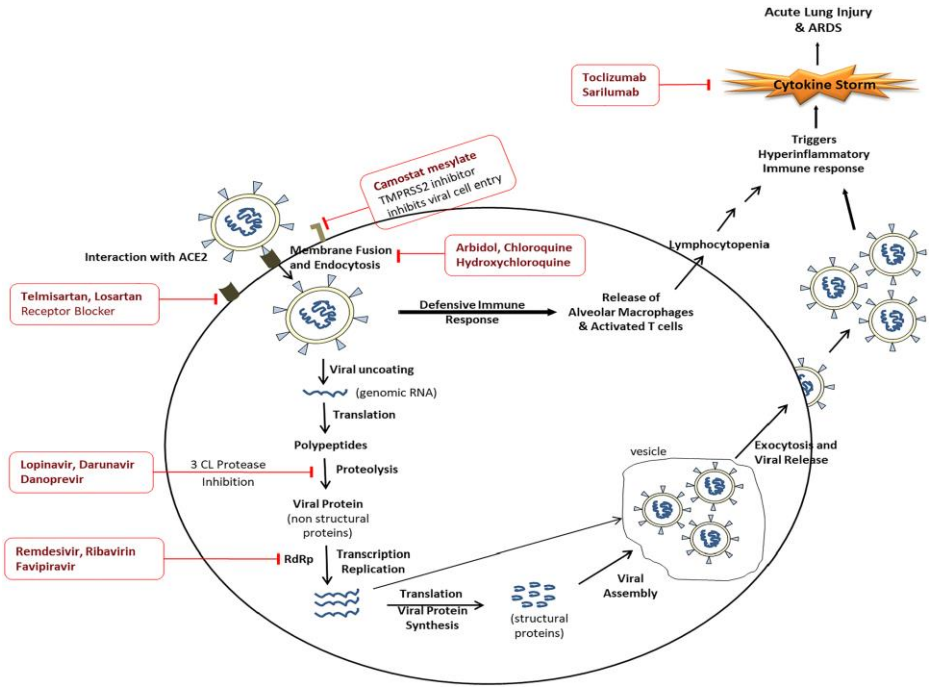

Figure 19. Mechanisms of action of different therapeutic agents in relation to viral life cycle (Kumari *et al*., 2020).

1.6.1. Hydroxychloroquine and chloroquine

In an earlier phase of the pandemic, due to the impressive SARS-CoV-2 spread and the huge number of severe cases of infection, it was difficult to test drugs in a conventional way of drug development. For this reason, many drugs that have been already used in other clinical conditions were included in clinical trials for COVID-19. For example, the antimalarial drugs hydroxychloroquine (HCQ) and chloroquine (CQ) were rapidly proposed as therapies for SARS-CoV-2 infection, as previously experienced for SARS and MERS (de Wilde *et al*., 2014; Keyaerts *et al*., 2004). Controversial results have been obtained over time. Indeed, despite an initial beneficial role of the drugs in reducing pneumonia severity (Gao *et al*., 2020), subsequent studies did not further confirm these results (Borba *et al*., 2020; Fiolet *et al*., 2020). Furthermore, even if the association between HCQ and azithromycin was also proposed and used during the earlier pandemic phase, no benefits have been proved (Echeverría-Esnal *et al*., 2021; Cavalcanti *et al*., 2020).

1.6.2. Remdesivir

Remdesivir (GS-5734) is an adenosine analogue active on other viruses, such as paramyxoviridae, filoviridae and pneumoviridae (Lo *et al*., 2017; Sheahan *et al*., 2017), able to inhibit RdRp and then block viral RNA replication. Results from a clinical trial revealed that remdesivir was associated with a reduction in time to recovery in treated patients respect to placebo group (Beigel *et al*., 2020) (Figure 20).

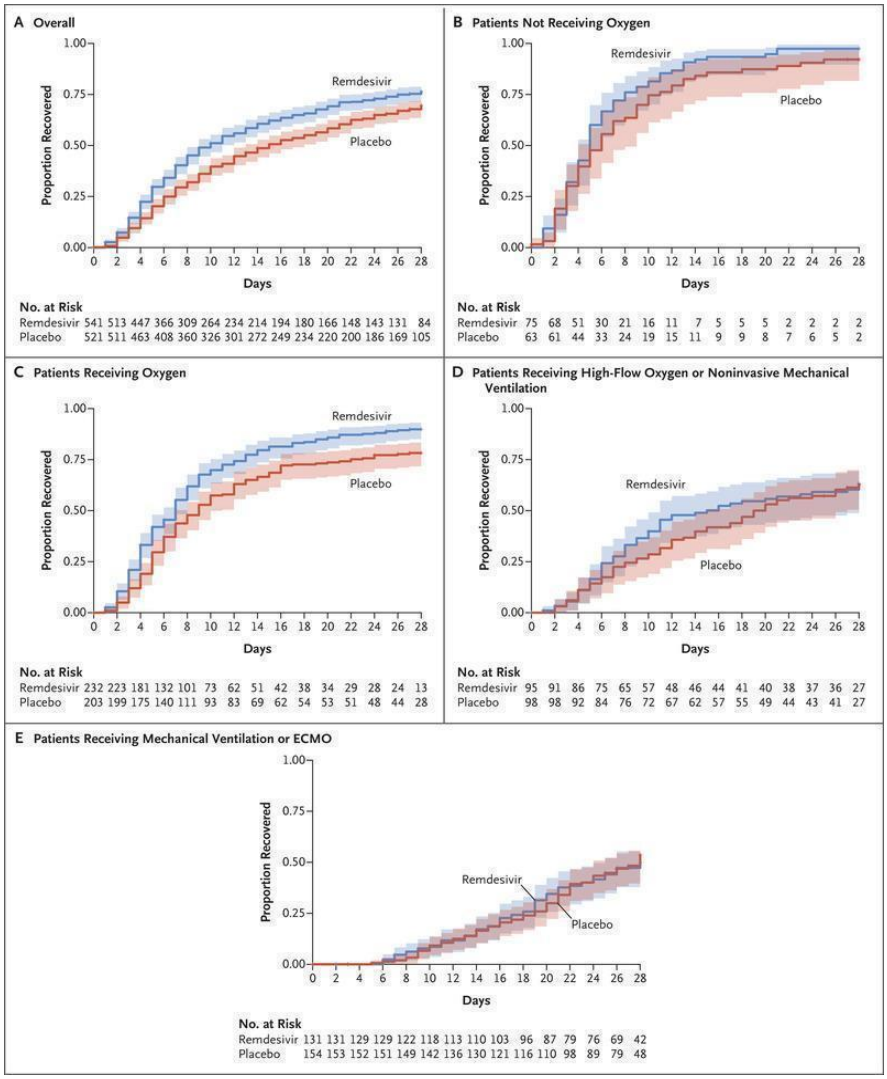

Figure 20. Kaplan-Meier estimating time to recovery in patients treated with remdesivir (blue line) respect to those treated with placebo (red line). Patients were overall analyzed (a) and then divided according to type of ventilation (Beigel *et al*., 2020).

1.6.3. Lopinavir and Ritonavir

Lopinavir and Ritonavir are two protease inhibitors used in combination for HIV therapy but also during SARS and MERS epidemic (Chu *et al*., 2004; de Wilde *et a*l., 2014; Sheahan *et al*., 2020). In normal conditions, viral protease leads to polyprotein proteolysis after translation and allows the generation of

fully functional proteins. As mechanisms of action, the protease inhibitors compete with the viral protease for binding substrate sites, resulting in production of incomplete or immature viruses (Lou *et al.*, 2014). So far, it has been reported that the combined therapy with lopinavir and ritonavir is not associated with improvement in outcome of patients with severe SARS-CoV-2 infection (Cao *et al*., 2020).

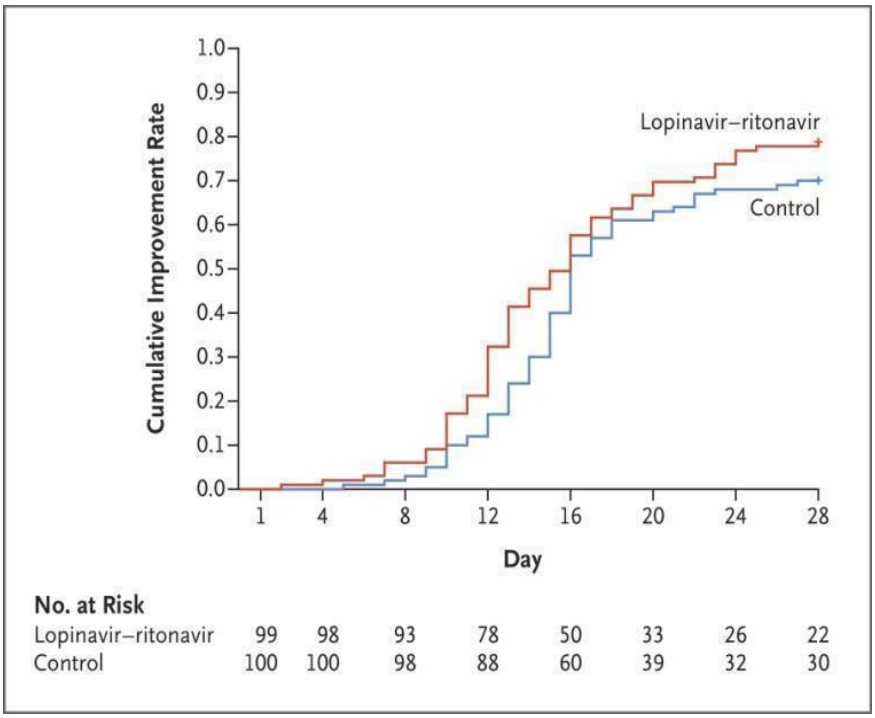

Figure 21. Time to Clinical Improvement in the Intention-to-Treat Population (Cao *et al*., 2020).

1.6.4. Molnupinavir and Paxlovid

Recently, new drugs have been developed in order to cure specifically SARS-CoV-2 infection. Among these, PF-07321332 (Paxlovid) from Pfizer and MK-4482/EIDD-2801 (Molnupiravir) from Merck have been recently studied with encouraging results. In detail, PF-07321332 is a 3C-like protease (3CLpro) inhibitor with potent *in vitro* antiviral activity against SARS-CoV-2 and other coronaviruses (NCT04756531) (Vandyck *et al*., 2021) while MK-4482/EIDD-2801 is the orally available pro-drug of the nucleoside analogue N4-hydroxycytidine (NHC). It has been reported that MK-4482/EIDD-2801 had a potent *in vivo* anti-influenza virus activity (Toots *et al.*, 2019). Furthermore, preliminary results demonstrated that early treatment with MK-4482/EIDD-

2801 might reduce the risk of progression to severe disease (Cox *et al.,* 2021). In this regard, further studies are required.

1.6.5. Monoclonal antibodies against IL6 and its receptor

The role of IL-6 is mainly related to the maintenance of homeostasis and activation of the immune system in case of tissue damage. Indeed, in case of tissue injury or infection, the production of IL-6 by macrophages and monocytes rapidly increases leading to a subsequent activation of a pro-inflammatory immune response (Tanaka *et al*., 2018). However, an abnormal activation of IL-6 and consequent increase of IL-6 serum level may lead to disorders and diseases. As previously reported in this thesis, COVID-19 is responsible of an abnormal pro-inflammatory response with dramatic increase of cytokines, including IL-6, which is related to the severity of the diseases and thus, introduced as prognostic markers in hospitalized patients (Han *et al*., 2020).

For this reason, one of the therapeutic options includes the use of monoclonal antibodies against IL6 and its receptor. In this setting, tocilizumab, a drug currently approved for treatment of Castelman disease, rheumatoid arthritis and juvenile idiopathic arthritis (Tanaka *et al*., 2018) has been largely studied in COVID-19 field.

Tocilizumab is a humanized anti-human IL-6 receptor (IL-6R) antibody able to bind IL-6R at IL-6 binding site level and then inhibit IL-6 pathway signal (Nishimoto *et al.*, 2008). Even if Stone and colleagues reported no significant benefits in moderately ill hospitalized patients after tocilizumab treatment (Stone *et al*., 2020), in critically ill patients tocilizumab was associated with prolonged survival (Gupta *et al*., 2020). Moreover, the association with glucorticoids, as dexamethasone, improved the benefits of tocilizumab treatment (Salama *et al*., 2020).

1.6.6. Hyperimmune plasma

Beside the emergence of COVID-19, the passive immunization using convalescent plasma for infectious disease treatment has been considered in the past as a valuable therapeutic option (Figure 22). In 1916 some patients affected by acute poliomyelitis were treated with plasma obtained by subjects recovered from polio with important benefits (Amoss and Chesney, 1917). In the following years, a similar approach was used for measles (Nabarro and Signy, 1931) and, more recently, for SARS in 2003 (Cheng *et al*., 2005), influenza pandemic of 2009 (Hung *et al*., 2011) and Ebola (van Griensven *et al*., 2016; Tedder *et al*., 2018; Winkler *et al*., 2015). Based on these encouraging experiences, convalescent plasma from recovered subjects was

considered for treatment of severe COVID-19 patients with different results. Indeed, while some studies reported that convalescent plasma therapy did not results in significant improvement of clinical condition when associated with standard treatment (Li *et al*., 2020; Zeng *et al*., 2020), several Italian research groups showed a mortality reduction when convalescent plasma with level of neutralizing antibodies higher than 1:160 was administered (Perotti *et al*., 2020: Perotti *et al*., 2020; Del Fante *et al*., 2021). The key of successful convalescent plasma therapy might be related to the screening of donors with high levels of neutralizing antibodies, as preliminarily reported (Duan *et al*., 2020).

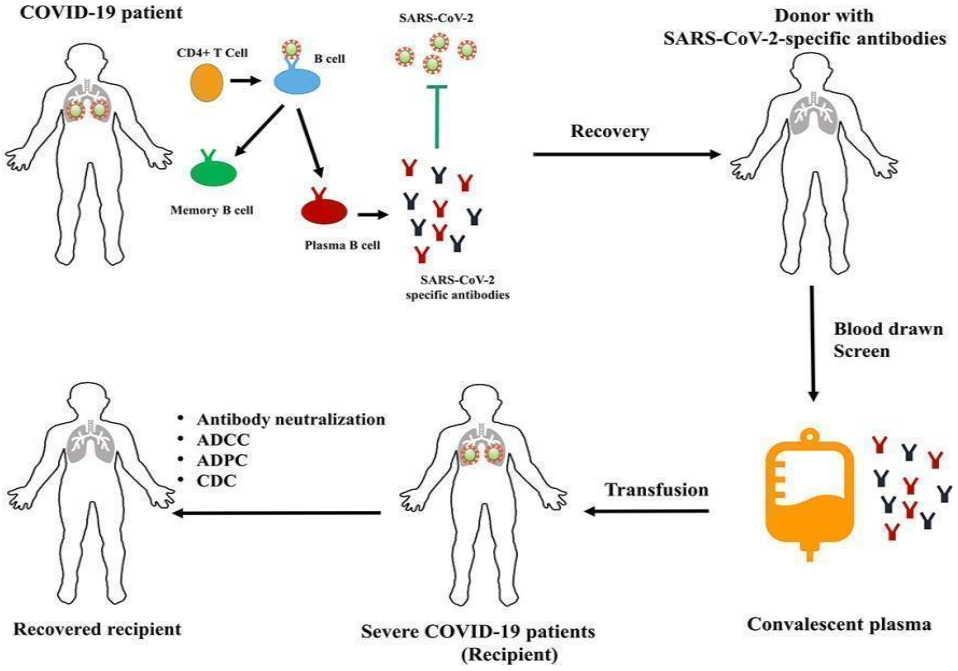

Figure 22. Schematic representation of plasma administration (Ouyang *et al.* 2020)

1.6.7. Monoclonal antibodies against Spike protein

Monoclonal antibodies are considered a valuable therapeutic strategy for malignancies, autoimmune or infectious diseases. After the publication of mouse monoclonal production using hybridoma technology in 1975, the number of researches and publications about monoclonal antibody generation and clinical utility increased dramatically (Köhler and Milstein,

1975). At the same time, the laboratory approaches changed in time leading to the synthesis of fully human monoclonal antibodies (Lonberg, 2005). In recent years, a number of neutralising monoclonal antibodies, including broadly active neutralizing antibodies (Durham ***et al***., 2019; Sajadi ***et al***., 2018; Corti ***et al***. 2013), have been developed and used for patient treatment, instead of convalescent plasma.

Overall, the efficiency of *in vitro* monoclonal antibodies generation has dramatically improved (Corti and Lanzavecchia, 2013). For example, Traggiai and colleagues in 2004 published a new and simple method for generating monoclonal antibodies with high neutralizing levels against SARS from memory B cells (Traggiai ***et al.***, 2004). This method, which allows efficient immortalization and cloning of human memory B cells, has been also used for generation of monoclonal antibodies against H5N1 influenza virus (Lanzavecchia ***et al.,*** 2007) and against paramyxovirus (Corti ***et al.,*** 2013). Recently Cao et al. published an efficient method of neutralizing antibody selection through high-throughput single cell sequencing starting from convalescent samples (Figure 23; Cao ***et al***., 2020). Generation of effective monoclonal antibodies by immunization of humanized mice is another valuable strategy (Akkina ***et al***., 2014).

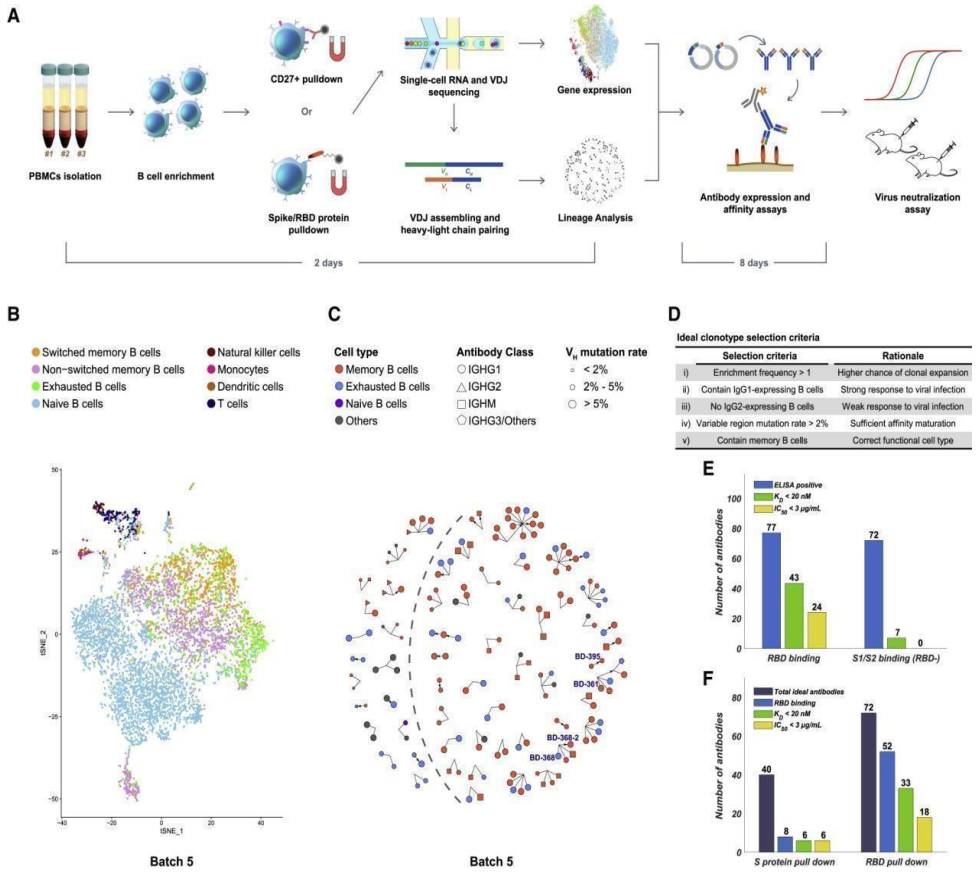

Figure 23. Efficient Neutralizing Antibody Identification through Antigen-Enriched High-Throughput Single-Cell RNA Sequencing (Cao *et al*., 2020).

Due to the paramount importance of the S-protein in SARS-CoV-2 cell entry, almost all monoclonal antibodies are directed against S-specific epitopes, in particular against the receptor binding domain (Shi *et al*., 2020; Wang *et al*., 2020; Ju *et al*., 2020), but also against N-terminal domain (Chi *et al*., 2020). Of note, monoclonal antibodies specific for SARS-CoV-1 might cross-react with SARS-CoV-2, as demonstrated in few papers (Tian *et al*., 2020; Pinto *et al*., 2020), thus increasing the potential therapeutic opportunities.

Results from clinical trials suggest that monoclonal antibodies act by preventing virus host cell entry thereby reducing SARS-CoV-2 viral load (Chen *et al*., 2020; Weinreich *et al.,* 2021). As such, they might be mainly useful for the treatment of patients earlier after symptoms onset, including subjects without neutralizing antibody response.

The results reported by clinical trials provided crucial information leading the USA Food and Drug Administration (FDA) to approve the use of bamlanivimab (FDA news release November 9, 2020 and casirivimab–imdevimab combination (FDA news release, November 21, 2020) in subjects with mild or moderate COVID-19, who are at high risk to develop severe disease.

The principal disadvantage of monoclonal antibodies may rely on the possible escape due to the emergence of SARS-CoV-2 variants (Weisblum et al., 2020) that might be avoided by using combination of different monoclonal antibodies, the so called "cocktails", which have been reported to be well tolerated and safe (Weinreich *et al.,* 2021). Bispecific antibodies have been also considered as potential candidates. They are defined as particular types of antibodies carrying two different antigen-binding sites in one molecule (Sedykh *et al*., 2018). Before the COVID-19 emergence, the therapeutic opportunities of bispecific antibodies have been also explored for treatment of diseases such Acute Myeloid Leukemia (Thakur *et al*., 2018; Guy *et al*., 2018; Clark *et al.,* 2020). In some cases, bispecific antibodies combining both neutralizing and non-neutralizing epitopes on RBD may improve the efficacy of SARS-CoV-2 antibodies (Lim *et al*., 2021), avoiding viral escape (De Gasparo *et al*., 2021; Cho H *et al*., 2021).

1.7. Prevention of SARS-CoV-2 infection: vaccination

Since the discovery of the new coronavirus, a great effort has been made for the development of prophylactic strategies against SARS-CoV-2 infection. The result was extraordinary: in less than eight months since the discovery of the new virus, 27 vaccines were evaluated for clinical study and 139 for preclinical phase (https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines 2020).

Different strategies have been considered for SARS-CoV-2 vaccine candidates, including the use of inactivated virus, virus-like particles, protein subunits, mRNA and vector virus (Kyriakidis *et al*., 2021). The mRNA is the intermediate product of gene expression, between DNA translation and protein production in the cytoplasm. As vaccine candidates, both non-replicating mRNA and self-amplifying RNA have been proposed. In the first case, the mRNA of chosen antigen is flanked by 5' and 3' untranslated regions, while, in the second case, both antigen and viral replication machinery are encoded in order to allow intracellular RNA amplification and increased antigen production (Pardi *et al*., 2018; Sahin *et al*., 2014). Obviously, naked RNA is easily degraded and vectors are necessary. When the mRNA reaches the cell cytoplasm protein is produced which after

appropriate post-translation modification develops in a fully functional antigen. This type of platform has several advantages, including safety, since vaccine mRNA is a non-infectious and non-integrating platform and no mutagenesis risk may occur. Using appropriate systems, half-life of mRNA may be regulated and the stability may be improved (Kariko 2019; Kariko *et al*., 2011).

Based on this experience and knowledge, several mRNA-based SARS-CoV-2 vaccines have been proposed and developed, and two of them, BNT162b2 and mRNA-1273, are now used in clinical practice, since they demonstrated more than 90% of efficacy in avoiding severe COVID-19 infection (Polack *et al.,* 2020; Baden *et al*., 2021). As for other vaccines, the choice of the antigen is crucial. Specifically, in the case of SARS-CoV-1 it was observed that neutralizing antibodies were mainly directed against S protein (Buchholz *et al*., 2004). In the case of SARS-CoV, it has been shown that only antibodies directed to S protein can neutralize the virus and prevent infection. As a result, all SARS-CoV-2 vaccines in development include at least a portion of the S protein. These may be restricted to only the S1 domain or the RBD. To date, two licensed vaccines are mRNA-based vaccines (BNT162b2 and mRNA-1273) approved by the FDA and European Medical Agency (EMA).

Before the SARS-CoV-2 pandemic, non-viral delivered nucleic acids, including mRNA vaccines, were largely studied, not only for prevention against infectious diseases but also for cancers. Briefly, non-viral delivered nucleic acids are divided into DNA and RNA vaccines that are processed through different pathways. Indeed, DNA based vaccines need to overcome both cellular and nuclear membranes for the transcription into mRNA and transfer to cytoplasm for the translation (Zhang *et al.,* 2019). mRNA vaccines have been studied since 1990 (Wolff *et al.,* 1990) and an effective optimization of mRNA vaccines synthesis occurred five years later when Ross and colleagues optimized the procedures for mRNA production and stabilization (Ross, 1995). Usually, mRNA based vaccines are divided into conventional and self-amplifying mRNA. The first type is characterized by the presence of the choice antigen flanked by an untranslated region and a polyadenylated (polyA) terminal region. On the other hand, so-called self-amplifying mRNA are able to self-replicate since they are based on an engineered viral genome including RNA replication machinery (Zhang *et al.,* 2019). It has been observed that self-amplifying mRNA-based vaccines are highly immunogenic compared to conventional mRNA (Vogel *et al.,* 2018). A strong immune response, both humoral and cell-mediated is normally elicited, as observed in the case of influenza virus (Pardi *et al.,* 2018).

The preliminary results of immunological and safety profile of the two first mRNA vaccine (BNT162b1 and BNT162b2, by BioNTech, with Fosun

Pharma and Pfizer) encodes for RBD and for the full length S protein, respectively, guided the authors to propose the second one for phase II/II trials and for further larger studies. Indeed, even if the immunogenicity of the two vaccines were comaprable, in terms of reactogenicity, mild systemic reactogenicity was linked to BNT162b2 vaccine (Walsh *et al.,* 2020). Moreover, both vaccines elicited a lower humoral response in older subjects. As a major limitation, data on the immune response elicited by the vaccine are limited to a low number of subjects analysed. i.e. 12 subjects immunized with the licensed formulation of two doses of 30μg (Walsh *et al.,* 2020). Further analysis performed by Polack, et al. confirmed the high immunogenicity induced by mRNA BNT162b2 vaccine (Polack *et al.,* 2020). Previous reports related to SARS-CoV have suggested a protective role of both humoral and cell-mediated immunity, and that T-cell response may confer long-term protection while the antibody response in humans was found to be relatively short-lived in convalescent individuals (Yang *et al*., 2004). The topic related to immune response elicited by mRNA BNT162b2 vaccine will be better explored in the thesis.

The second mRNA licensed vaccine, produced by Moderna, with National Institute of Allergy and Infectious Diseases, is also based on full-length S protein. Preliminary results obtained in non-human primates suggested a robust immune response elicited in response to mRNA-1273 vaccine (Corbett *et al.,* 2020) that was confirmed by clinical trials (Jackson *et al.,* 2020), with an efficacy higher than 94% in preventing COVID-19 illness (Baden *et al.,* 2020). For both BNT162b2 and mRNA-1273 vaccines, two doses are required and necessary for eliciting a strong immunogenic response.

The other two vaccines licensed in Italy, ChAdOx-1 and Ad-26 are two vector-based vaccines, distributed by University of Oxford with AstraZeneca and Janssen Pharmaceutical Companies, respectively. The first vaccine also named AZD1222 consists of the replication-deficient simian adenovirus vector ChAdOx1, containing the full-length S-protein and a tissue plasminogen activator leader sequence (Folegatti *et al.,* 2020). Reported vaccine efficacy in randomized clinical trials was 70.4% (Volsey *et al.,* 2020). Similarly, efficacy and immunogenicity of Ad-26 vaccine has been demonstrated after a single dose administration in pre-clinical (Mercado *et al.,* 2020; Bos *et al.,* 2020; Tostanoski *et al.,* 2020) and clinical studies (Sadoff *et al.,* 2021; Stephenson *et al.,* 2021). As reported for monoclonal antibodies, efficacy of licensed SARS-CoV-2 vaccines, both mRNA-based and vector-based, against viral variants is still a debated theme.

1.7.1. SARS-CoV-2 vaccination in cancer patients

Although evidences from randomized clinical trials are still lacking, it is plausible that the safety and efficacy of vaccination against SARS-CoV-2 for cancer patients may be similar or lower to those of patients without cancer, considering data extrapolation from other vaccines and the mechanism of action of most COVID-19 vaccines (Ward *et al.*, 2017). For example, considering anti-influenza vaccination, seroconversion and seroprotection appear to be lower in patients receiving chemotherapy than in the general population, but not in patients receiving single-agent immune checkpoint inhibitors (ICI) (Bayle *et al.,* 2020).

ICIs included mainly drugs that inhibit the pattern PD1/PD-L1. In the tumorigenesis, the PD-1/PD-L1 pathway is involved in the inhibition of T effector response promoting the occurrence of tumour escape and progression of cancer (Figure 24). Thus, anti-PD-1/PD-L1 therapy act in preventing the progression of advanced metastatic tumour and improving the progression the survival rate of patients (Yang *et al*., 2021; Darvin *et al.,* 2021).

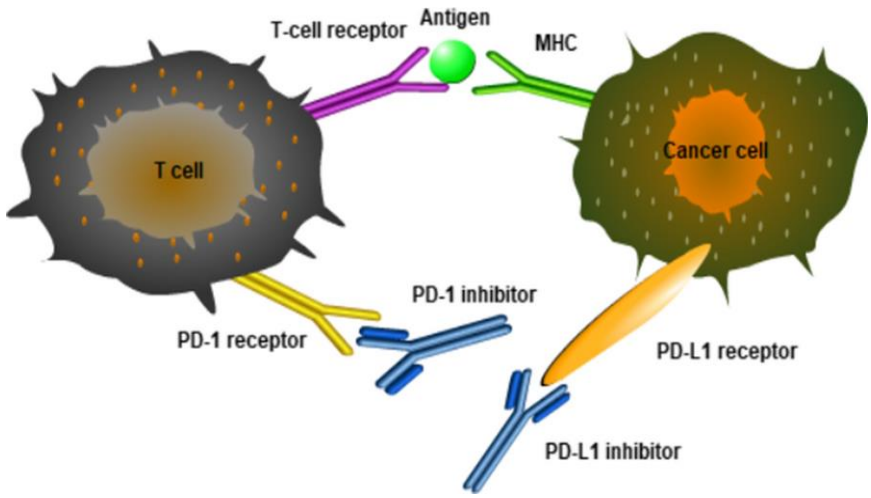

Figure 24. Tumor cells inhibit T cell activation by expressing PD-L1 on their cell surface, and PD-1/PD-L1 inhibitors reactivate T cells by specifically binding to PD-1/PD-L1 (Yang *et al.*,2021)

Based on their mechanism of action and on preclinical data, ICIs are likely to enhance rather than diminish immune response against vaccines (Liao and Zhang, 2021). Recently, Waissengrin and colleagues reported the safety of the BNT162b2 mRNA vaccine in a cohort of patients treated with ICIs (Waissengrin *et al.,* 2021) but the immunogenicity of vaccination needs to be

further explored. Otherwise, reduced immunogenicity of SARS-CoV-2 vaccine in immunocompromised patients, in particular in transplant recipients has been observed (Miele *et al*., 2021; Grupper *et al*., 2021; Marinaki *et al.,* 2021), especially in those cases of antimetabolite immunosuppression use (Boyarsky *et al.,* 2021). This topic is important, since inadequate immunological response may increase the risk of COVID-19 infection in vaccinated immunocompromised subjects (Ali *et al.,* 2021).

1.8. SARS-CoV-2 variants: new challenges in therapy and prevention

From a biological point of view, the occurrence of mutation at genomic level in viruses are frequently observed. Even if RNA viruses show higher mutation frequency compared to DNA viruses, HCoVs make fewer mutations than other RNA viruses due to a viral enzyme able to correct replication errors (Lauring and Hodcroft, 2021). The first significant mutation detected at S-protein level was the D614G mutation, which determines the substitution of aspartic acid for glycine at 614 position. After March 2020, the mutation was detected worldwide, including Italy (Alteri *et al*., 2021). Interestingly, D614G mutation was associated with increased replication and transmissibility (Volz *et al*., 2021; Plante *et al*., 2021; Zhou *et al.,* 2021 Hou *et al*., 2020). At the same time, the presence of viruses carrying D614G mutation was associated with increased levels of neutralizing antibodies (Weissman *et al.,* 2021). In September 2020, in England a new variant of concern, carrying N501Y mutation, was identified (Wise, 2020) and became rapidly dominant. Based on epidemiological analysis, the first 501Y lineage, also called 501 variant 1 circulated between September and November 2020 in Wales, but the proportion was much lower compared to 501N lineage in the sequenced samples. However, the second 501Y lineage, also defined as B.1.1.7 and recently called “alpha” by WHO, rapidly spread reaching 49.7% of circulating viruses at the end of November (Leung *et al.,* 2021). Of note, 501Y mutation seems to increase the binding affinity between RBD and ACE2 receptors (Starr *et al*., 2020). Even if this strain reported higher transmissibility, it does not seem to be resistant to neutralizing antibodies elicited by SARS-CoV-2 vaccines (Collier *et al.,* 2021).

The P.1 variant or, as now called, “gamma strain” showed two additional mutations at RBD level (K417T and E484K) beyond the N501Y; it emerged in Manaus (Brazil) at the beginning of 2021 (Faria *et al.,* 2021) and rapidly spread in Brazil and South America, where almost 40% of sequenced genomes were identified as P.1 strain in February 2021. One of the major concerns about P.1 strain is related to reduced efficacy of monoclonal antibodies (Starr *et al.,* 2021) and neutralizing antibodies elicited after natural infection or vaccination (Hoffmann *et al.,* 2021; Wang *et al.,* 2021). Even if a

decrease in terms of neutralization was observed against P.1 strain, this variant seems to be less resistant respect to B.1.135 or “beta” (Tegally *et al*., 2021). Indeed, recent studies reported that monoclonal antibodies showed less efficacy against the strain and that level of neutralizing antibodies in convalescent and vaccinated subjects was dramatically decreased (Hoffmann *et al.,* 2021; Wang *et al.,* 2021). Even if a marked reduction is observed in terms of neutralizing activity against B.1.351 variant, sera from vaccinated subjects with BNT162b2 vaccine seems to efficiently neutralize the variant (Zani et al., 2021). Moreover, the efficacy of Ad26 vaccine against South African variants has been reported in macaques (Yu et al., 2021). Finally, the global attention is now focused on the newly emerged variant, named as B.1.617.2 or Delta, firstly emerged in Indian, United Kingdom and then in the rest of Europe where it is now the dominant strain (Torjesen, 2021). Even if the variant seems to be highly transmissible and contagious, vaccination strategy might confer adequate protection against disease. As reported in preliminary studies, BNT162b2 vaccination is able to elicit neutralizing antibodies against B.1.617 (Liu *et al.,* 2021). In this setting, the role of cell-mediated response should be better investigated. Interestingly, preliminary studies reported that cell-mediated response against variants is less affected than humoral response (Geers *et al.,* 2021; Tarke *et al.,* 2021; Woldemeskel *et al.,* 2021). The topic is highly relevant and, in this thesis, the most salient results in terms of humoral and cell-mediated response elicited by vaccination against the main SARS-CoV-2 variants of concern have been reported (VOC).

2. Aims of the work

In the context of COVID-19 emergence, the Molecular Virology Laboratory of the IRCCS Policlinico San Matteo and University of Pavia has been involved in SARS-CoV-2 molecular and immunological investigations for diagnostic and research purposes. In this setting, my PhD work had three main aims.

- Design and application of a microneutralization assay for the quantification of anti-SARS-CoV-2 neutralizing antibodies in different clinical and epidemiological settings. This approach was rapidly introduced for general population screening, such as asymptomatic blood donors of the Lodi Red Zone, firstly involved in pandemic. Moreover, characterization of SARS-CoV-2 neutralizing antibodies helped in identification of “hyperimmune” convalescent plasma donors for the treatment of severe COVID-19 cases. The assay was also adapted for defining the *in vitro* efficacy of monoclonal antibodies, including a bispecific monoclonal antibody, in collaboration with Institute for Research in Biomedicine of Bellinzona (Switzerland), in the context of the Antibody therapy against coronavirus (ATAC-Horizon 2020).

- Design and application of a new ELISpot assay for the quantification of cell-mediated response against SARS-CoV-2 antigens in SARS-CoV-2 infected subjects and vaccinated healthcare workers and oncologic patients treated with immune checkpoint inhibitors. In a subset of healthcare workers, humoral and cell-mediated responses elicited by vaccination were challenged against the most relevant SARS-CoV-2 VOC.

- Dissection of the role of monocytes and macrophages as possible “Trojan Horse" in the viral dissemination. For this aim, we designed *in vitro* experiments using monocytes and/or differentiated macrophages directly infected with SARS-CoV-2 strains or cocultivated with VERO E6 cell lines, in order to demonstrate the active infection of immune cells and transfer of infection to other susceptible cells.

3. Material and Methods

3.1. Design of microneutralization assay for the characterization of SARS-CoV-2 neutralizing antibodies and its applications

3.1.1. SARS-CoV-2 viral strain isolation and sequencing analysis

In our laboratory different SARS-CoV-2 strains have been isolated and titrated during the pandemic period in BLS3 facility. Briefly, SARS-CoV-2 strains, firstly including original A strain (D614), B.1. strain (PV10734 with D614G mutation), and then alpha strain (501Y.V1 lineage B.1.1.7), beta strain (501Y.V2 lineage B.1.351), gamma strain (501Y.V3 lineage P.1) and delta strain (lineage B.1.617.2) were isolated from nasal swabs collected from infected patients.

In detail, 200 µl of each sample was inoculated and propagated into VERO E6 (VERO C1008 (Vero 76, clone E6, Vero E6; ATCC® CRL-1586™) permissive cell line and titrated to prepare cell free virus for neutralization assay as following described. Two-fold dilution of virus preparation (50 µl) were plated with 50 µl of VERO E6 for measurement of cytopathic effect. Median Tissue Culture Infectious Dose (TCID50) was calculated according to Reed and Muench formula (Ramakrishnan, 2016).

All the strains were sequenced in order to confirm the presence of variant-defining mutations (Table 1). Complete genome sequencing (Alteri *et al.,* 2021) was performed in order to confirm the presence of variant-defining mutations and sequences were submitted to Global Initiative on Sharing Avian Influenza Data (GISAID) under the following reference numbers (EPI_ISL_568579; EPI_ISL_1403609-11).

Table 1. Mutations of the VOC used in the experiments (based on ECDC, 2021)

Who label	Lineage +additional mutations	Spike mutations of interest	Country first detected (community)	Year/month first detected
Alpha	B.1.1.7	N501Y, D614G, P581H	United Kingdom	September 2020
	B.1.1.7+E484K	E484K N501Y, D614G, P581H	United Kingdom	December 2020
Beta	B.1.351	K417N, E484K, N501Y, D614G, A701V	South Africa	September 2020
Gamma	P.1	K417T, E484K, N501Y, D614G, H655Y	Brazil	December 2020
Delta	B.1.617.2	L452R, T478K, D614G, P681R	India	December 2020

3.1.2. Quantification of neutralizing antibodies in COVID-19 convalescent subjects and asymptomatic blood donors from Lodi Red Zone

For SARS-CoV-2 neutralizing antibodies quantification, 50 µl of sera were four-fold diluted starting from 1:10 to 1:640 and added in two wells of a flat bottom tissue culture microtiter plate (COSTAR, Corning Incorporated, NY 14831, USA). Then, 50 µl of 100 TCID50 of SARS-CoV-2 virus (PV10734 D614G was used as reference strain), previously titrated, was added. After 1-hour incubation at 33 °C 5% CO_2, VERO E6 cells were added to each well. After 72 hours of incubation, plates were scored for cytopathic effect (CPE) detection in comparison to the virus control. In detail, plates were stained with Gram's crystal violet solution (Merck KGaA, 64271 Damstadt, Germany) with the addition of 5% formaldehyde 40% m/v (Carlo ErbaSpA, Arese, Italy) for 30 min and washed under running water. Blue staining of wells indicated the presence of neutralizing antibodies. Neutralizing titer was defined as the maximum dilution with the reduction of 90% of CPE. Neutralizing titres higher or equal to 1:10 were considered as positive (Figure 25). Positive and negative controls were included in all plates (Percivalle *et al.,* 2020).

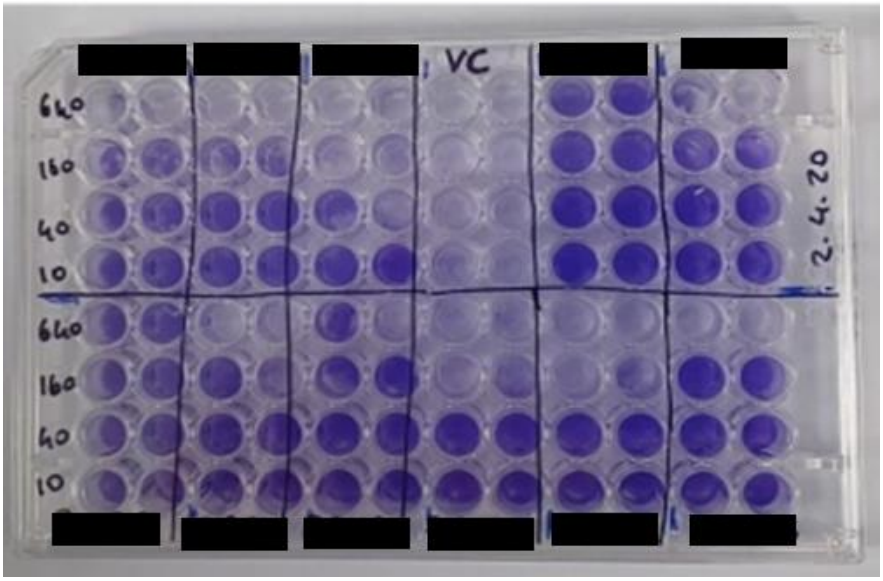

Figure 25. A representative microneutralization plate after staining. Eleven serum samples were four-fold diluted starting from 1:10 dilution to 1:640 and challenged with viral preparation, as described in main text. VC: virus control (Percivalle *et al.,* 2020).

The assay was firstly performed using 30 sera collected in the pre-pandemic period (21 females and 9 males; median age: 43 years, range 24–74), including 10 sera positive for other common coronaviruses (229E, OC43, HKU1, NL63), in order to evaluate the specificity of the assay. Then. the sensitivity of the assay was challenged using 40 sera collected in the period 15–30 March 2020 from 40 RT-PCR confirmed COVID-19 patients (14 females and 26 males median age: 61 years, range 45–81) during their convalescence (median 25 days after first SARS-CoV-2 positive nasal swab; range: 9–44 days).

Serum samples from 390 blood donors of Lodi Red Zone (118 females and 272 males; median age 46 years; range 19–70 years) were prospectively collected at the Blood Transfusion Centre of the Lodi Hospital from 18 March to 6 April 2020 and analysed for SARS-CoV-2 NT Abs. In parallel, nasal swabs were collected and SARS-CoV-2 RT-PCR was performed according to diagnostic protocol used at Virology Unit of IRCCS Policlinico San Matteo (Corman *et al.,* 2020).

History of symptoms or high-risk contacts during the previous 30 days was recorded. In order to evidence SARS-CoV-2 circulation before the identification of the “index case” on 20 February 2020, stored serum samples

from 300 blood donors of the same area and collected between 27 January 2020 and the first 20 days of February 2020 were analysed.

3.1.3. Design of neutralizing assay for monoclonal antibodies challenging

Six monoclonal antibodies (MoAbs) provided by the Institute for Research in Biomedicine (University of Italian Switzerland, USI of Bellinzona) were analyzed *in vitro* against SARS-CoV-2 strains. One of these MoAbs was a bispecific MoAb, CoV-X2, that is a human-derived IgG1-like bispecific antibody in the CrossMAb format that is the result of the combination of the fragment antigen binding (Fab) of the monoclonal antibodies C121 and C135, which are two potent neutralizers of SARS-CoV-2 (Robbiani *et al., 2020*). In a second part of the study, MoAbs were challenged *in vivo*. Complete data derived from computational biochemical studies and *in vitro/in vivo* analyses have been recently published (De Gasparo *et al.,* 2021).

In this thesis, data derived from *in vitro* challenging of the tested monoclonal antibodies are discussed. Neutralizing activity of monoclonal antibodies was tested as previously described. Fifty µl of two-fold scalar dilution of monoclonal antibodies starting from 10 or 180 µg/mL were added in 96-well microplates; thereafter an equal volume of 100 TCID50 of SARS-CoV-2 strain. Monoclonal antibodies were challenged for Chinese-derived D614 and D614G PV10734 strains. For each monoclonal antibody inhibitory concentration 50% (IC50) was given.

3.2. Characterization of cell-mediated immune response in SARS-CoV-2 infected subjects

3.2.1. Subjects' enrolment and ethical statement

Eighty-seven COVID-19 positive subjects proven by positive SARS-CoV-2 RNA detection in nasal swab by RT-PCR were enrolled for assessment of SARS-CoV-2 humoral and cell-mediated response elicited by natural infection. All the subjects were enrolled when SARS-CoV-2 RNA became negative (convalescence) and median day after symptoms' onset was 30 days (range 7-239 days). Demographic and clinical characteristics of enrolled patients are shown in Table 2.

Table 2. Characteristics of enrolled COVID-19 subjects

Sex	**N subjects**	**%**
Female	44	51
Male	43	49
Age (years)	**no subjects**	**%**
18-29	9	11
30-39	14	16
40-49	21	24
50-59	17	20
60-69	15	17
≥70	10	12
Symptoms	**no subjects**	**%**
Fever	62	71
Anosmia/Ageusia	19	22
Asthenia	30	35
Cough	24	28
Dyspnoea	10	11
Diarrhoea	5	6
Hospitalization	**no subjects**	**%**
Yes	23	26
No	64	74
Assisted ventilation*	**no subjects**	**%**
Yes	10	12
No	77	88

*Highflow nasal cannulae, ventimask and/or continuous positive airway pressure therapy (CPAP)

Serum samples and heparinized whole blood samples were collected for the assessment of SARS-CoV-2 neutralizing antibodies (NT Abs) as well as for the quantification of SARS-CoV-2 specific T-cell response, as described. The study was approved by the ethical committee of IRCCS Policlinico San Matteo (P-20200041154 and P-20200029440). As control, stored PBMC collected in the pre-pandemic period were used in order to assess pre-existing SARS-CoV-2 immunity.

3.2.2. Peripheral blood mononuclear cells isolation

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood samples collected in heparinized tubes by density gradient centrifugation (Lymphoprep, Axis-Shield, Norway). Then, PBMC were resuspended in RPMI 1640 with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml

streptomycin, and 10% of heat inactivated fetal bovine serum (FBS; Euroclone). After cell count, isolated PBMC were cryopreserved in freezing medium (90% FBS and 10% DMSO from Sigma-Aldrich, MO, USA). Each vial, containing $10x10^6$ PBMC, was stored overnight at -80 °C using cryovial with isopropanol and then kept in liquid nitrogen until analysis.

3.2.3. Peptide pools and viral lysate for immunological assays

For the analysis of convalescent patients and for the setting of the new ELISpot assay we used peptide pools (consisting of 15-mer sequences with 11 amino acids overlap) representative of Spike (315 peptides), VME1 (53 peptides), NCAP (102 peptides), NS7B (8 peptides) and NS8 (28 peptides) were used (0.25 µg/ml per well). Additionally, whole lysate obtained from 10^5 plaque-forming unit (PFU)/ml SARS-CoV-2 viral strain isolated in our laboratory was inactivated at UV-light and used as antigen.

3.2.4. Enzyme-linked immunospot assay

Enzyme-linked immunospot (ELISpot) assay was performed as follows (Figure 26). Membrane-bottomed 96-well plates (Multiscreen-IP) from Merck Millipore, Germany, were coated with anti-interferon (IFN)-γ monoclonal capture antibody against from Human IFN-γ ELISpot kits (Diaclone, France) and kept at 4°C overnight. Then, after 2-hr blocking with culture medium, PBMC ($2x10^5$/100µl 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. Phytoheamagglutinin (PHA; 5 µg/mL) was used as positive control, and medium alone as negative control. Culture medium was RPMI 1640 supplemented with 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, and 10% of heat inactivated fetal bovine serum (FBS, Euroclone). Plates were maintained overnight at 37°C in a 5% CO_2 humidified atmosphere. After multiple wash, anti–IFN–γ biotinylated antibody was added and incubated overnight at 4°C. Finally, streptavidin–alkaline phosphatase conjugate was added, and after 60 min incubation at 37°C in a 5% CO_2, substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) was added for 20 min at room temperature. Plates were washed under running water kept overnight at room temperature before spot counting.

Spots were counted using an automated AID ELISPOT reader system (AutoImmunDiagnostika GmbH, Strasburg, Germany). The mean number of spots from duplicate wells were adjusted to million of PBMC. Moreover, the background response (negative control) was subtracted by the antigen response in order to obtained the net spots per million PBMC or IFNγ spot forming unit (SFU)/million PBMC. Based on mean of IFNγ SFU/million PBMC

obtained with negative control plus two standard deviations, responses higher or equal than 10 IFNγ SFU/million PBMC were considered positive.

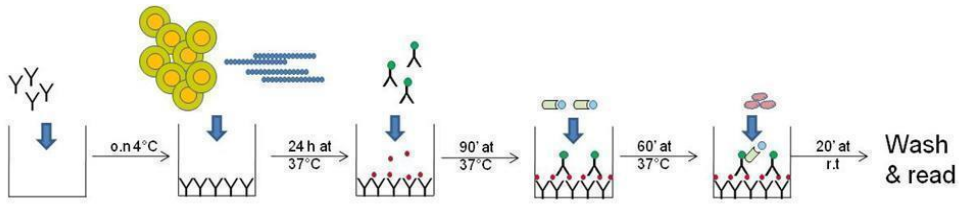

Figure 26. Schematic representation of ELISpot assay procedure

3.2.5. Selective depletion of CD4 and CD8 T cells

CD4 and CD8 T cells were depleted Human CD8 or CD4 MicroBeads from MiltenyiBiotec (Bergisch Gladbach, Germany) were used for depleting CD8 and CD4 cells, respectively in 11 COVID-19 positive subjects according to manufacturer's instructions. MS columns from Milteny Biotec were used for magnetic cell isolation and flow cytometry assay confirmed that the depleted fractions contained less than 5% of target cells. ELIspot assay was then performed in parallel using total PBMC, CD4-depleted PBMC and CD8-depleted PBMC.

3.3. Characterization of humoral and cell-mediated immune response in healthcare workers vaccinated with mRNA BNT162b2 vaccine against PV10734 D614G reference strain and SARS-CoV-2 variants

3.3.1. Subjects' enrolment and ethical statement

One-hundred forty-five healthcare workers at Fondazione IRCCS Policlinico San Matteo volunteer to participate to a prospective study for assessment of SARS-CoV-2 immune response elicited by mRNA BNT162b2 vaccine (31 males and 114 females; median age 44; range 21-69 years). Of them 18 (12.4%) experienced a previous SARS-CoV-2 infection The project was approved by ethical committee of IRCCS Policlinico San Matteo (P-20210000232). Sampling was performed at time of vaccination (day 0, T0), at time of second dose administration (day 21, T1) and after 21 days after complete vaccination schedule (day 42, T2). For assessment of long-term immune response, sampling will be performed after six and twelve months. In this thesis, data of short-term immune response are shown.

Serum samples and heparinized whole blood samples were obtained for assessing the level of SARS-CoV-2 NT Abs and SARS-CoV-2 specific T-cell response, as previously described. The ELISpot assay was adapted using only S peptide pool in order to quantify cell-specific response elicited by vaccination. Moreover, the N peptide pool was also used for assessing pre-existing immunity, especially in those cases of previous SARS-CoV-2 infection. In a subgroup of subjects, characterization of cell-mediated response was assessed by flow cytometry. Moreover, total IgG level was assessed in all the subjects at each time point and, in a subgroup of subjects, immune response elicited by vaccination against variants was assessed. Additional methodologies are described below.

3.3.2. Flow cytometry analysis and intracellular cytokine staining

To evaluate T-cell subsets proliferation, PBMC (600,000/200 µl culture medium per well) were stimulated in triplicate in 96-well round-bottom plates with peptide pools representative of the S and N proteins, at the final concentration of 0.1 µg/ml for 7 days. Peptide pool from human actin, was used as a negative control antigen. Culture medium and peptide pools were the same used for ELISpot assay. After culture, cells were washed with PBS 0,5µM EDTA and stained in PBS with Live/Dead Fixable Violet Dye (Invitrogen), and in PBS 5% FCS with anti-CXCR5 (BLR1 clone), anti-IgG2b (biotinylated) and Streptavidin BV421, CD3 PerCP 5.5, CD4 APC Cy7, CD8 FITC, CD25 PECy7, CD278 (ICOS) APC antibodies. Finally, cells were washed and suspended in 1% paraformaldehyde. The frequency of CD25+ICOS+ expanded CD3+CD4+, CD3+CD4+CXCR5+ and CD3+CD8+ T-cells was determined by subtracting the frequency of PBMC incubated with actin peptides from the frequency of PBMC incubated with SARS-CoV-2 S and N peptides. Flow-cytometry analyses were performed with a FACS Canto II flow cytometer and DIVA software (BD Biosciences) (Figure 27).

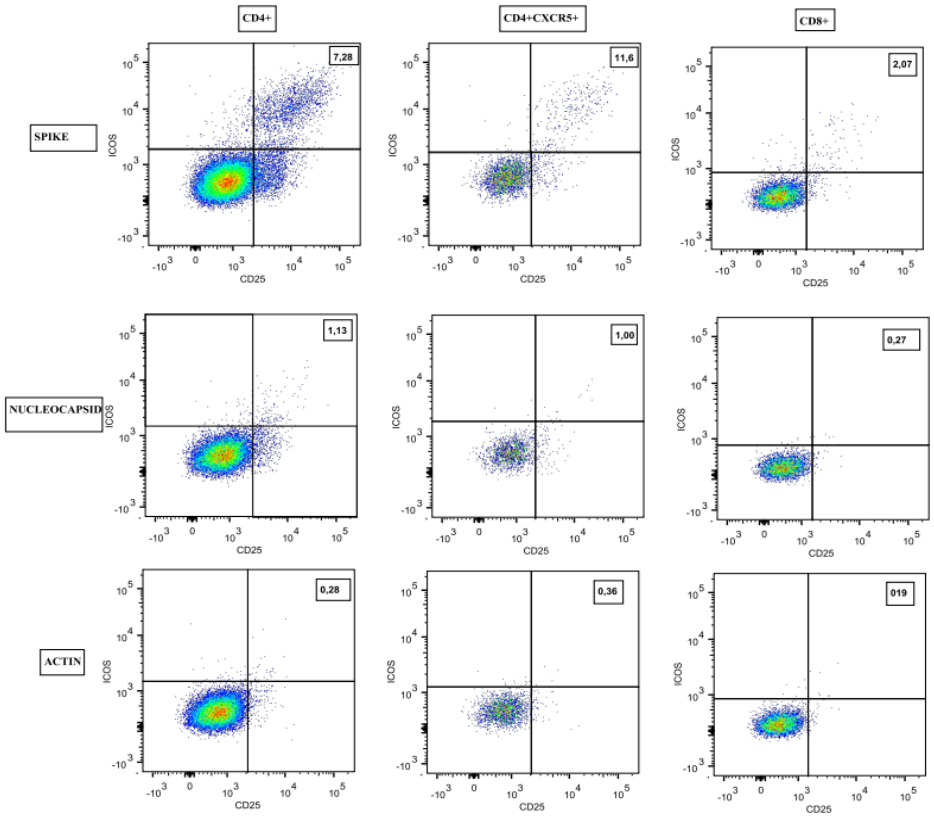

Figure 27. Example of T cell subsets detected after stimulation with appropriate antigens. Pseudocolor plots of CD4+, CD4+ CXCR5+ (TFH) and CD8+ T cells from a representative subject after stimulation with Spike, Nucleocapsid and Human Actin (negative control) protein overlapping peptide pools. Cells are gated on viable CD3+ lymphocytes. Activated antigen-specific cells in each subset were identified by ICOS and CD25 co-expression. Numbers in the right-top quadrant indicate the percentage of antigen-specific cells.

3.3.3. Assessment of humoral and cell mediated response against SARS-CoV-2 variants

Half-area 96-well microplates were coated for 1 h with 5µg/ml recombinant RBDs of the B.1 strain, B.1.1.7 strain, or the B.1.351/501Y.V2 strain. After overnight (or 1 h) blocking with 5% (wt/vol) skimmed milk, the plates were washed and incubated for 1 h with human serum four-fold serial dilutions (starting from 1:50), then for 45 min with horseradish peroxidase-labeled goat

IgG to human IgG and, finally, for 25 min with 30mg/ml orthophenylendiamine before the addition of 4 N sulphuric acid. The optical density (OD) value of the serum incubated without RBD was subtracted from the OD value of the serum incubated with RBD. Cut-off of 0.100 net OD was calculated based on mean+2SD results of SARS-CoV-2 seronegative subjects at the serum dilution 1:50. Serum dilution yielding 0.100 net OD value was considered as the RBD-binding serum titre. In parallel, variant SARS-CoV-2 strains, including B.1.1.7, B.1.351, P.1., B.1.617.2 strains were used for quantification of SARS-CoV-2 NT Abs as previously described. Finally, for the evaluation of cell-mediated response against the SARS-CoV-2 variants, supernatant of Vero E6 cells cultured in $25cm^2$ flasks and infected for 72 h with 100 TCID50/ml of different strains. In parallel, SARS-CoV-2 strains were UV-inactivated for 1 hour and used as antigen formulation. PBMC ($4x10^5$/100µl culture medium per well) were cultured in duplicate in the presence of inactivated SARS-CoV-2-infected (or mock-infected as negative control) cell supernatant diluted 1:50 in culture medium.

3.4. Immunogenicity of mRNA BNT162b2 vaccine in immunogenically fragile patients: analysis of a cohort of cancer patients treated with immune checkpoint inhibitors

3.4.1. Patients' enrolment and ethical approval

In collaboration with Oncology Unit of IRCCS Policlinico San Matteo, cancer patients who received a full course of vaccine during immunotherapy (anti-PD-1 or anti-PD-L1), in combination or not with chemotherapy were enrolled. The Inclusion Criteria were:

i) patients aged 18 and older, regardless of gender;
ii) life-expectancy (as estimated by treating physician) ≥ 6 months;
iii) confirmed histological diagnosis of solid tumors;
iv) treatment with immunotherapy alone or in combination with chemotherapy;
v) signing of informed consent;
vi) patients with a history of a previous PCR- or serologically-confirmed diagnosis of SARS-CoV-2 infection will be also enrolled. Patients with psychiatric illness/social situations that would limit compliance with study requirements were excluded from the study. All the clinical and demographic characteristics of the enrolled patients are enclosed in Table 3.

Table 3. Characteristics of 82 enrolled cancer patients

Sex	**no subjects**	**%**
Female	24	30
Male	58	70
Age (years)	**no subjects**	**%**
18-29	1	1
30-39	1	1
40-49	3	4
50-59	9	11
60-69	38	46
70-79	27	33
≥80	3	4
Comorbidities	**no subjects**	**%**
Cardiovascular	15	18
COPD/Asthma	10	12
Coronary heart disease	4	13
Diabetes mellitus	12	14
Autoimmune disorders	3	4
HCV	2	2
HBV	7	8
HIV	1	1
No comorbidities	41	50
One or more comorbidities	41	50
Previous SARS-CoV-2 exposure*	**no subjects**	**%**
Yes	12	15
No	70	85
Type of tumor	**no subjects**	**%**
Lung Cancer	64	78
Melanoma	7	9
Renal cancer	6	7
Head&Neck cancer	3	4
Bladder cancer	1	1
Squamous cell skin cancer	1	1
Type of oncological treatment	**no subjects**	**%**
ICIs+Chemotherapy	19	23
ICIs	61	75
ICIs+Targeted therapy	2	2
Type of ICIs	**no subjects**	**%**
Pembrolizumab	49	60
Nivolumab	19	23
Durvalumab	11	13
Atezolizumab	2	3
Cemiplimab	1	1

Legend.no subjects: number of subjects; %: percentage of subjects; COPD: chronic obstructive pulmonary disease; HCV: Hepatitis C virus HBV: Hepatitis B virus; HIV: Human Immunodeficiency virus; ICI: immuno checkpoint inhibitors; *documented by positive SARS-CoV-2 RNA in nasal swab and/or positive serology for Spike or Nucleocapsid.

The study was approved by the ethical committee of IRCCS Policlinico San Matteo (P-20210023530). Sampling was performed at time of vaccination (day 0, T0), at time of second dose administration (day 21, T1) and after 21 days after complete vaccination schedule (day 42, T2). For assessment of long-term immune response, sampling will be performed after six and twelve months. In this thesis, data of short-term immune response are showed (Lasagna *et al.,* 2021).

3.4.2. Assessment of humoral and cell-mediated response elicited by vaccination

SARS-CoV-2 NT Abs elicited by vaccination as well as cell-mediated immune response were analysed as previously described for healthcare workers. In order to allow simplest analysis only S peptide pool was used for stimulating PBMC in an *ex vivo* ELISpot assay.

3.4.3. Statistical analyses

Descriptive data were described as median and interquartile range (quantitative data) or frequency and percentage (qualitative data). In the first case, data were compared using Mann-Whitney test while Fishers' exact test was used for qualitative data. Geometric mean titer (GMT) and 95% confidence interval (CI) of geometric mean was used for analysis of NT Abs levels and comparison was made using Mann-Whitney test. Correlation between antigen-specific cell-mediated response and NT Abs was described using the Spearman test. For the analysis of paired data in vaccinated subjects over the three time points (T0, T1 and T2) Wilcoxon test was used. Categorical variables, including type and stage of tumour, type and timing of therapy were analyzed in a bivariate scheme for assessing the role of clinical variables in vaccine immunogenicity. All the tests were two-tailed and p value less than 0.05 were given as significant. All the analyses were performed using GraphPad 8.3.0 software (GraphPad, La Jolla CA, USA).

3.5. *Macrophages: role of innate immune system cells in SARS-CoV-2 infection*

3.5.1. Cell culture

Heparinized blood samples from blood donors were collected and PBMC were isolated as previously described. PBMC were plated at a concentration of $5x10^6$ into 24 well microplates (COSTAR, Corning Incorporated, NY 14831, USA) for 3h at 37 °C 5% CO_2 to let cells adhere. Non-adherent cells were removed and macrophages were differentiated using Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) 800 U/ml for two weeks. In parallel, macrophages were plated in 24 wells with a round glass coverslip for cell staining after virus infection. For monocytes assays, PBMC were maintained in polypropylene tubes (Greiner PP-TUBE-Steril, Greiner Bio-One International GmbH) to avoid adhesion. PBMC collected from SARS-CoV-2 positive subjects were plated in 24 wells with coverslip for staining after virus infection with the SARS-CoV-2 Chinese-derived D614, PV10734 D614G strains and B.1.1.7 variant. As controls, residual stored PBMC from five unexposed individuals collected before 2019 were used. Blood samples for the five COVID-19 positive patients were obtained after symptom onset at time of discharge from the hospital or at time of negative nasal swab. The study was approved by the Ethics Committee of IRCCS Policlinico San Matteo (P-20200041154 and P-20200029440). Additionally, five buffy-coat units from healthy blood donors were given by "Blood Bank" Processing and validation center DMTE/SIMT Fondazione IRCCS Policlinico San Matteo according to the Italian law decree of the Ministry of Health, November 2, 2015. "Provisions relating to the quality and safety requirements of blood and blood components".

3.5.2. Flow cytometry

The characterization of differentiated human macrophages was performed at Humanitas University of Rozzano via flow cytometry with the following mAbs: CD11b BV 786 (Biolegend, clone IRCF44), CD14 APC (BD Biosciences, clone M5E2), CD206 FITC (BD Biosciences, clone 19.2), HLA-DR BV711 APC (BD Biosciences, clone G46-6), CD16 PE (BD Biosciences, clone 3G8), CD4 BV 421 (BD Biosciences, clone RPA-T4),CD8FITC (BD Biosciences, clone RPA-T8), CD19 APC (BD Biosciences, cloneHIB19), and CD69PECy7 (Biolegend, clone G10F5). Live dead staining (ThermoFisher) was performed as control for vitality. Labelled cells were fixed in PBS-/- 1X 1% formalin. A minimum of 50.000 events were acquired for each sample using a BD Lyric (BD Biosciences) and analysed by FACS Diva 8.0.1 (BD Biosciences).

3.5.3. Direct infection of macrophages and monocytes

Differentiated macrophages grown in 24 well tissue culture plates were infected with the three SARS-CoV-2 strains at 0.05 multiplicity of infection (MOI), 100 µl for 2 h at 33 C°5% CO_2. After removal of virus inoculum, cells were washed and maintained in serum free EMEM with addition of 1% penicillin, streptomycin, glutamine and 5γ/mL of trypsin. After 48h macrophages grown on glass slides were fixed in methanol/acetone 2:1 for 5 minutes and stained with SARS-CoV-2 (2019-Cov) Nucleoprotein/NP antibody, Rabbit Mab (Sino Biological, 1400 Liberty Ridge Drive, Wayne, PA 19087) followed by a donkey anti-rabbit Alexa Fluor 488 (ThermoFisher, Waltham, Massachusetts, US). To study the different stages of macrophage infection with D614 Chinese-derived, D614G PV10734 and alpha strains after 1, 4, 8, 24, 72, and 96h, cells grown on glass slides were fixed and stained as previously reported. At 96 h macrophages were also stained with SARS-CoV-2 (2019-Cov) anti-S antibody, Rabbit Mab (Sino Biological, 1400 Liberty Ridge Drive, Wayne, PA 19087) followed by the same donkey anti-rabbit. Nuclei were stained with DAPI (Sigma-Aldrich, St. Louis, Missouri, United States) and slides were examined at 40X with Nikon's C1 Digital Eclipse Modular Confocal Microscope System (software Ez-C1, vers 3.70 Nikon Instruments Inc. Melville, NY, U.S.A) for the expression of N and S protein. In parallel, supernatants obtained at the same time points were titrated in VERO E6 cells to detect virus replication. As control, VERO E6 were infected in parallel with the same MOI of the three viral strains and supernatants were titrated. The same experiment was performed also with monocytes that were kept in polypropylene tubes for all the time-course.

3.5.4. Macrophages infection through co-culture with infected VERO E6

Macrophages and monocytes were co-cultured with VERO E6 infected with the three viral strains. The unequivocal demonstration that macrophages and monocytes co-cultured with SARS-CoV-2 infected cells did not only express N antigens, but could also transmit the infectious virus, required removal of all SARS-CoV-2 infected cells present in the cells co-culture. This was achieved by taking advantage of the capacity of macrophages and monocytes to migrate through natural and artificial barriers in response to chemotactic peptide N-formylmethionyl-leucyl-phenylalanine (FMLP). Control experiments showed that VERO E6 infected or mock-infected were not able to migrate in response to FMLP. The experiment was conducted as follows: VERO E6 cells were cultivated in 24 well microplates and infected with the three strains of SARS-CoV-2 at 100 TCID50 for 1h at 33 C°5% CO_2. The inoculum was removed and serum-free EMEM with the addition of 1% penicillin, streptomycin, glutamine and 5γ/mL of trypsin was added. After 48h

$5x10^6$ macrophages or monocytes were co-cultured overnight. Then, macrophages or monocytes were removed, washed and migrated through a 5µm transwell filter (Costar –Corning Incorporated, NY 14831, USA) in the presence of 10-8FMLP chemoattractive. Migrated cells were washed abundantly, counted and $2x10^5$ cytospin slides were prepared. The same amount of migrated cells were also inoculated into VERO E6 cells for virus isolation through the development of cytopathic effect (CPE). Supernatants from the last washing were inoculated on VERO E6 as control (Figure 28).

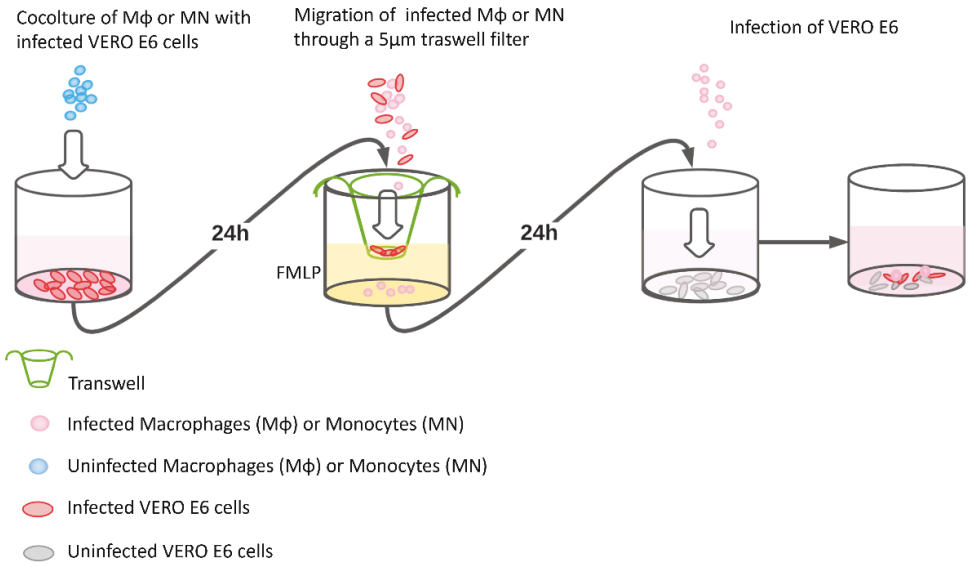

Figure 28. Graphical representation of the macrophages/monocytes infection through co-culture with infected VERO E6 and virus transmission to uninfected VERO E6 from infected macrophages/monocytes after migration (Percivalle et al., 2021).

3.5.5. Virus transmission assay

To demonstrate virus transmission from infected macrophages to VERO E6 during coculture, two vital fluorescent dyes were used: 5-chloromethylfluorescein diacetate (CMFDA, Invitrogen) and 4-({[4-(chloromethyl)phenyl]carbonyl}amino)-2-(1,2,2,4,8,10,10,11-octamethyl-10,11-dihydro-2H-pyrano[3,2-g:5,6-g']diquinolin-1-ium-6-yl)benzoate (CMTPX, Invitrogen). Each dye was used as a 5 mM stock solution in dimethylsulfoxide (Sigma). Infected and uninfected macrophages were stained with 1 mM CMTPX (Cell Tracker Red), whereas VERO E6 were stained with 10 mM CMFDA (Cell Tracker Green) according to a reported procedure (Jaroszeski *et al.,* 1994). Microscopic observation of macrophages

and their dual fluorescing fusion products was achieved by using a fluorescent microscope (model DM RBE; Leica, Wetzlar, Germany) with filter G/R 513803 (Leitz) designed for simultaneous excitation by 490 +/- 20 and 575 +/- 30 nm fluorescent light.

3.5.6. Kinetic of N protein uptake and RNA replication on macrophages and VERO E6 permissive cell line

The kinetics of nucleoprotein expression in macrophages from the previous experiments was evaluated with a confocal microscope after 1, 4, 8, 24, 48, 72 and 96h. In parallel, infected macrophages at the same time-course were detached, washed, and frozen for RNA quantification in real-time RT-PCR according to our protocol (Corman *et al.,* 2020). Also, VERO E6 cells were infected with the same amount of virus and at the same time trypsinized, washed and frozen to be tested. To compare the amount of RNA replication in macrophages and VERO E6, the ß2 globulin was quantified to calculate the number of cells tested. Supernatants from each time point of both macrophages and VERO E6 were tested in SARS-CoV-2 real-time RT-PCR.

3.5.7. Mechanism of virus entry into macrophages

To study the mechanism of virus entry into macrophages, the virus was treated with a pool of human post-COVID 19 sera with a SARS-CoV-2 neutralizing titer higher than 1:640 and with a bi-specific human monoclonal antibody anti-S protein (De Gasparo *et al.,* 2021) for 1 h at 33 °C. The blocked virus was then inoculated into differentiated macrophages and in VERO E6. After 24h macrophages and VERO E6 were stained for the expression of N protein to verify the entry of the virus.

3.5.8. Light microscopy and light microscopy immunochemistry

For light microscopy, cell pellets were fixed in 10% buffered formalin solution, dehydrated, and embedded in paraffin. Serial sections, 3-mm-thick, were cut and stained with hematoxylin-eosin; unstained sections were used for immunohistochemistry. Sections were de-paraffinized and brought to TRIS phosphate-buffered saline solution (TBS O,l5M, pH 7.35). After blocking endogenous peroxidase with 3% H_2O_2, and pretreatment with trypsin 0,05 % the slides were incubated overnight with anti-S and anti-N antibodies (Table 4). The immunohistochemical staining was performed with the peroxidase-antiperoxidase method and diaminobenzidine was used as chromogen substrate. Specificity tests were performed using appropriate negative controls. All the experiments were carried out at the Transplant Research

Area and Center for Inherited Cardiovascular Diseases of IRCCS Policlinico San Matteo.

Table 4. Antibodies used in the study

Antibody	Dilution	Pre-treatment	Secondary	Code	Specificity
SARS-CoV-2 Spike S1	1:200	2 cycles MW (900 W) + 3 cycles MW (720 W), pH 9.9	DR	Sino Biological 40150-R007	2019-nCoV Spike
SARS-CoV-2 Nucleocapsid	1:2500	2 cycles MW (900 W) +3 cycles MW (720 W), pH 9.9	DR	Sino Biological 40143-R019	2019-nCoV Nucleocapsid
CD68 (PGM1)	1:100	15' Trypsin	DM	Dako M0876	Subcellular fraction of human Mø.
CD68 (KP1)	1:100	15' Trypsin	DM	Santa Cruz SC-20060	Subcellular fraction of human Mø.
CD163	1:200	no	DM	ThermoFisher Scientific MA5-11458	Human MN and Mø. Circulating MN and most tissue Mø.

Legend. DR: donkey-rabbit; DM: donkey-mouse; MN: monocytes; Mø: macrophages.

3.5.9. Ultrastructural study

For electron microscopy, samples were fixed with Karnovsky's solution in cacodylate buffer 0,2 M (pH 7.3) for 4 hours at 4°C; then postfixed with 1% osmium tetroxide in cacodylate buffer 0,2 M (pH 7.3) for 1 hour at RT, dehydrated in ethanol and propylene oxide and embedded in epon-araldite resin. Ultrathin sections were stained with uranyl acetate and Reynolds's lead citrate and observed with a JEOL JEM 1011 electron microscope. Pre-embedding immunogold staining was performed in infected VERO E6 cells at 72 hours from inoculation to validate the specificity of the commercial antibodies. The pellet containing VERO E6 cells in PBS solution was subjected to three washing cycles in Tris buffer (10 min each), immersed in 0,05% TritonX for 20 min, washed in Tris buffer, threatened in 10% Normal Goat Serum (30 min), and incubated with the primary antibody (1:50 for 92 h at 4°C). After five washing cycles in Tris buffer (10 min each), the pellet was incubated with the secondary antibody conjugated to 15-nm gold particles (72

h, 4°C) (BBI Solutions), washed in Tris buffer, fixed in Karnovsky's solution (15 min, 4°C), post-fixed in 1% osmium tetroxide (1 h, room temperature), washed in Tris buffer, and dehydrated in a graded series of ethyl alcohols. Finally, the specimen was embedded in Epon-Araldite overnight at 60°C. Ultra-thin sections were stained with uranyl acetate 5% and Reynold's solution, and observed using a JEOL JEM 1011 electron microscope. All the microscopy experiments were carried out at the Transplant Research Area and Center for Inherited Cardiovascular Diseases of IRCCS Policlinico San Matteo.

4. Results

4.1. *Assessment of microneutralization assay for seroepidemiological studies and evaluation of SARS-CoV-2 monoclonal antibodies*

4.1.1. Sensitivity and specificity of SARS-CoV-2 neutralizing antibody assay

A new microneutralization assay using SARS-CoV-2 reference strain (PV10734 D614G) was assessed in our laboratory. Sensitivity and specificity were determined using 40 sera of COVID-19 positive patients and 30 sera collected before the pandemic period, respectively. We observed that none of the sera collected in the pre-pandemic period was positive for SARS-CoV-2 NT Abs, including sera of subjects with proven HCoVs infection, since all of them showed NT Abs level lower than 1:10. Thus, a specificity of 100% was demonstrated. On the other side, 38/40 (95%) COVID-19 positive patients tested positive for SARS-CoV-2 NT Abs, since titres were higher than 1:10. On the basis of these data, the sensitivity of our SARS-CoV-2 NT Abs assay was 95%. Levels of response in convalescent subjects are shown in Figure 29.

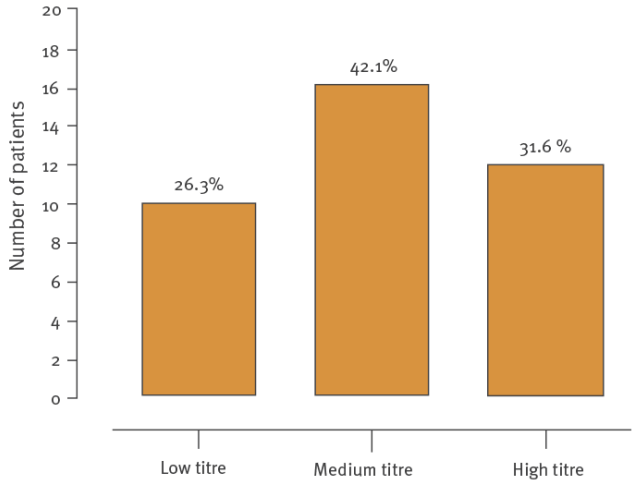

Figure 29. NT Abs levels in 38 convalescent subjects, classified as low titre (1:10-1:40), medium titer (1:40-1:160) and high titer (>1:160)

4.1.2. Seroprevalence of SARS-CoV-2 in Lodi Red Area between 18 March and 6 April 2020

Overall, 390 blood donors (BDs) enrolled between 18 March and 6 April, 2020 represented 17% of the 2,272 registered blood donors residing in the Lodi Red Zone. The geographical distribution as well as the number of subjects tested positive for SARS-CoV-2 NT Abs is shown in Figure 30.

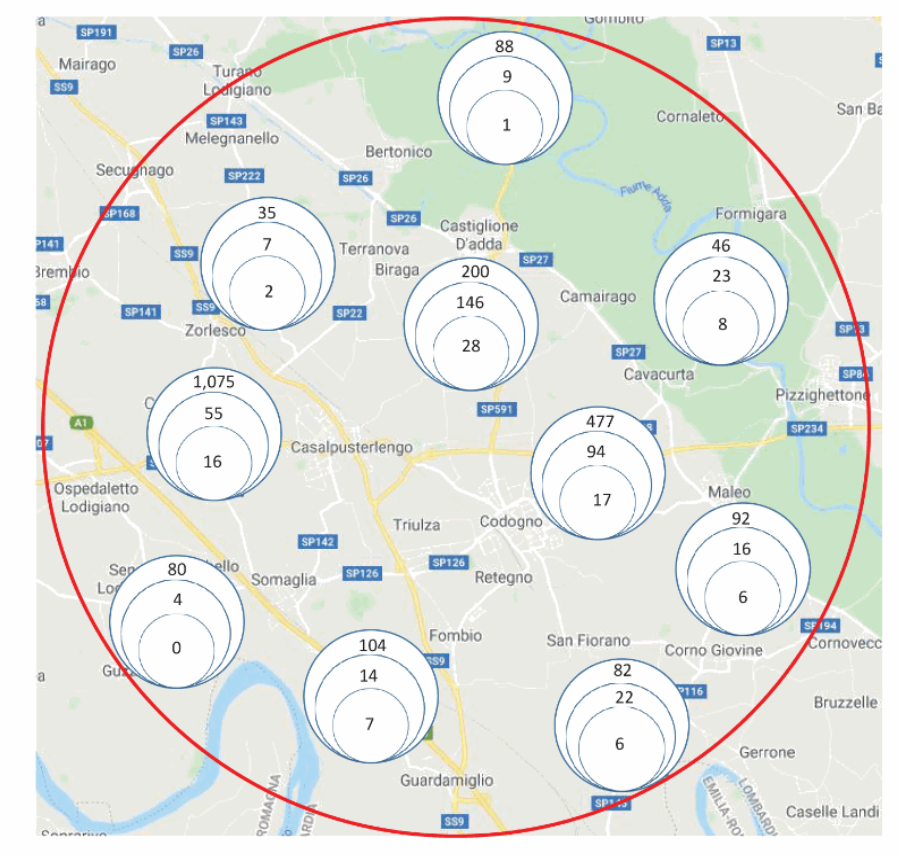

Figure 30. The numbers of BDs in each municipality are shown in the outer blue circles. The distribution per municipality of the total 390 BDs recruited for the study and tested for NT-Abs and by nasal swab real-time RT-PCR is reported via the middle circles, while the internal circles indicate the numbers of NT-Ab positive BDs per municipality among the total 91 BDs found positive for NT-Abs. The red circle indicates the limits of the Lodi Red Zone (Percivalle *et al.,* 2020)

Overall, 370/390 blood donors (95%) were negative by SARS-CoV-2 real-time RT-PCR, and only 20/390 tested positive (5%). On the other side, looking at SARS-CoV-2 NT-Abs, 91/390 (23%) blood donors were positive for SARS-CoV-2 NT-Abs while 299/390 (77%) tested negative. Only three out of 91 (3%) SARS-CoV-2 NT-Abs positive blood donors were also positive for RT-PCR (Table 5). Of note most donors were asymptomatic and only 8/390 (2%) reported mild symptoms during the 30 days before the enrolment.

Table 5. SARS-CoV-2 real-time RT-PCR and NT-Abs in 390 blood donors

	SARS-CoV-2 NT Abs positive	SARS-CoV-2 NT Abs negative	Total
SARS-CoV-2 RT PCR positive	3	17	20
SARS-CoV-2 RT PCR negative	88	282	370
Total	91	299	390

Legend. SARS-CoV-2: severe acute respiratory syndrome virus 2; NT Abs: neutralising antibodies; RT PCR: retrotranscription polymerase chain reaction

4.1.3. SARS-CoV-2 NT Abs titre is related to SARS-CoV-2 severity

We classified the 38 COVID-19 positive patients and 91 SARS-CoV-2 NT Abs positive blood donors according to SARS-CoV-2 NT Abs level, defining low, medium and high NT Abs level when NT Abs were between 1:10 and 1:40, between 1:40 and 1:160 and higher than 1:160, respectively. Of note, SARS-CoV-2 NT Abs detected in COVID-19 patients were higher than that detected in blood donors. Indeed, as shown in Table 6, the large majority of COVID-19 positive patients showed a medium-higher NT-Abs titre (74%) while only 37% of blood donors reported medium-high level of SARS-CoV-2 NT Abs ($p=0.0007$ by Fishers' exact test).

Table 6. NT Abs level in 38 COVID-19 positive patients and 91 blood donors

	Low titer (1:10-1:40)	Medium titer (1:40-1:160)	High titer (>1:160)
COVID-19 pts (N=38)	10 (26%)	16 (42%)	12 (32%)
Blood donors (N=91)	57 (63%)	29 (32%)	5 (5%)

4.1.4. SARS-CoV-2 NT Abs positive sera collected before the "index case" diagnosis suggest a previous SARS-CoV-2 circulation in Lodi Red Zone

We found that 5 out of 300 (2%) stored sera collected from blood donors before February 20, 2020 were positive for SARS-CoV-2 NT-Abs, thus suggesting a previous overlooked SARS-CoV-2 circulation before the diagnosis of the first COVID-19 case, the so called "index case". Of note, all these 5 positive samples had been collected between 12 and 17 February, 2020.

4.1.5. Efficacy of monoclonal antibodies against SARS-CoV-2 variants

Our microneutralization assay was used for assessment of monoclonal antibodies designed against SARS-CoV-2 infection. All the monoclonal antibodies were provided by Institute for Research in Biomedicine, Università della Svizzera italiana (USI), Bellinzona, Switzerland and all the relative data have been published (De Gasparo *et al.,* 2021). All the six mononclonal antibodies (121, 144, 135-144, 135, 121-135, 121-135-144) were analyzed against D614G PV10734 strain and D614 Chinese-derived strain (Figure 31), observing an IC50 ranging from 0.10 to 0.47 µg/mL and 0.16 to 1.41 µg/m, respectively. Overall, a better efficacy was observed against D614G PV10734 strain for all the monoclonal antibodies. A Table with IC50 for each virus was provided (Table 7).

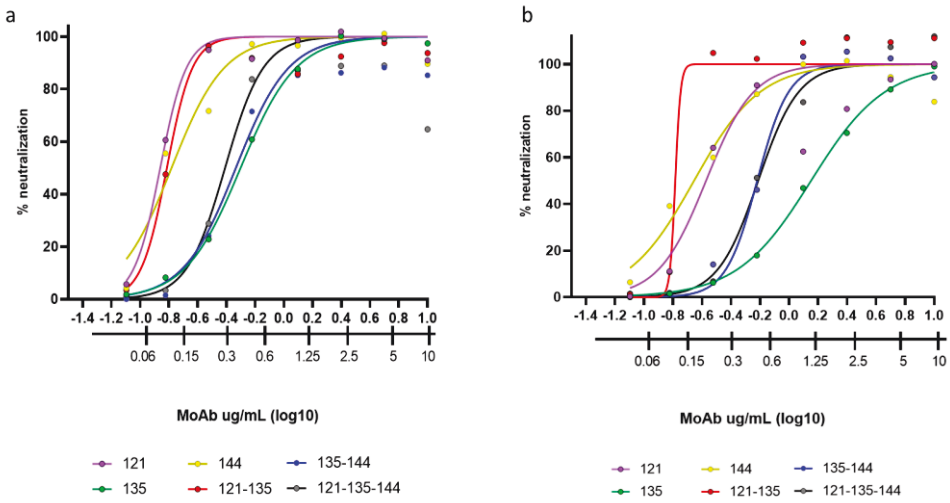

Figure 31. Percentages of neutralization for six monoclonal antibodies at different dilutions were given against D614G PV10734 strain (a) and D614 Chinese-derived strain (b).

Table 7. IC50% of six monoclonal antibodies against two viral strains

Monoclonal antibody	D614G PV10734 strain	D614 Chinese-derived
121	0.10	0.27
135	0.42	1.41
144	0.17	0.22
121-135	0.11	0.16
135-144	0.47	0.60
121-135-144	0.16	0.61

IC50: 50% inhibition concentration; all the results are given as µg/mL.

4.2. Humoral and cell-mediated immune response to SARS-CoV-2 natural infection

4.2.1. Characteristics of enrolled cohort of COVID-19 positive subjects

Mononuclear cells derived from 87 convalescent subjects (median age 47 years; 43 males and 44 females) with RT-PCR proven SARS-CoV-2 infection were retrospectively analysed (Table 2). Sixty-four out of 87 (73.6%) were mostly mild symptomatic, showing fever, cough, asthenia; 23/87 (26.4%) were hospitalized for moderate or severe SARS-CoV-2 infection and ten of them (43.5%) required assisted ventilation. As controls, stored residual samples obtained from 33 healthy subjects (median age 40 years, range 26–62; 12 males and 21 females) collected by August 2019 were used.

4.2.2. Pre-existing SARS-CoV-2 T-cell response in healthy unexposed donors

PBMCs from 33 SARS-CoV-2 unexposed donors collected in the pre-pandemic period were challenged against SARS-CoV-2 specific peptide pools and whole viral lysate. Overall, median T-cell response was 2.0 (IQR 0.0- 6.5) IFN-γ SFU/10^6 PBMCs and 1.0 (IQR 0.0-10) IFN-γ SFU/10^6 PBMC for S- and N-peptide pools, respectively. Moreover, we detected a median of 1.0 (IQR 0.0- 5.0) IFN-γ SFU/10^6 PBMC for Membrane and of 1.0 (IQR 0.0-5.0) IFN-γ SFU/10^6 PBMC for NS8 peptide pools. Additionally, median T-cell response was 1.0 (IQR 0.0- 6.0) IFN-γ SFU/10^6 PBMC and 0.0 (IQR 0.0-6.0) IFN-γ SFU/10^6 PBMC for NS7B while the median T-cell response to the whole lysate was almost undetectable (median 0.0 IQR 0.0- 4.0 IFN-γ SFU/10^6 PBMCs). Fourteen out of 33 (42.4%) unexposed donors showed a positive T-cell response for at least one SARS-CoV-2 peptide pool (Figure 32). Of them, 4/14 (28.6%) experienced a proven HCoV infection in the past, while no information was available for the other ten subjects.

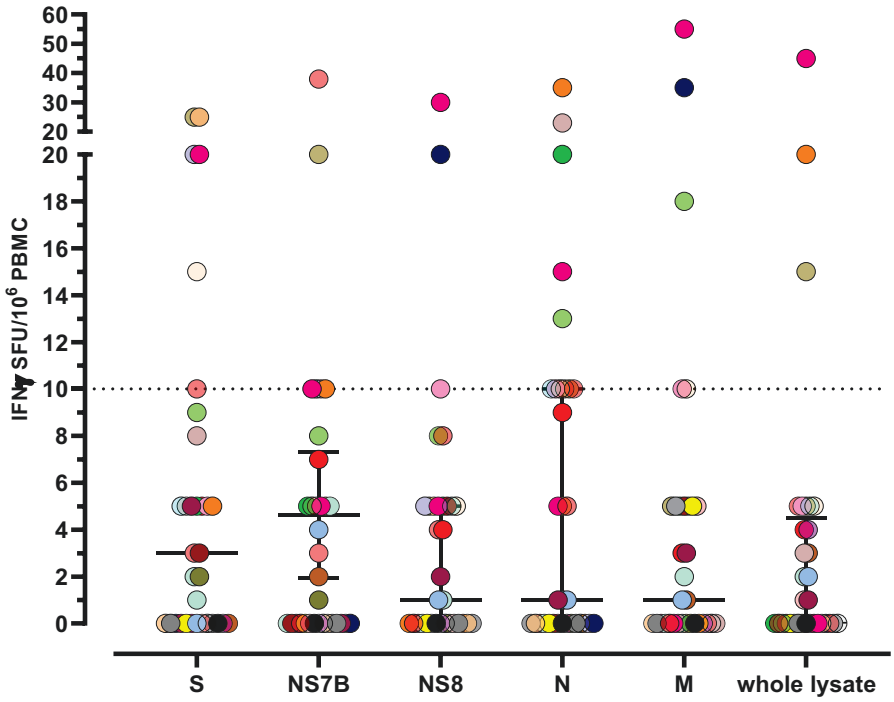

Figure 32. SARS-CoV-2 T-cell response in unexposed subjects. Each colour represents a single subject. S: spike; M: membrane; N: nucleoprotein. The horizontal dotted line indicate the cut-off of positive response

4.2.3. SARS-CoV-2 T-cell response was detectable in almost all SARS-CoV-2 exposed subjects regardless the symptoms severity

In the large majority of exposed donors (85/87; 97.7%), a positive T-cell response against at least one SARS-CoV-2 antigen was detected. Briefly, the large majority of patients showed a positive T-cell response against S-peptide pool (82/87; 94.3%) with a median response of 65 IFNγ SFU/10^6 PBMC, IQR 30-150. Seventy-four out of 87 (85.1%) patients developed a positive response for Membrane-specific peptide pool (median 40 IQR 15-160 IFNγ SFU/10^6 PBMC); the same number of subjects showed a positive response against N-protein (median 40 IQR 12-127 IFNγ SFU/10^6 PBMC). In 65/87 (74.7%) subjects a positive T-cell response against the whole lysate was detected with a median T-cell response of 20 IFNγ SFU/10^6 PBMC (IQR 5-65 IFNγ SFU/10^6 PBMC). Finally, 29/87 (33.3%) and 40/87 (46%) subjects showed a positive T-cell response against NS7B (median 5, IQR 0-25 IFNγ SFU/10^6 PBMC) and NS8 (median 5, IQR 0-25 IFNγ SFU/10^6 PBMC) peptide

pools was observed (Figure 33). The difference in terms of SARS-CoV-2 specific T-cell response between mild and severe COVID-19 cases was not statistically significant, except for NS8 peptide pool (median T-cell response 10 IQR 0-30 IFNγ SFU/10^6 PBMC vs 0 IQR 0-10 IFNγ SFU/10^6 PBMC, respectively; p=0.0075).

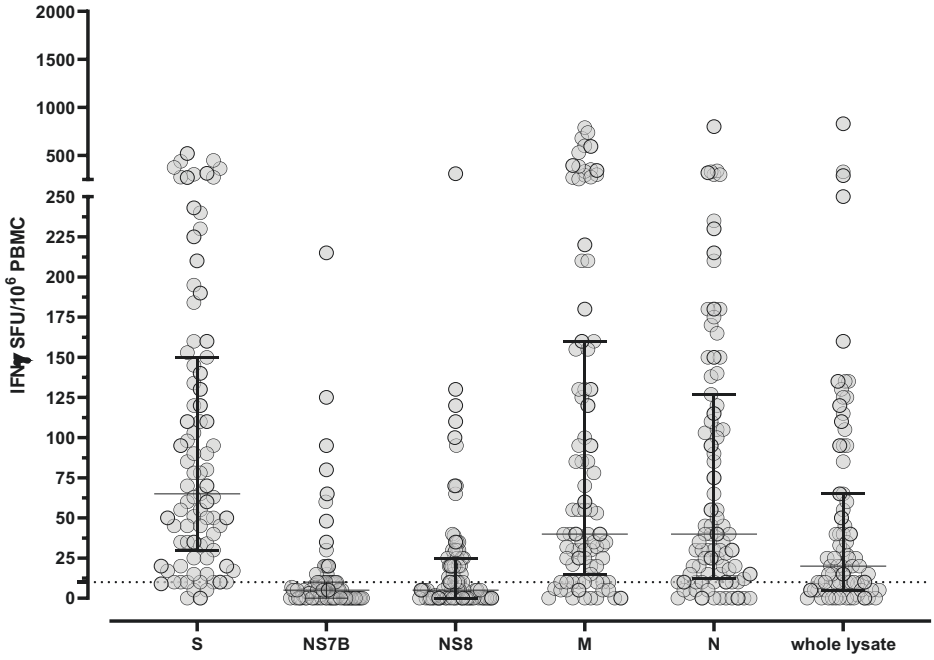

Figure 33. SARS-CoV-2 T-cell response in exposed subjects. S: spike; M: membrane; N: nucleoprotein. The horizontal dotted line indicate the cut-off of positive response

4.2.4. SARS-CoV-2 T-cell response was mainly mediated by CD4+ T cells

The overall SARS-CoV-2-specific T-cell response dramatically decreased by about 95% after CD4+ T-cell depletion. Additionally, N, M and whole lysate-specific T-cell response were almost undetectable after CD4+ T-cell depletion, while the S-specific T-cell response was reduced to 7.4%. Otherwise, the overall SARS-CoV-2 T-cell response was reduced of approximatively 20% after CD8+ T-cell depletion, suggesting that SARS-CoV-2 T-cell response was mainly mediated by the T helper response. Unfortunately, due to the low number of cells and to the retrospective nature of the work, we were able to perform pre-depletion assay in only eleven COVID-19 positive subjects with sustained SARS-CoV-2 specific T-cell

response, thus differences between mild and severe COVID-19 patients were not appreciable.

4.2.5. S-specific T-cell response weakly correlates with SARS-CoV-2 NT Abs level

SARS-CoV-2 NT Abs were measured in 79/87 (90.8%) SARS-CoV-2 exposed subjects. Despite the absence of strong correlation between antigen-specific T-cell response and SARS-CoV-2 NT Abs titre, a weak positive correlation between SARS-CoV-2 NT Abs titre and S-specific T-cell response was observed, as well as between SARS-CoV-2 NT Abs and whole lysate-specific T-cell response, as shown in Figure 31. Of note, in 8 out of 9 patients (88.9%) with negative SARS-CoV-2 NT Abs titre a sustained SARS-CoV-2 specific T-cell response was observed. No correlation was observed between SARS-CoV-2 specific T-cell response and days after symptoms' onset, since r calculated with Spearman's test ranged between 0.0464 and 0.2052; p values were always not significant (Figure 34).

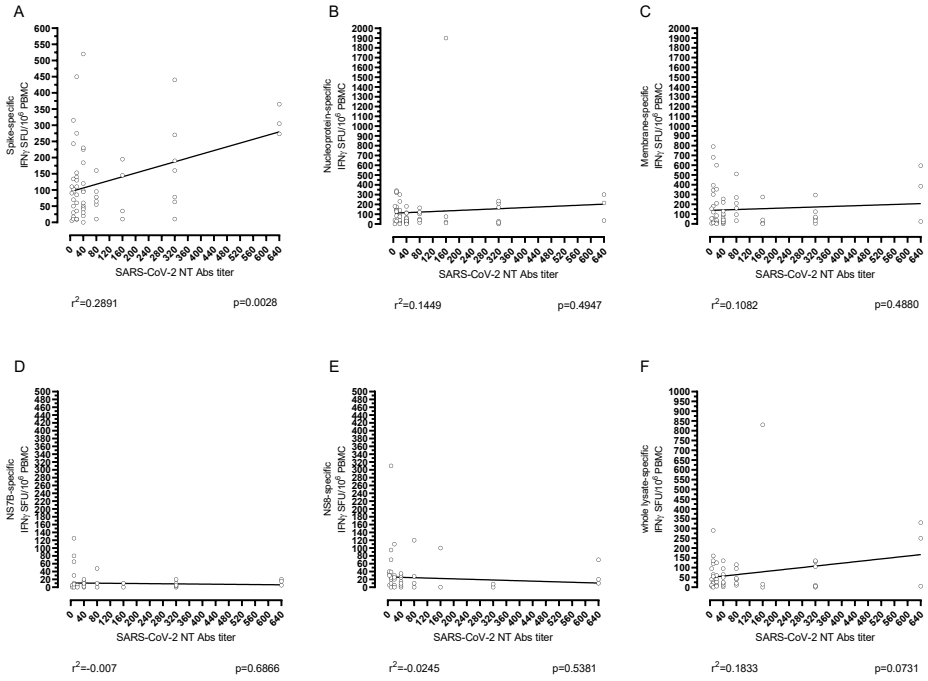

Figure 34. Correlation between Spike (A), Nucleocapsid (B), Membrane (C), NS7B (D), NS8 (E) and whole lysate (F) against SARS-CoV-2 NT Abs was shown

4.2.6. SARS-CoV-2-specific T-cell response persisted for almost one year

Persistence of SARS-CoV-2 specific T-cell response at median 56 days after symptom onset (range 20-84 days; T1) and at 246 days after symptom onset (range 118-362 days; T2) was evaluated in ten subjects (Figure 35). Median T-cell response for S-protein was stable and maintained at T1 and T2 since medians were 62.5 (IQR 34.8-110.8) and 65.0 (IQR 37.5-95.5) IFN-γ SFU/10^6 PBMCs (p 0.6074); the M-protein and N-protein median T-cell response at T1 were 65.0 (IQR 10- 216.3) and 44.0 (IQR 12.5-103.5) IFN-γ SFU/10^6 PBMCs and declined to 25.5 (IQR 5-80) and to 26.5 (IQR 5-70.0) IFN-γ SFU/10^6 PBMCs, respectively; however, the differences were not statistically significant (p=0.6875 and p=0.1602, respectively). At T1, NS7B and NS8 median T-cell responses were 7.5 IQR (0-12.5) and 5.0 (IQR 0-10.7) IFN-γ SFU/10^6 PBMCs, respectively, while at T2 median T-cell responses were 5.0 (IQR 0-3.5) and 5 (IQR 0-10) IFN-γ SFU/10^6 PBMCs and no statistical difference was reported (p=0.0625 and p=0.6875, respectively). Finally, a significant difference between T1 and T2 was observed only for whole lysate T-cell response, since the median level of response decreased from 29 (IQR 12-133.8) to 10 (10-27.5) IFN-γ SFU/10^6 PBMC (p 0.0313). Of note, for the latter antigen only eight paired samples were analysed, due to low amount of cells available.

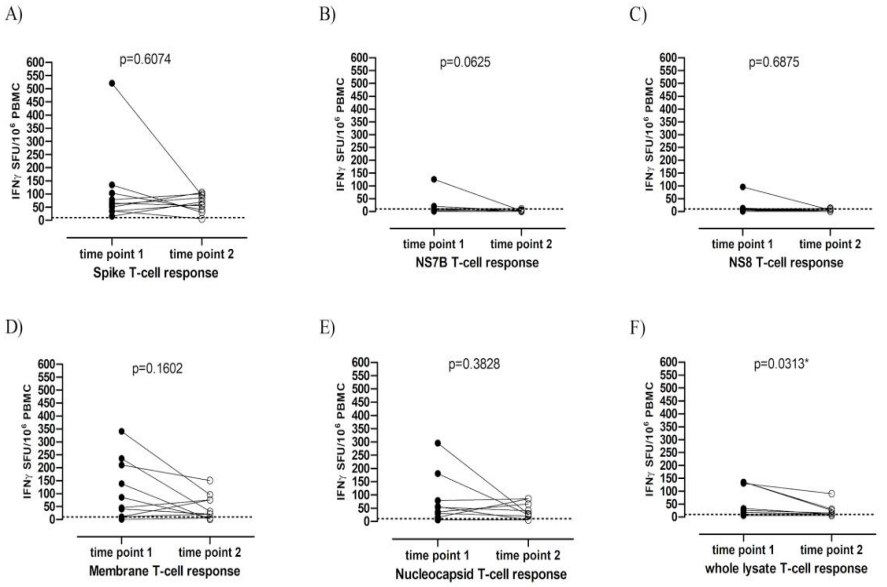

Figure 35. Longitudinal analysis of SARS-CoV-2 specific T-cell response in COVID-19 subjects analysed for each antigen. Time point 1 is the time of enrolment, time point 2 is the time of follow up. P value was indicated for each comparison

4.3. mRNA BNT162b2 vaccine elicits a sustained immune response against VOC in healthcare workers

4.3.1. Demographic characteristic of enrolled healthcare workers vaccinated with BNT162b2 mRNA vaccine

The immune response to the mRNA vaccine BNT162b2 was evaluated in 145 healthy healthcare workers receiving vaccination between December 27, 2020 and February 11, 2021. Among the subjects enrolled in the study, 127 (87.6%) were SARS-CoV-2 naïve (30 males and 97 females) and 18 (12.4%) SARS-CoV-2 experienced (1 male and 17 females) before vaccination. The median age was 44 years (range 21-69).

4.3.2. Humoral response elicited by vaccination

At T1 (21 days after the first dose), in all but three SARS-CoV2 experienced subjects, SARS-CoV-2 NT Abs at the upper limit of the assay was reported. However, all the subjects showed an increase to the upper limit of the assay after the second dose, without a significant increase of GMT titre (T2).

On the other hand, GMT of SARS-CoV-2 NT Abs in SARS-CoV-2 naive healthcare was tenfold lower than respect to SARS-CoV-2 experienced HCW (36 95%CI 29-47 vs 458 95%CI 301-719) at T1. When looking at T2, GMT SARS-CoV-2 NT Abs was 306 (95%CI 267-355) vs 640 (95%CI 640-640), respectively (Figure 36).

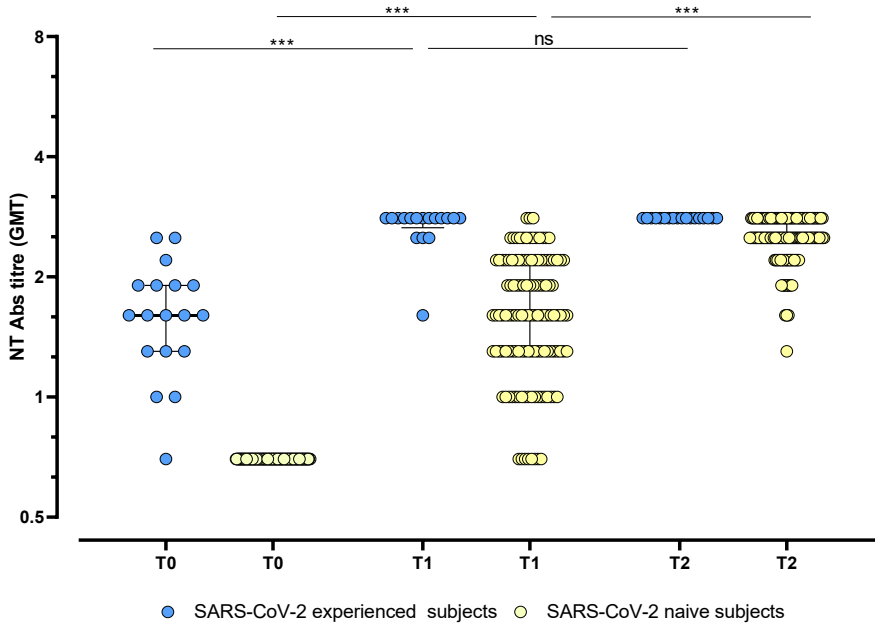

Figure 36. SARS-CoV-2 NT Abs elicited by vaccination in SARS-CoV-2 seropositive subjects (previously COVID-19 experienced; light blue dots) and SARS-CoV-2 seronegative subjects. Horizontal dotted line represents the cut off level of positive response. GMT: geometric mean titre

4.3.3. Cell-mediated response elicited by vaccination in healthcare workers

ELISpot assay was used for detecting S-specific and, as control, N-specific cell-mediated response in vaccinated subjects at baseline, at T1 (21 days after the first dose) and T2 (21 days after the second dose). All but one SARS-CoV-2 experienced subjects (17/18, 94.4%) showed S-specific T cells at baseline (median 27.50 IFNγ SFU/million PBMC; IQR 13.8-52.5), and levels of S-specific T cells increased at T1 (median 80.0 IFNγ SFU/million PBMC; IQR 47.5-315; p<0.001) and did not further increase at T2 (median 172.5 IFNγ SFU/million PBMC; IQR 85-255; p=0.510). Focusing on SARS-CoV-2 naive subjects, the percentage of responders was 69.3% at T1 (median level 20 IFNγ SFU/million PBMC; IQR 5-57.50) and 98.4% at T2 (median level 110 IFNγ SFU/million PBMC; IQR 60-192.5). Levels of S-specific T cells

significantly increased from T1 to T2 ($p<0.001$); even if the level of S-specific T-cells at T2 in experienced subjects was higher than that observed in naïve subjects at the same time point, the difference was not statistically significant ($p=0.095$; Figure 37A). No significant correlation was observed between age and S-specific T-cell response at both T1 ($r=-0.02$ IC95% -0.21-0.17; $p=0.8214$) and T2 ($r=-0.17$; IC95% -0.32-0.04; $p=0.107$). Levels of N-specific T cells did not change with time in both experienced and naïve subjects (Figure 37B). Of note, 30% of SARS-CoV-2 naïve subjects showed detectable SARS-CoV-2 specific T-cell response against S and 18.2% against N at baseline.

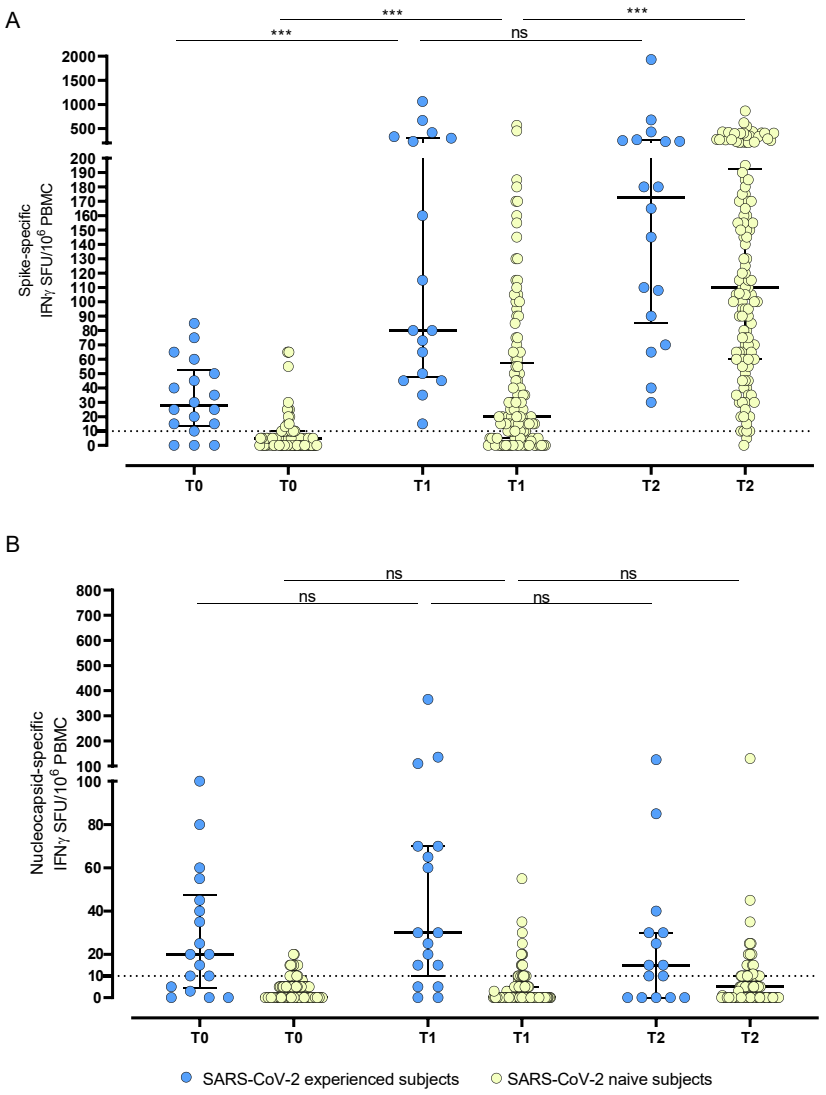

Figure 37. Spike-specific T-cell response (A) and Nucleoprotein (B) evaluated by ELISpot assay in SARS-CoV-2 seropositive subjects (previously COVID-19 experienced; light blue dots) and SARS-CoV-2 seronegative subjects. Horizontal dotted line represents the cut off level of positive response

The phenotype of S-specific T cells elicited by the vaccine was analysed in a subgroup of 20 naïve subjects at T2 by flow-cytometry analysis of T-cells proliferating in response to S and N peptide pools and co-expressing CD25 and ICOS activation markers. The vaccine elicited CD4+ T-cell (median 7.0%, IQR 2.1-8.2% CD25+ICOS+ cells) and, particularly (p=0.005), CD4+ TFH-cell response (median 7.8%, IQR 6.2-12.4% CD25+ICOS+ cells). In addition, also S-specific CD8+ T cells (median 2.6%, IQR 1.4-6.1% CD25+ICOS+ cells) were detected in vaccinated subjects. This pattern is similar to that observed in 9 SARS-CoV-2-infected subjects with mild symptoms collected a median time of 38 (range 28-90) days after infection, who showed a slightly lower number of proliferating CD4+ T and CD4+ TFH cells than vaccinated subjects (p=0.044 and 0.047, respectively). As observed also with the ELISpot assay, the response to N protein was detected in few vaccinated subjects. Overall, after the first dose, the overall percentage of full responders (i.e. subjects developing both T cells specific for SARS-CoV2 S protein and NT antibody) was 77.8% (95% CI: 70.1-84.0), corresponding to 100% of experienced and 68.8% (95% CI: 60.2-76.3) of naïve subjects. After the complete vaccination schedule, the overall percentage of full responders was 98.6% (95% CI: 95.0-99.8), corresponding to 100% of experienced and 98.4% (95% CI: 94.4-99.7) of naïve subjects (Figure 38).

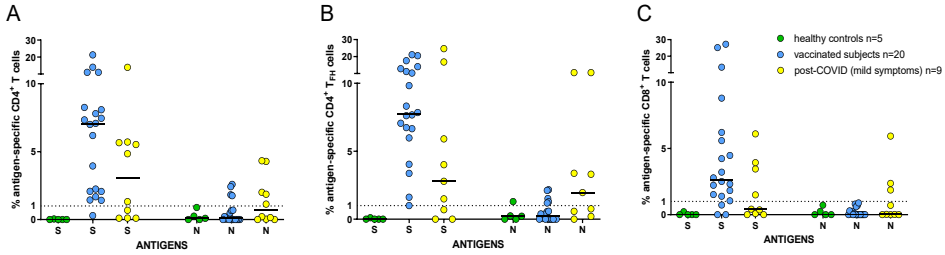

Figure 38. Percentage of Spike-specific Nucleoprotein-specific CD4 T cells (A), CD4 THF cells (B) and CD8 cells (C) were evaluated in healthy controls (green dots), vaccinated subjects (blue dots) and subjects recovered from mild COVID-19 (yellow dots). Horizontal dotted line represent the cut off level of positive response

4.4. mRNA BNT162b2 vaccine immunogenicity in a cohort of cancer patients treated with immune-checkpoint inhibitors

4.4.1. Demographic and clinical characteristics of enrolled patients

Eighty-two subjects (24 females and 58 males; median age 67 range 26-82 years) were enrolled between 24th March and 23rd April 2021 in two Oncology Units of Northern Italy (Fondazione IRCCS Policlinico San Matteo, Pavia and AUSL Ospedale Guglielmo Da Saliceto, Piacenza). Sixty-four (78%) of the 82 patients had lung cancer, 7 (9%) had melanoma, 6 (7%) had kidney cancer, while the remaining five patients had head&neck cancer, bladder cancer and squamous cell skin cancer, respectively 3 (4%), 1 (1%) and 1 (1%). Based on positive serology results at baseline and/or documented positive SARS-CoV-2 RNA PCR in nasal swabs, 13/82 (15.9%) were considered as positive for previous SARS-CoV-2 infection (SARS-CoV-2 experienced patients) while 69/82 were considered naïve for SARS-CoV-2 infection. The most common treatment was ICIs alone (61 patients, 75%) and the most common ICI was pembrolizumab (49 patients, 60%). Forty-one (50%) out of 82 patients had no comorbidities; in those with comorbidities, the most common were cardiovascular diseases (15/41; 36.6%) and diabetes mellitus (12/41; 29.3%). All the clinical and demographic variables have been included in Table 3.

4.4.2. Humoral response elicited by mRNA BNT162b2 vaccine in cancer patients

Neutralizing antibodies (NT Abs) against SARS-CoV-2 reference strain were measured at T1 and T2 in the two groups of subjects. Thirty-three out of 68 SARS-CoV-2 naïve subjects developed a positive NT Abs level at T1 [GMT 9 (IC95% 7-12)]; on the other side all the SARS-CoV-2 experienced subjects but one tested at T1 reached the maximum level of SARS-CoV-2 NT Abs (GMT 420 IC95% 190-1049). At T2, 44/46 (95.7%) naïve subjects developed a positive SARS-CoV-2 NT Abs response. At the same time point, all the SARS-CoV-2 experienced subjects showed SARS-CoV-2 NT abs level at the upper limit of the assay (Figure 39). A weak inverse correlation between age and NT Abs level was observed at T1 ($r= -0.40$; IC95% -0.54 to -0.23 $p<0.001$), but not at T2 ($r= 0.02$; IC95% -0.16 to 0.20 $p=0.829$).

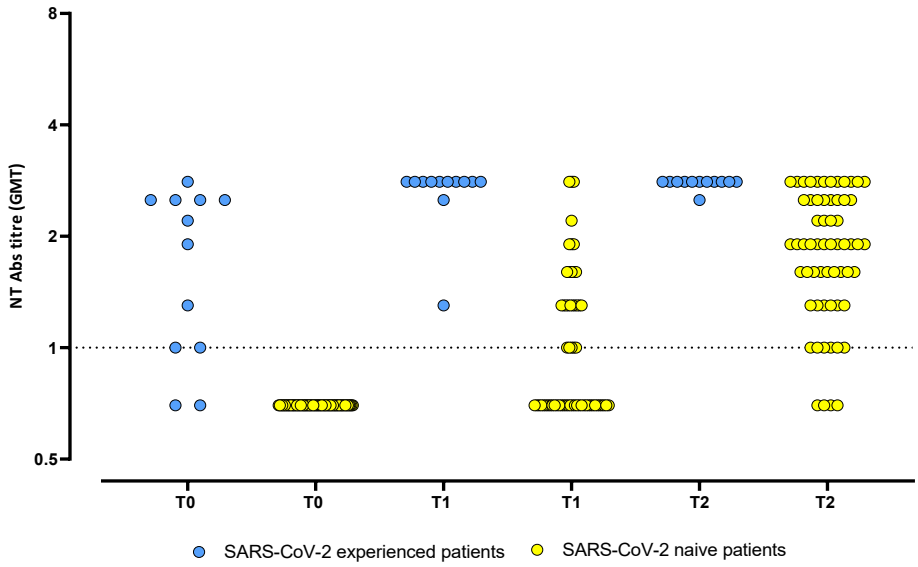

Figure 39. SARS-CoV-2 NT Abs titre elicited by vaccination in SARS-CoV-2 seropositive patients (previously COVID-19 experienced; light blue dots) and SARS-CoV-2 naïve patients (yellow dots). Horizontal dotted line represent the cut off level of positive response. GMT: geometric mean titre

4.4.3. Cell-mediated immune response elicited by mRNA BNT162b2 vaccine in cancer patients

All but one SARS-CoV-2 experienced subjects (11/12, 91.7%) showed S-specific T cells at baseline (median 77.5 IFNγ SFU/million PBMC; IQR 36.3-155), and levels of S-specific T cells increased at T1 (median 352.5 IFNγ SFU/million PBMC; IQR 96.3-522.5; p=0.001) and did not further increase at T2 (median 362.5 IFNγ SFU/million PBMC; IQR 236.3-2059; p=0.156). Focusing on SARS-CoV-2 naïve subjects, S-specific T-cell response was almost undetectable at T0 (median 0 IFNγ SFU/million PBMC IQR 0-7.5) and significantly increased at T1 and T2 (median 15 IFNγ SFU/million PBMC IQR 0-40 vs 90 IFNγ SFU/million PBMC IQR 32.5-224; respectively) (p<0.001). Overall, the percentage of responders in the group of SARS-CoV-2 naïve subjects was 67.2% at T1 and 90.2% at T2 (Figure 40).

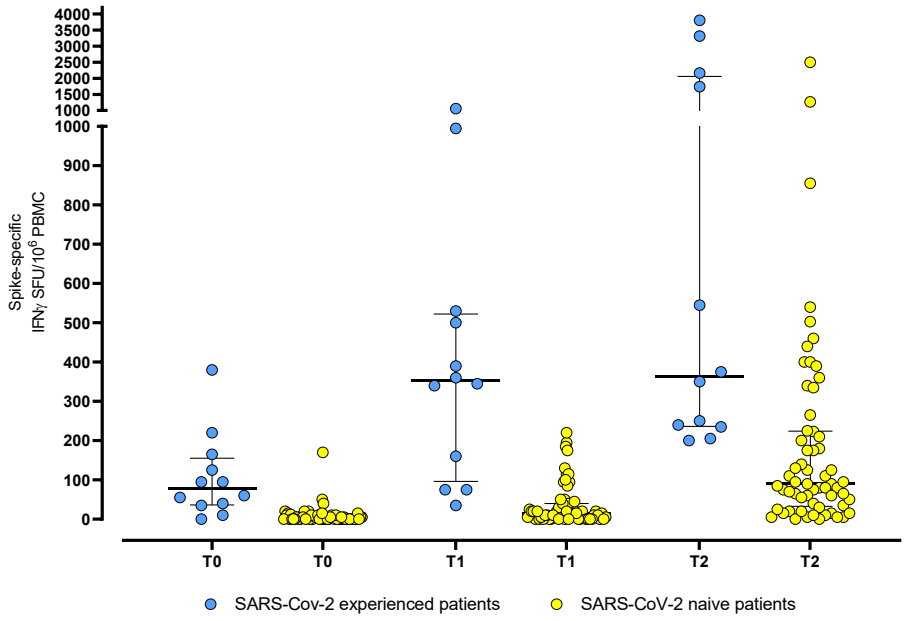

Figure 40. SARS-CoV-2 Spike-specific T-cell response elicited by vaccination in SARS-CoV-2 seropositive patients (previously COVID-19 experienced; light blue dots) and SARS-CoV-2 naïve patients (yellow dots). Horizontal dotted line represent the cut off level of positive response.

4.4.4. Chemotherapy may impact on immunogenicity of vaccine

None of the analysed clinical variables (PD-L1 status, TNM staging, histology, type of treatment, with or without chemotherapy, treatment setting, time gap between the start of immunotherapy and vaccine administration, history of a previous laboratory-confirmed diagnosis of SARS-CoV-2 infection), showed a statistically significant correlation with the increase in SARS-CoV-2 specific T cells. Only in patients treated with chemo-immunotherapy T-response seems to be lower, even if the difference was not statistically significant (odds ratio 0.20 95% CI 0.04-1.14, p = 0.0778).

4.5. BNT162b2 vaccine elicited a sustained humoral and cell-mediated response against VOC

4.5.1. Assessment of humoral response against VOC in BNT162b2 vaccinated healthcare workers

Sera collected at T2 from 31 healthy subjects who were naïve for SARS-CoV-2 infection were challenged against RBD of the wild type (WT) strain, B.1.1.7 and B.1.351 variants. RBD from WT and 614G variant strains are similar, since the D614G mutation is located on the stem of the S protein. Overall, median RBD-specific reciprocal antibody titres were 5838 (IQR 2675-16351) for WT strain and 3220 (801-9263) and 60.5 (<50-196) for B.1.1.7 and B.1.351 strains, respectively (Figure 41A). A reduction of about 50% and 99% of RBD-specific antibody titres was observed from WT to B.1.1.7 and B.1.351 strains, respectively (Figure 41B-C).

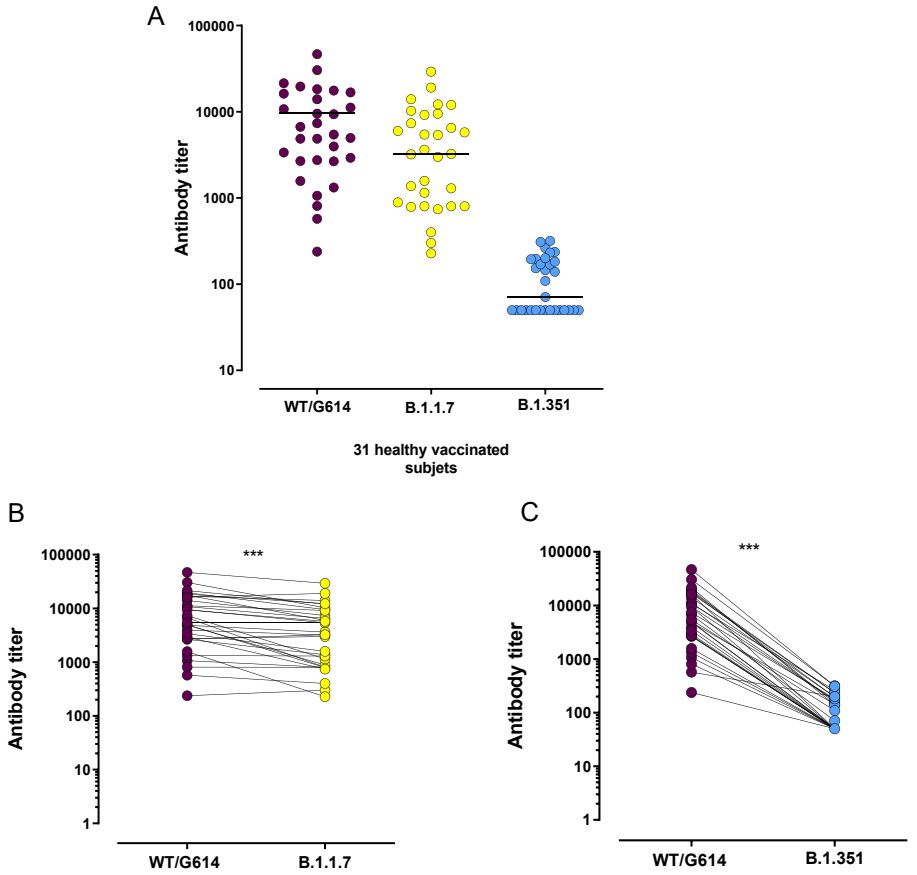

Figure 41. Median RBD-specific antibody levels against wild type, B.1.1.7 and B.1.351 strains are shown (A). Comparison between wild type vs the B.1.351 (b) and between wild type vs the B.1.1.7 (c) RBD-specific antibody titres. WT/G614 are referred to a common RBD between wild type (D614 strain) and D614G strain, (***) p value<0.001.

Retrospectively, residual sera collected at T2 from naïve and experienced subjects were tested against WT strain by neutralization assay and challenged G614 strain were challenged against D614 strain, alpha B.1.1.7, beta B.1.351, gamma P.1 and delta B.1.617.2 variants (Figure 39). Among the SARS-CoV-2 naïve subjects, an increase in four-fold dilution for SARS-CoV-2 NT antibodies was observed against the D614G strain with respect to D614 strain ($p<0.001$) while the level of SARS-CoV-2 NT antibodies was not affected when sera were tested against B.1.1.7 and P.1. strains. A five-fold and twenty-fold reduction in median titre of SARS-CoV-2 NT antibodies was observed in response to B.1.351 strain in comparison to D614 virus and

D614G strains, respectively ($p<0.001$). However, although at lower levels, NT antibodies against the B.1.351 variant were still detectable in 80.9% subjects. Similarly, a decrease of five-fold and twenty-fold of median titre of SARS-CoV-2 NT antibodies was observed in response to B.1.617.2 strain in comparison to the A Chinese derived D614 and D614G PV10734 strains. A five-fold and twenty-two-fold reduction in median titre of SARS-CoV-2 NT antibodies was observed in response to B.1.351 strain in comparison to D614 and G614 strains, respectively ($p<0.001$). However, although at lower levels, NT Abs against the B.1.351 variant were still detectable in 80.9% subjects. Similarly, a decrease of five-fold and twenty-fold of median titre of SARS-CoV-2 NT Abs was observed in response to B.1.617.2 strain in comparison to the D614 and D614G strains ($p<0.001$); overall, response was positive for B.1.617.2 variant in 86.2% naïve subjects.

Interestingly, among the 16 SARS-CoV-2 experienced subjects, a sustained NT level against the all tested variants was observed. The median reduction of SARS-CoV-2 NT against the B.1.351variant was four-fold with respect to D614 (median 1:160 vs 1:640; respectively $p=0.008$) and no reduction was observed when sera were challenged against the other variants (Figure 42). These results suggest that a triple exposure to SARS-CoV-2 antigens may increase the SARS-CoV-2 NT level against the VOC.

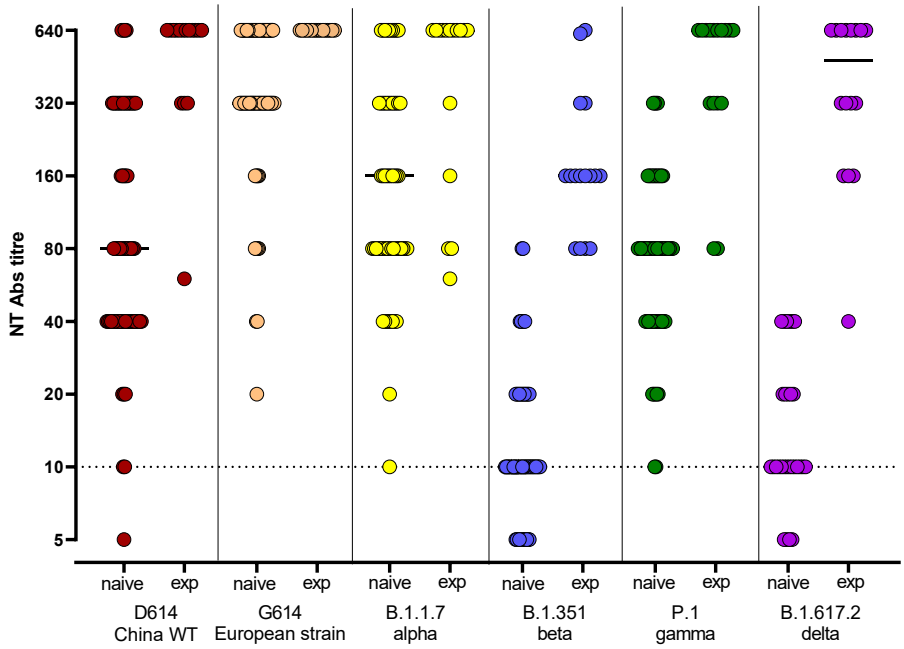

Figure 42. SARS-CoV-2 NT Abs elicited against the main SARS-CoV-2 strains, including VOC B.1.1.7, P.1, B.1.351 and B.1.617.2 were measured in SARS-CoV-2 vaccinated subjects, including naïve and experienced subjects (exp). Median response was given for each strain and the number of subjects tested for each variant was indicated.

4.5.2. Cell-mediated immune response elicited by BNT162b2 vaccine is not affected by SARS-CoV-2 VOC

Additionally, in 36 vaccinated subjects residual PBMC isolated at T2 were challenged against inactivated virus preparations from supernatant of VERO E6 cells infected with the five SARS-CoV-2 strains and cell-mediated response was analysed by ELISpot assay. The ELISpot assay was less sensitive in measuring T-cell response using WT virus than using peptide pools from the homologous S protein: median IFNγ SFU/million PBMC were 112.5 (IQR 71.3-233.8) and 47.5 (IQR 23-91), respectively. However, even if a slightly lower level of cell-mediated T-cell response was observed against B.1.1.7, B.1.351 and P.1 variants (Figure 43A), SARS-CoV-2 specific T cells were detected in the large majority of the subjects, with no significant difference in the frequency of responders. In detail, 34/36 (94.4%) were positive for Chinese-derived D614 SARS-CoV-2 T-cell response and 33/36 (91.7%) were positive for PV10734 D614G SARS-CoV-2 T-cell response.

B.1.1.7, B.1351 and P1-specific T-cell response was detectable in 30/36 (83.3%), 29/36 (80.5%) and 31/36 (86.1%) vaccinated subjects, respectively (Figure 43B), indicating that the BNT162b2 vaccine was able to elicit T-cells able to recognize conserved epitopes from any virus variant tested.

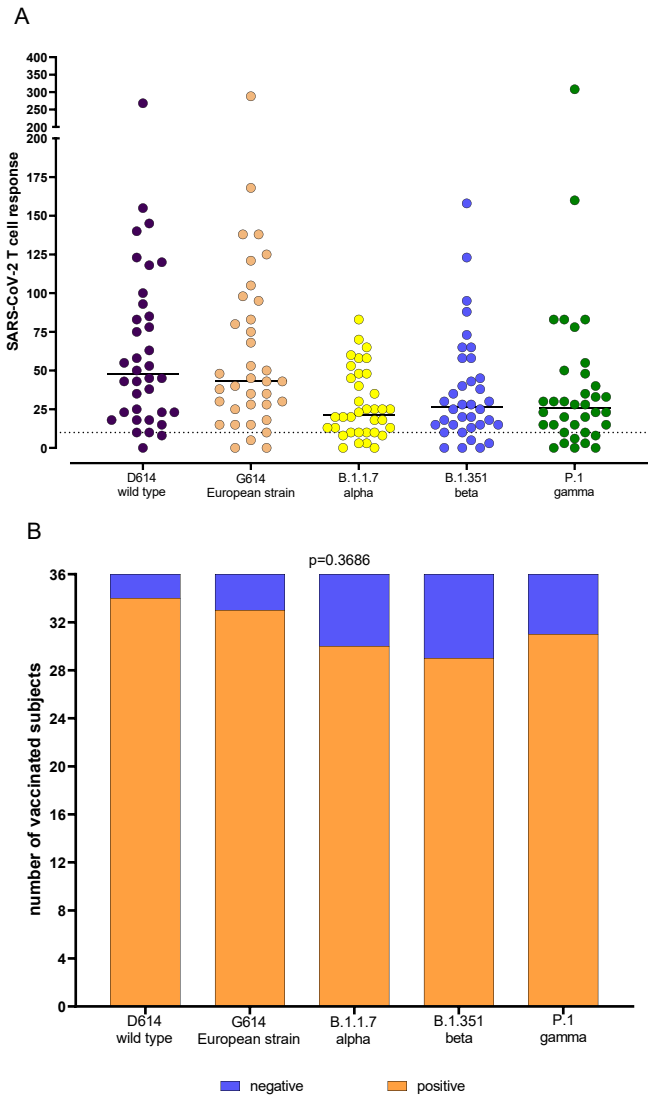

Figure 43. ELISpot T-cell response measured against SARS-CoV-2 variants. Median T cell response against SARS-CoV-2 variants are shown in section A, while number of subjects with positive SARS-CoV-2 T-cell response and negative SARS-CoV-2 T-cell response is shown in section B.

4.6. *Monocytes and macrophages act as "Trojan Horse" in spread of SARS-CoV-2 infection*

4.6.1. Macrophages and monocytes infection with SARS-CoV-2 strains

We examined if different strains of SARS-CoV-2 were able to directly infect differentiated macrophages and monocytes from blood donors. The presence of N protein was analysed in macrophages and monocytes using the three SARS-CoV-2 strains. No CPE was observed after inoculation of VERO E6 cells with supernatants from infected macrophages at each time of the experiment. As control, supernatants of the three viral strains inoculated into VERO E6 and collected at the same time points were tested and CPE was observed at each time point (Figure 44).

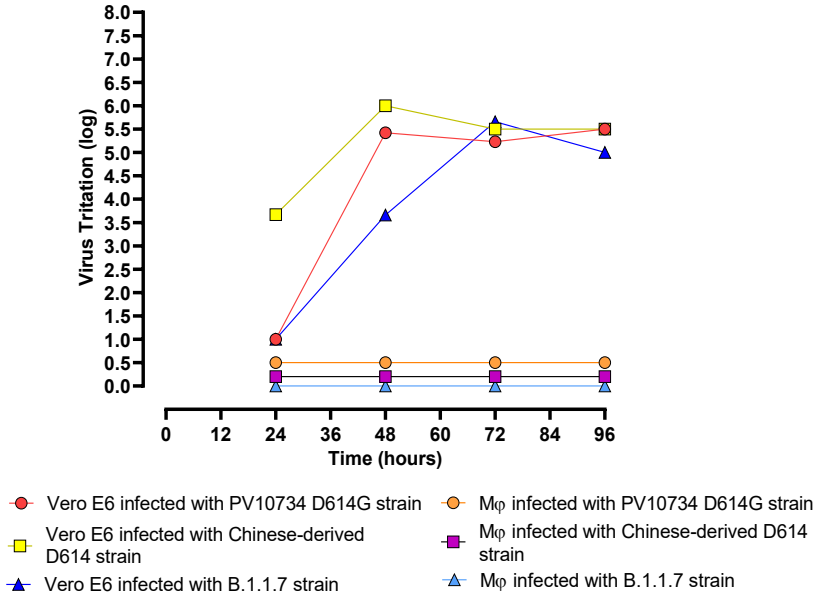

Figure 44. Time course of virus production in supernatants from macrophages and VERO E6 infected using three different viral variants; Mø: macrophages.

The same results were obtained using monocytes and macrophages from the 10 post-COVID-19 and naive donors infected with the five variants (data not shown). To further investigate the absence of viral replication in macrophages, we quantified the intracellular RNA in these cells after infection with the three variants (Figure 45A) compared to that produced in VERO E6 (Figure 45B).

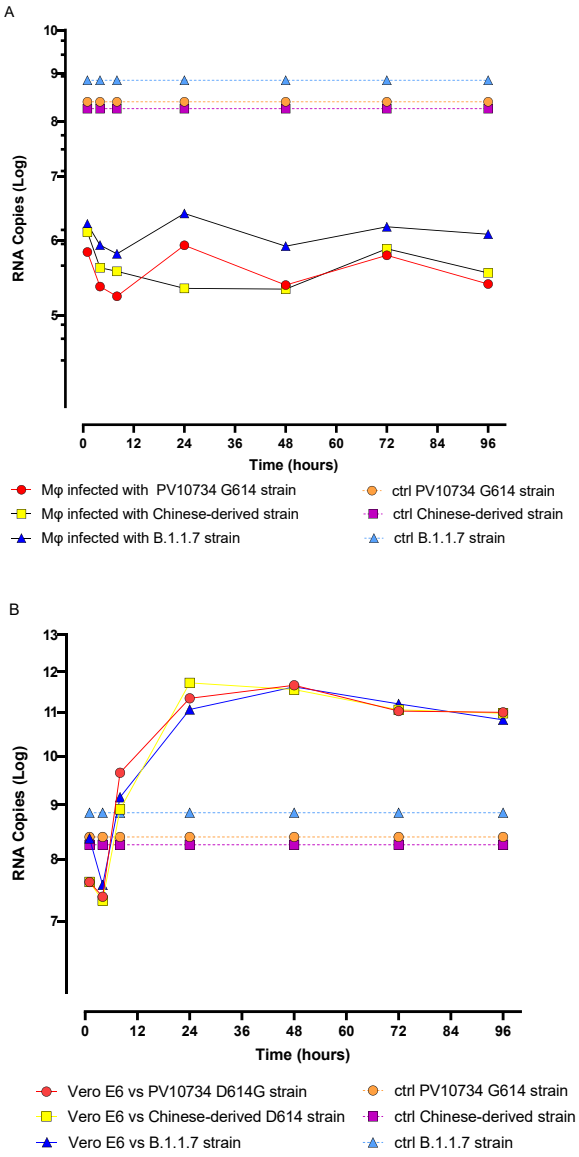

Figure 45. Time-course for RNA quantification in infected macrophages (A) and VERO E6 (B) with the three virus strains at different time-points. Virus input used for infection is reported (dotted lines). Mø: macrophages.

4.6.2. Analysis of macrophages and monocytes infection co-cultured with infected VERO E6

After co-cultivation with infected VERO E6 with PV10734 D614G strain, macrophages and monocytes migrated through a 5µm filter and were used for infection of uninfected VERO E6 cells. CPE was detected as soon as after 48h p.i. on VERO E6 and increased until complete detachment of the cells at 96 h p.i (Figure 46). Virus transmission to VERO E6 was detected at all time-points. When we looked to the mechanism of virus entry into macrophages, we found that the virus treated with the pool of post-COVID 19 patients' sera and anti-S monoclonal antibody, was detected into macrophages stained for N protein in equal number of cells as in untreated control.

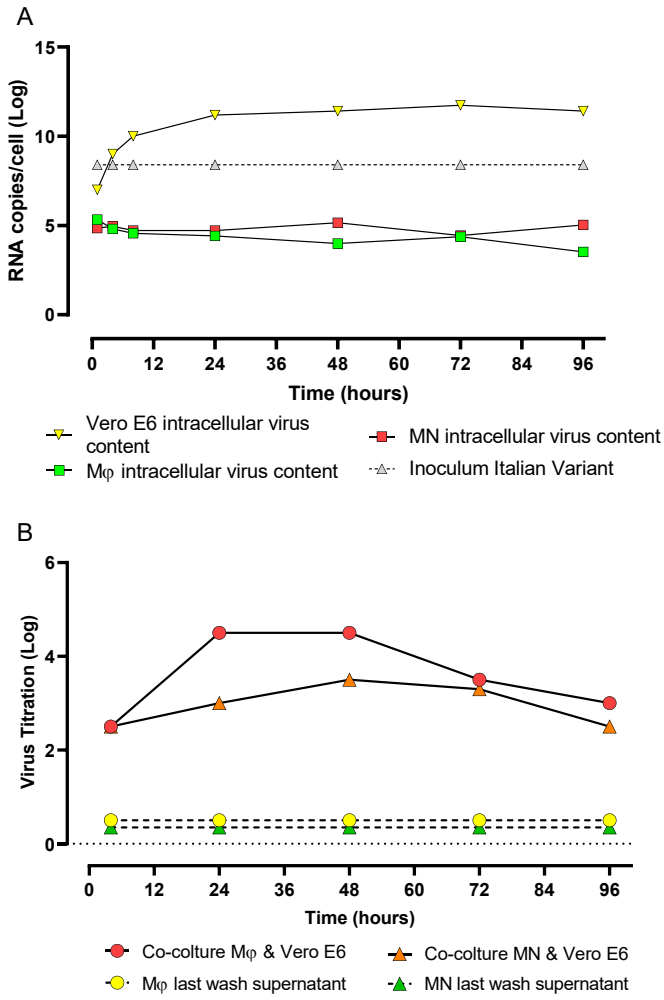

Figure 46. Time-course of intracellular RNA quantification of macrophages and monocytes in comparison with VERO E6 (A) and virus release in supernatants (B) from macrophages and monocytes infected with PV10734 D614G strain and co-cultivated with VERO E6 at different time-points in comparison with supernatants of the last wash of the same cells inoculated into VERO E6. Mø: macrophages; MN: monocytes

4.6.3. Viral transmission assay

Infected macrophages labelled with red fluorescent probe allowed tracing of infected VERO E6 there were separately labelled with the green probe with the fusion of the two fluorocromes. This fusion was observed starting from 24h after co-cultivation of infected macrophages with uninfected VERO E6 (Figure 47). No fusion of fluorescent probes was observed in VERO E6 co-cultured with uninfected macrophages.

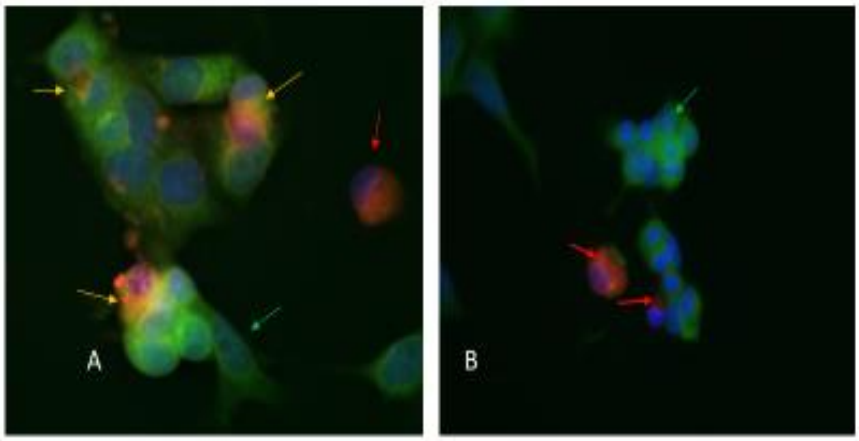

Figure 47. Virus transmission assay of: A) infected macrophages stained in red (red arrow) co-cultured with uninfected VERO E6 stained in green (green arrow). The merging orange colour (yellow arrows) is representative of virus transmission to VERO E6. In blue, the nuclei stained with DAPI. B) Uninfected macrophages stained in red (red arrow) co-cultured with uninfected VERO E6 stained in green (green arrow).

4.6.4. Kinetics of N protein uptake by macrophages and monocytes after co-cultivation.

Macrophages and monocytes infected during co-cultivation on VERO E6 infected with the three viral strains and migrated through transwell filter were examined for N protein expression after 1, 8, 24, 48, 72 and 96 h. N antigen expression was detected as early after 1 h in 30% of the macrophages. The number of positive cells increased involving all the cells present in the slides 8 h p.i. with an increase also in the intensity of the expression of the N protein. Macrophages start to become larger and in the nuclei, some oval structures appear in parallel with some structures located outside of the macrophages that we called «blackberry-shaped » structures, as soon as 8 h p.i for Chinese-derived D614 strain and from 24 h p.i. for the other two strains.

These structures increased in number in parallel with the enlargement of macrophages as reported in Table 8. From 72 to 96 h p.i the number of macrophages decreased because the engulfed cells lysed with an increase of «blackberry-shaped» forms. The survived macrophages increased their dimension by three log in comparison with 1 h p.i. S-protein expression was detected only at 96 h p.i. The above characteristics and the time-course of these events in macrophages are reported in Table 8 and Figure 48.

Table 8. Phenotypic characteristics and percentage of infected macrophages during the time course infection with the three SARS-CoV-2 variants

Characteristics	Virus Variant	1h	4h	8h	24h	48h	72h	96h
Infected cells	D614G/B.1.1.7	30%	70%	TMTC	TMTC	TMTC	TMTC	TMTC
	D614	30%	80%	TMTC	TMTC	TMTC	TMTC	TMTC
CPE	D614G/B.1.1.7	-	-	-	±	+	+	+
	D614	-	-	±	±	+	+	+
Average Cell dimension (μm^2)	D614G/B.1.1.7	562,5	837,5	1261,9	2246,1	4521,1	6009,6	11521,7
	D614	189,2	616,4	761,5	2415,9	3750,5	5003,7	8508,7
«blackberry-shaped» forms*	D614G/B.1.1.7	0	0	0	1	2	2	2
	D614	0	0	2	5	2	2	2
Multinucleated Cells	D614G/B.1.1.7	<10%	<10%	10%	30%	50%	50%	50%
	D614	<10%	10%	30%	30%	50%	50%	70%
Spike protein	D614G/B.1.1.7	-	-	-	-	-	-	+
	D614	-	-	-	-	-	-	+

Legend: * «blackberry-shaped» formations per field; TMTC: Too Many To Count; CPE: cytopathic effect

In panel A and B of Figure 48 the anti-N immunostain of infected macrophages fixed in formalin and embedded in paraffin, demonstrate that this immunostain can be used on routinely processed samples for pathology. Immunostains specifically labels cytoplasm, and shows a punctuated morphology, with single cells that tend to coalesce, appearing as multinucleated (red arrows, B). The macrophage activation is morphologically supported by the large number of pseudopodia that confer a hairy-like appearance to non-coalescent individual cells (black arrows, B). Similar results were obtained with monocytes: at 1 h p.i. a lower number of cells (10%) were infected compared to macrophages and increased in number and intensity of N protein expression. At 8 h p.i. all the cells were infected and at 72/96 h become bigger. No «blackberry-shaped» forms were detected at any time examined.

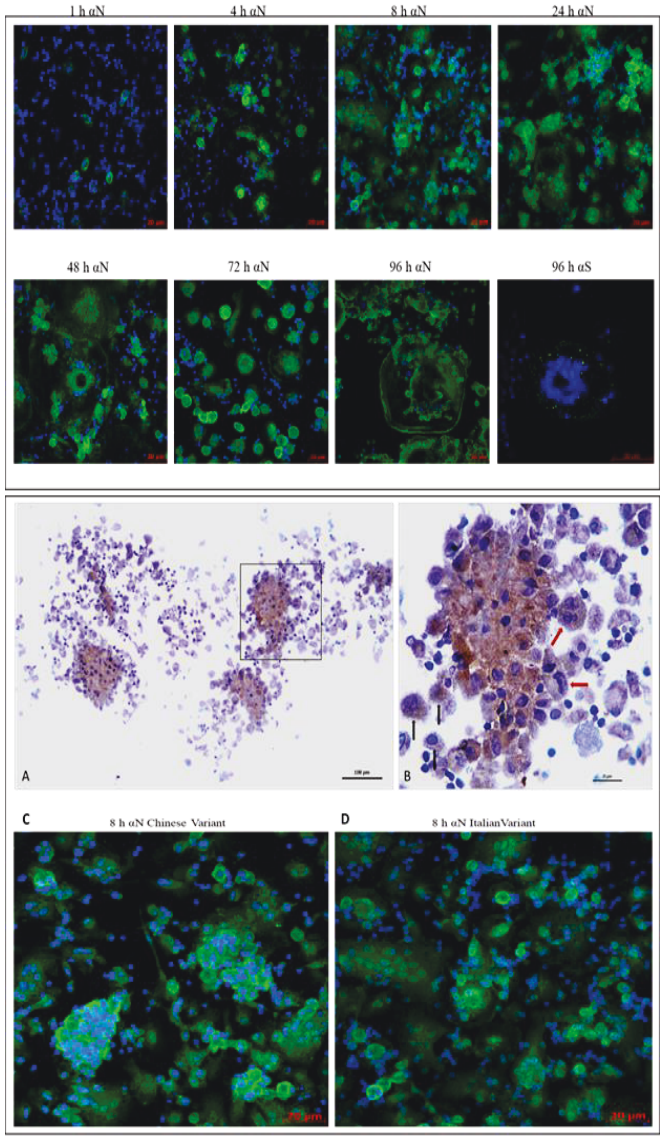

Figure 48. Time-course of SARS-CoV-2 infected macrophages. In the upper panel is reported the PV10734 D614G variant time course stained with the anti-N and anti-S protein (green) and with DAPI (blue), at 40 X magnification in confocal microscopy. In the lower panel, anti-N immunostain (brown) of infected macrophages (A-B) and the comparison of «blackberry-shaped» formations in the Chinese-derived D614 (C) and PV10734 D614G strains (D) at 8 h p.i. with confocal microscopy was reported. The area squared in A is enlarged in panel B with multinucleated macrophages (red arrows) and individual cells (black arrows).

4.6.5. Pathologic study of infected macrophages and VERO E6 cells

Thanks to the collaboration with Prof Arbustini, light microscopy study done at the different intervals from viral inoculation on macrophages showed early syncytial like cells and later (>24 h) multiple cells aggregation, resulting in «blackberry-shaped» forms observed with fluorescence studies as shown in the upper panel of figure 48. The ultrastructural study of infected macrophages demonstrated the presence of intracytoplasmic vesicles containing viral particles that we called “persistence vesicles”. These macrophages showed activation and preservation of cytoplasmic membranes. Moreover, the absence of replication vesicles similar to those seen in infected VERO E6 cells was reported (Figure 49F). The persistence vesicles, each containing a few viral particles, were observed in macrophages infected after 1 h, as well as after 24, 48, 72 and 96 h from virus inoculation, suggesting that viral particles included in membrane-bounded vesicles persist, irrespective of the interval from viral inoculation in macrophages (Figure 49, A-E). Indeed, in infected VERO E6 cells, viral particles were Too Many To Count (TMTC), both free in cytoplasm and clustered in replication vesicles. In addition, infected VERO E6 cells showed numerous viral particles adherent to cell membranes, with high concentration on cytoplasmic pseudopodia (Figure 49 F). The pre-embedding immunoelectron microscopy study shows specific anti-N immunostain of both viral particles and nucleocapsid material free in the cytoplasm, with a high amount of viral proteins and a low number of mature viral particles (Figure 49 F). The dark osmophilic cytoplasmic protein masses that are specifically labelled by the anti-N antibodies demonstrate that actively infected VERO E6 cells produce more viral proteins than viral particles. The small light grey part of a cytopathic adjacent cell (Figure 49 F upper left corner) also shows immunolabelling without particles. The specificity of the immunostain is proven by the absence of immunogold particles in the extracellular background (Figure 49 F). This feature was not observed in infected macrophages (Figure 49 A-E).

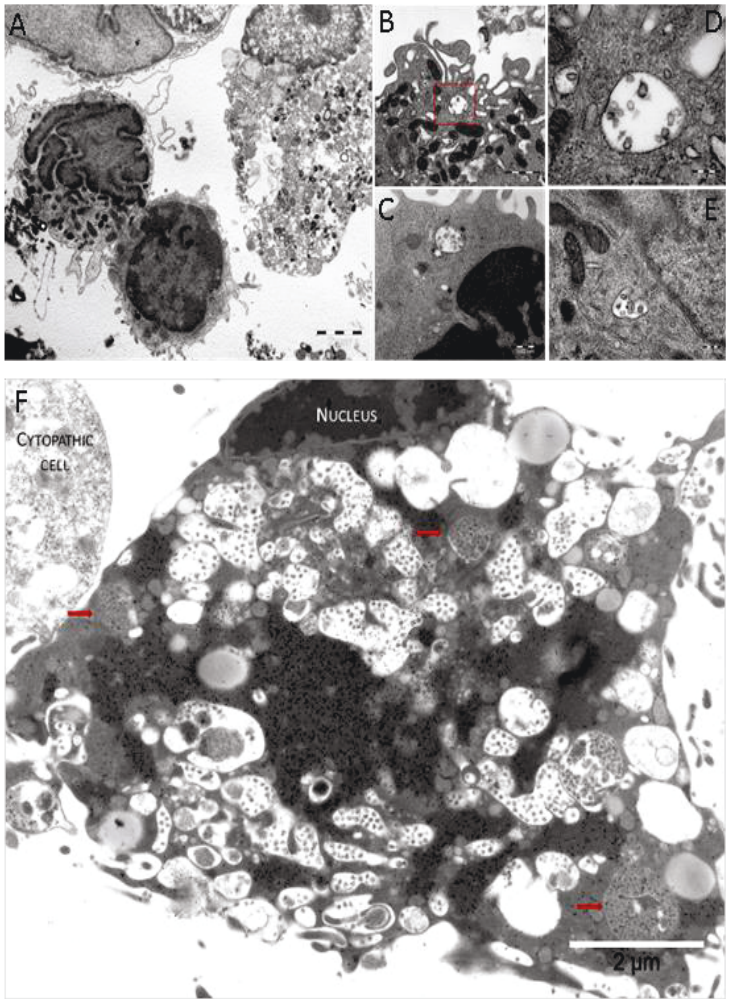

Figure 49. Morphofunctional features of SARS-CoV-2 *in vitro* infected macrophages (A-E) and VERO E6 (F) were reported. A) Low magnification view of in vitro infected macrophages at 24 h p.i.; B) Red squared area-intracytoplasmic vesicles containing viral particles, enlarged in panel C. D, E). Examples of vesicles in macrophages that still preserve their morphologic integrity but show features of cell activation (pseudopodia) (D) and preserved organelle morphology (E). F) Pre-embedding anti-nucleocapsid immunostaining of VERO E6 cells, at 72 h p.i. with multiple replication vesicles and loose viral particles (those within the vesicles with the white background), or more compacted vesicles (red arrows) with multiple smaller virions.

5. Discussion

The role of immune response in SARS-CoV-2 infection and vaccination represents one of the “hot topics” related to COVID-19 pandemic, also in relation to the spread of VOCs that are responsible of rapid and unpredictable changes in epidemiology of the pandemic. Thus, a comprehensive investigation of immune response kinetics and mechanisms related to both SARS-CoV-2 infection and vaccination against all SARS-CoV-2 VOCs should be implemented. In this context, since the emergence of first SARS-CoV-2 cases in February 2020, our research team has been actively involved in SARS-CoV-2 research in collaboration with national and international research groups, designing new assays for evaluation of humoral and cell-mediated response and analysing pathogenic mechanisms of viral spread.

5.1. Neutralization assay against SARS-CoV-2

In the first part of the study, we designed a microneutralization assay for the evaluation of SARS-CoV-2 NT Abs, using SARS-CoV-2 strain isolated from an Italian hospitalized patient and carrying D614G mutation (strain PV10734). We decided to use D614G mutated strain as reference strain instead of the original Chinese-derived D614 strain, since the G614 strain rapidly spread in Europe during the first wave of pandemic (February-April 2020), replacing D614 original strain and was dominant in Lombardy Region (Alteri *et al.,* 2021). Thus, it was more adherent to the real epidemiological situation. Of note, it has been reported that D614G mutated strain showed a slightly higher sensitivity to neutralization (Weissman *et al.*, 2021). Using this approach, we found that more than 20% of subjects referred to the first Red Zone in Lombardy region (the so-called Lodi Red Zone) experienced SARS-CoV-2 infection in the period ranging from February and April 2020, even if in asymptomatic or mild symptomatic way (Percivalle *et al.,* 2020). These data were further confirmed and extended in a larger population of about 2,000 blood donors from Lodi province enrolled between March and June 2020 (Cassaniti *et al.*, 2021). Furthermore, our data demonstrated that SARS-CoV-2 NT Abs level are related to SARS-CoV-2 severity symptoms, since median level of SARS-CoV-2 NT Abs are higher in symptomatic hospitalized patients than respect to asymptomatic or mild symptomatic subjects, as reported by others (Garcia-Beltran *et al.,* 2021; Bruni *et al.,* 2020).

The duration of SARS-CoV-2 antibody protection represents a crucial point, since other authors suggested that a decline of RBD-S antibodies in asymptomatic to moderate COVID-19 subjects might occur in about 4-8 weeks (Bruni *et al.,* 2020; Milano *et al.*, 2020). Otherwise, other studies reported a longer duration (up to six months) of IgG antibodies against S-

protein, especially in symptomatic severe patients (den Hartog *et al.,* 2021). The durability of antibodies' protection seems to the linked to reinfection risk in subjects recovered from COVID-19. Interestingly, in a six-month survey performed in healthcare workers enrolled in three hospitals of Northern Italy, including IRCCS Policlinico San Matteo of Pavia, the risk for positive SARS-CoV-2 RT-PCR was significantly lower in SARS-CoV-2 seropositive healthcare workers compared to SARS-CoV-2 seronegative healthcare workers, thus suggesting a protective role of SARS-CoV-2 antibodies (Rovida *et al.,* 2021). These results are in line with those obtained by other research groups (Lumley *et al.*, 2021) and provide a basis for COVID vaccination. Otherwise, despite a more rapid decline of SARS-CoV-2 antibodies, it is conceivable that cell-mediated response persists longer during time (Sherina *et al.,* 2021; Cassaniti et al., 2021), also on the basis of previous experience with SARS-CoV-1 (Yang *et al*., 2007). Our approach designed for SARS-CoV-2 NT Abs quantification, which was successfully used for population screening and seroepidemiological studies (Percivalle *et al.,* 2020; Cassaniti *et al.,* 2021) as well as for monitoring of SARS-CoV-2 NT Abs level in convalescent subjects was successfully applied to identify suitable donors of hyperimmune plasma to transfuse in severe COVID-19 patients (Del Fante *et al.,* 2020).

Furthermore, microneutralization assay was used for *in vitro* MAbs screening. Briefly, we assessed the efficacy of six MAbs using both PV10734 D614G and Chinese-derived D614 strains. The rate of neutralization was measured for each concentration and compared between the two strains. Our data contributed to identification of a bispecific MAb (CoV-X2) that bound at low nanomolar affinity to RBD and S trimeric of wild type virus and variants (De Gasparo *et al.,* 2021). An *in vivo* mouse model was later used to prove the in vivo efficacy. One of the main concern related to therapeutic MAbs use is linked to the possible emergence of resistant variants. Normally, SARS-CoV-2 viruses that can mutate its S protein in specific points that allow them can mutate its S proteins in order to escape MAbs are able to survive (Schmidt *et al*., 2020). To mitigate this possibility, combinations of monoclonal antibodies have been tested (Weisblum *et al*., 2020). One alternative might be represented by the use of multispecific antibodies, which have the advantages of cocktails with a single-molecule strategies. Indeed, the use of antibody cocktails requires a significant increase of production capacity than with respect to a single Mab. Overall, data reported by De Gasparo and colleagues showed that the use of bispecific MAb may represent a valuable strategy instead of the use of antibody cocktail to prevent viral escape from epitope-specific immunity. Further evaluations are required and clinical trial are mandatory to prove the efficacy in the human population.

5.2. Immune response against SARS-CoV-2 in convalescent and vaccinated subjects

In the second part of my PhD program, an in-house *in vitro* assay for the characterization of SARS-CoV-2 T-cell response was designed and applied in different settings of populations (Cassaniti *et al.*, 2021). In detail, in SARS-CoV-2 convalescent subjects, the role of humoral response was investigated in combination with SARS-CoV-2 T cell memory response, in order to better characterize long-term protective mechanisms against possible reinfection. Our investigative study involved 82 convalescent patients, affected by either mild or severe COVID-19. Overall, we observed that more than 97% subjects showed a sustained T-cell response measured by *ex-vivo* ELISpot assay to at least one SARS-CoV-2 antigen. Interestingly, a detectable SARS-CoV-2 T-cell response was also reported in about 40% of unexposed donors sampled before the pandemic period, in agreement with previous observations (Grifoni *et al*., 2020; Le Bert *et al.,* 2020; Meckiff *et al.,* 2020). The pre-existing cell response against SARS-CoV-2 antigens in unexposed subjects might be probably related to previous infection with “common cold” HCoVs (Sette and Crotty, 2020). So far, the data obtained in healthy population reflect the endemic circulation of HCoVs since they account for about 20% of common cold cases and are ubiquitous (Li *et al.,* 2008; Braun *et al*. 2020); thus, the possible cross-reactivity between HCoVs might be due to the recognition of conserved epitopes. Thus, it could be possible that previous exposure to HCoVs antigens might be involved in protection mechanisms against severe COVID-19 or, eventually, in faster and stronger development of immune protection in case of vaccination (Sette and Crotty, 2020). Thus far, according to some authors, the presence of a pre-existing cross-reacting immune response seems to correlate with milder symptoms as observed among influenza viruses (Wilkinson *et al.*, 2012; Hancock *et al*., 2009). Additionally, we demonstrated that SARS-CoV-2 reactive T cells were mainly CD4+, in agreement with previous observations (Braun *et al.,* 2020; Matues *et al*., 2020; Grifoni *et al*., 2020). Then, it is conceivable that the induction of a SARS-CoV-2-specific CD4+ T cell response might be involved in the generation of neutralizing antibodies, being T helper cells, especially Th1, and humoral response interdependent (Mitchison, 2004). It has been also reported that subjects who experienced severe COVID-19 showed sustained NT Abs level with rapid increase of CXCR3 TFH cells that might have a dominant role in the generation or maintenance of NT abs in COVID-19 patients (Zhang *et al*., 2021).

Looking at persistence of immune response against SARS-CoV-2 after natural infection, we learned from SARS-CoV-1 epidemic in 2003 that memory T cells could be detectable even 11 (Ng *et al.,* 2016) and 17 years (Le Bert *et al.,* 2020) after primary infection, suggesting a long-lasting memory

response. The longer persistence of T-cell response despite a decline of SARS-CoV-2 antibodies has also been observed in a cohort of Italian and Swedish patients enrolled in the same study (Sherina *et al.,* 2021). This trend was also observed in other clinical settings, including HBV vaccination (Cassaniti *et al.,* 2016) and Flaviviruses infection (Percivalle *et al.*, 2020). In light of these results, it is conceivable that a long-term SARS-CoV-2 response might be present even in absence of detectable SARS-CoV-2 antibodies, thus suggesting a long-term protection against reinfection. Indeed, based on our experience, the rate of SARS-CoV-2 infection in previously infected healthcare workers is less than 2% while it rose to 6.6% in SARS-CoV-2 seronegative subjects (Rovida *et al.,* 2021). However, prospective long-term studies are mandatory for better understanding of the protection conferred by natural infection and for designing future vaccination strategies.

After the emergence of vaccination and the massive immunization of healthcare workers, a prospective and comprehensive evaluation of the development of antibody and T-cell responses elicited by the mRNA vaccine BNT162b2 for SARS-CoV-2 in a cohort of 145 immunocompetent healthcare workers of our institute was performed (Cassaniti *et al.*, 2021). As most relevant result, we reported that all vaccinated subjects developed a sustained SARS-CoV-2 NT Abs level, and 98.6% a specific T-cell response after complete vaccination schedule both antibody and T-cell response were not affected by age after the second dose. The mRNA-based vaccine was highly effective in eliciting both antibody and T-cell responses. Regarding the T-cell subsets induced, we observed both CD4+ and CD8+ T cells proliferative responses. This pattern is similar to that observed in a preclinical study for another mRNA-based vaccine against human cytomegalovirus (Jonh *et al*., 2018). Among CD4+ T cells, we observed an effective response of the TFH subset, which is involved in providing help for B-cell differentiation and antibody affinity maturation.

In this study, we observed that all SARS-CoV-2 naïve individuals developed a humoral response higher than that observed after natural infection after two vaccine doses but not after one vaccine dose, when about 6% of individuals was still negative for SARS-CoV-2 NT Abs. The cell-mediated response was detectable in 98.4% naïve individuals after two doses of vaccine, whereas one vaccine dose was not sufficient for eliciting detectable SARS-CoV-2 T-cell response in 32.1% of subjects. Conversely, both humoral and cellular responses were strongly induced after the first dose of the BNT162b2 vaccine in previously SARS-CoV-2 infected subjects. Therefore, it appears that the first vaccine dose acts as a boost in these subjects. Moreover, both antibody and T-cell responses did not appear to increase after the second dose in these individuals. According to previous observation (Manistry *et al.,* 2021; Prendecki *et al.,* 2020), this evidence strengthens the concept that just one

vaccine dose may be sufficient in subjects with previous SARS-CoV-2 infection (Bradley *et al*., 2021). In addition, while the NT Abs level observed after the first dose was inversely correlated with age, thus resulting in a potentially ineffective immunization in elderly subjects that are at higher risk for developing the more severe consequences of SARS-CoV-2 infection, the second dose was effective in boosting the response in all subjects, regardless age. These results are of paramount importance for defining the criteria of a possible booster dose in healthy subjects.

Furthermore, potential cross-reactive T-cells originally elicited by the common cold HCoVs may be boosted by the vaccine and contribute to protection. In our study, about 20-30% SARS-CoV-2 naïve subjects showed a T-cell response specific for S or N proteins. This might be related to the presence of cross-reactive epitopes from common cold coronaviruses that seasonally circulates (Cassaniti *et al.,* 2021; Grifoni *et al.,* 2020; Le Bert *et al.,* 2020; Meckiff *et al.,* 2021; Sette and Crotty, 2020). In conclusion, our results supported that the BNT162b2 vaccine is able to induce robust antibody and T-cell responses in all immunocompetent individuals and pave the way for the clinical evaluation of other mRNA vaccines currently in development (Liu, 2019). Although the effectiveness of the antibody response may be partially reduced by some VOC, the T-cell response seems to be less affected. Although we only analysed the T-cell response induced by BNT162b2 mRNA vaccine, it is likely that the other SARS-CoV-2 mRNA vaccine licensed showed a similar broadly reactive T-cell response. These data support that in the future evaluation of SARS-CoV-2 vaccines persistence of the memory response induced remained a crucial issue. This aspect will be prospectively addressed by our ongoing study. Indeed, in our prospective longitudinal study, immune response elicited by BNT162b2 vaccinated healthcare workers will be challenged at six and twelve months after vaccination.

5.3. BNT162b2 vaccination in cancer patients treated with ICIs

It is well known that cancer patients are at increased risk of morbidity and mortality from SARS-CoV-2 infection (Garassino *et al.,* 2020). Despite this evidence, very few patients with cancer were enrolled in COVID-19 vaccine studies and so many unanswered questions remained about the risk-benefit ratio of these vaccines in this frail population. As there is an urgent need to protect cancer patients from COVID-19, the main professional societies and organizations, e.g., the American Association of Clinical Oncology (ASCO), the European Society of Medical Oncology (ESMO) and the Associazione Italiana di Oncologia Medica (AIOM) strongly endorsed prioritization of such patients for SARS-CoV-2 vaccination (ASCO,2021; Garassino *et al.,* 2021; AIOM 2021), although there are still many unclear issues about their efficacy and safety. Recently, Thakkar and colleagues evaluated anti-S IgG titers in

200 cancer patients including solid tumours and hematologic tumours showing a significantly lower seroconversion rate in patients with hematologic malignancies than respect to those with solid tumours. Moreover, the highest levels of seroconversion were observed in patients receiving immune checkpoint inhibitor therapy (Thakkar *et al.,* 2021).

In our cohort study, we aimed to evaluate the humoral and cell-mediated immune response in cancer patients treated with PD-1/PD-L1 inhibitors and receiving BNT162b2 anti-SARS-CoV-2 vaccine (Lasagna *et al.*, 2021). Our data confirmed that the rate of SARS-CoV-2 naïve subjects developing positive antibody level is high as expected following two vaccine doses, even if only one third of patients developed a positive antibody response after the first dose, confirming previous results obtained in cancer patients (Fong *et al.,* 2021). Furthermore, we also investigated the development of Spike-specific cell-mediated immune response using an in-house *ex-vivo* ELISpot assay. Interestingly, over 90% of patients developed a sustained S-specific T-cell response at T2, suggesting that adaptive immune response is not compromised in this cohort of subjects. Furthermore, a sustained CD4 and CD8 T cell response was elicited by vaccination. Thus, in our cohort of subjects with solid cancer, the administration of a full course of an mRNA vaccine provides good immunogenicity regardless the type of cancer and/or ICIs.

Comparable to healthcare workers, previously SARS-CoV-2 exposed cancer patients mounted a robust neutralizing immune response even after a first dose suggesting that the past infection may be an immune enhancer condition. Goshen-Lago et al reported that the adverse events after the two doses of BNT162b2 vaccine in cancer patients were comparable with those observed in healthy population (Goshen-Lago *et al.,* 2020). In addition, Waissengrin and colleagues described the safety of the BNT162b2 mRNA vaccine in a cohort of cancer patients treated with ICIs. Comparing side-effects in the patients treated with ICIs with a healthy control group matched by sex and year of birth, they observed no new immune-related side-effects or exacerbation of existing immune-related side-effects; a side-effect profile similar to healthy controls was reported (Waissengrin *et al*., 2021). Since both ICI treatment and COVID-19 vaccines stimulate the immune response, it has been hypothesized that these vaccines may increase the incidence of immune-related adverse events with ICI treatment. In our study, only one patient reported two immune-related side effects (hepatitis and colitis) 10 days after the first dose of vaccine.

5.4. Impact of SARS-CoV-2 VOCs on SARS-CoV-2 vaccination

Looking at the emergent problem of SARS-CoV-2 VOC, we analyse the immunogenicity elicited by vaccination against the most important VOC (Cassaniti *et al.,* 2021). Among the SARS-CoV-2 variants tested, only B.1.351 and B.1.617.2 significantly impacted NT Abs levels in vaccinated subjects, while SARS-CoV-2 T-cell response was not significantly affected. Since the beginning of the pandemic, about 250 million individuals have been infected by SARS-CoV-2 and NT Abs titres are extremely variable (Robbiani *et al.*, 2020). Those with low titre could be re-infected, and due to the presence of incomplete protective neutralizing antibodies might develop escape variants (Van Elslande *et al.,* 2020; Larson *et al.,* 2020). The accumulation of mutations in the RBD and N-terminal domain of S-protein might be associated with increased escape from neutralization. In particular, mutation at 484 position in the spike has been related to the reduction in neutralization sensitivity (Greaney *et al.,* 2021; Weisblum *et al.,* 2020). S-protein mutations in the B.1.351 strain are linked to immune escape from several classes of anti-SARS-CoV-2 monoclonal antibodies, and a significant decrease in neutralization titer of plasma from convalescent subjects was observed when sera were challenged with B.1.351 variant (Wibmer *et al.,* 2021).

Usually, after natural infection or vaccination a polyclonal immune response arises against multiple antigenic epitopes. Consequently, small numbers of variations in antigen sequence should have little impact on recognition by the immune system, including both NT Abs and T cells. Our results show that vaccinated individuals developed an equally effective NT antibodies response against the Chinese-derived D614 strain and B.1.1.7 variant, as well as against P.1 variant. However, according to previous observation, sera from vaccinated subjects showed a significant reduction in SARS-CoV-2 NT antibodies when challenged against the B.1.351 and B.1.617.2 variants. Nevertheless, serum from the majority (almost 80%) of vaccinated subjects maintained its neutralizing activity and was effective against these variants that could be effective in preventing the development of severe disease. These results are in contrast with another report showing a higher reduction in neutralization titres against pseudoviruses with S protein from B.1.1.7, B.1.351 and P.1, with respect to Chinese-derived D614 strain, and no increase in NT Abs against PV10734 D614G (Garcia-Beltran *et al.,* 2021). Although the binding titer to B.1.351 RBD was highly reduced with respect to D614 strain (about 100 folds), the NT titer was less affected, showing a five-fold reduction. Importantly, our studies rely on the use of wild type viruses rather than pseudoviruses. So far, the use of pseudoviruses could give discrepant results with respect to natural strains, as reported (Wang *et al.,*

2021), likely because the artificial lentiviral particles cannot resemble the complete biology of actual clinical isolates.

Interestingly, the level of NT Abs against SARS-CoV-2 variants in SARS-CoV-2-experienced vaccinated subjects was robust. This preliminary observation might be helpful in the design of vaccination strategies, suggesting that three consecutive exposure with SARS-CoV-2 S antigen could increase the level of neutralizing antibodies against SARS-CoV-2 variants. However, and most considerably, our studies on cell-mediated response revealed that SARS-CoV-2 T-cell response is minimally affected by the mutations occurring in SARS-CoV-2 variants, as reported (Tarke *et al.,* 2021), and most subjects tested showed a detectable T-cell response against each virus strain. Differently from Tarke and colleagues, we used inactivated preparations of different SARS-CoV-2 variant isolates in order to recapitulate realistically antigen presenting mechanisms and natural epitope recognition from T cells. The levels of T-cell response and NT Abs level elicited by the vaccine were not correlated; therefore, a low NT Abs titer does not exclude the presence of a protective T-cell response able to control SARS-CoV-2 infection and avoid the development of a severe disease.

5.5. Macrophages and monocytes in SARS-CoV-2 infection: potential "Trojan Horse"?

In the last part of this PhD project, we contributed to the understanding of *in vitro* mechanisms of SARS-CoV-2 transmission through cells of innate immune system that can have a dual role, since they can contribute to immune response against SARS-CoV-2 but they can also act as "Trojan Horses", favouring the spread of the virus (Percivalle *et al.,* 2021). Our study aimed to unravels the bidirectional trajectory of reciprocal infection of VERO E6 cells and macrophages/monocytes. In this trajectory, infected VERO E6 cells with active viral replication transmit the virus to both macrophages and monocytes, where the virus persists but seems to not replicate. On the other hand, infected monocytes and macrophages, with persistent but not replicating viruses, are able to infect VERO E6 cells that, in turn, demonstrate active viral replication. Therefore, macrophage and monocytes could host viable viruses but seems to be not permissive for complete viral replication. These *in vitro* observations suggest that macrophages might be key players of viral dissemination and persistence as circulating mononuclear cells that could potentially transfer viable viruses when co-localizing with epithelial cells. This bidirectional mechanism has been observed up to 96 hours in our experimental setting. Several authors showed that SARS-CoV-2 can infect macrophages and monocytes without virus production (Boumaza *et al.,* 2020) but the transmission of the virus from infected macrophages is still debated.

To the best of our knowledge, this is the first study reporting that macrophages and monocytes infected by different variants of SARS-CoV-2 are able to transmit the infection to permissive VERO E6 cells possibly acting as "Trojan horse" in *in vivo* infection. In the presence of all components of viral binding and activation, the virus can infect macrophages and monocytes without replication stimulating the production of proinflammatory cytokines and chemokines, as described for MERS-CoV (Zhou *et al.,* 2015). It could be speculate that, upon infection, macrophages migrate into tissue and become infected resident cells, enabling virus anchoring to susceptible cells. Infected macrophages, paradoxically, might facilitate invasion of different organs by SARS-CoV-2. Importantly, we demonstrated that macrophages and monocytes are abortively infected by SARS-CoV-2, both directly or through co-cultivation with infected VERO E6. A similar mechanism was previously observed in Human Cytomegalovirus (HCMV) infection. In the case of HCMV, polymorphonuclear leukocytes (PMNL) could be infected only through co-cultivation with infected endothelial primary cells without production of the virus by PMNL, whereas they were able to transmit the infectious virus to uninfected endothelial cells (Revello *et al.,* 1998).

These observations indicate that virus infectivity can be preserved inside macrophages, even if these cells are not permissive for viral replication. Indeed, an high level of viral proteins was observed even in presence of a low number of mature viral particles, that might be a consequence of abortive viral replication. Otherwise, viral proteins seems to be sufficient to transmit the infection to the target cells. Moreover, our hypothesis seems to be furtherly confirmed by the lack of RNA increase during time-course experiments. Interestingly, no difference in terms of viral infection with the three SARS-CoV-2 strains was observed when macrophages and monocytes from naïve and post-COVID-19 donors were used. On the contrary, Yilla et al. reported that only interferon-ɣ producing macrophages could be infected with SARS-CoV-2 (Ylla *et al.,* 2005).

The kinetics of the expression of the N-protein in the time-course showed that after 8 hours all the macrophages were infected by the three variants with increasing fluorescence, fragmentation of the nuclei and formation of “blackberry-shaped” structures full of viral proteins. These “blackberry-shaped” formations start to appear as early as 8 hours with the Chinese-derived D614 strain and in the next 24 hours for the other strains. We can speculate that these structures could be identified as microvesicles in coalescent cells that are produced and released at high concentration in inflammatory conditions. These microvesicles formation, at the confocal microscopy, react with the SARS-CoV-2 N protein and could act as extracellular shuttles for the virus dissemination. Looking at viral transmission to VERO E6 from infected macrophages through co-cultivation or direct

infection in the time-course, we could isolate the three variants at each time-point. These data might suggest that the virus survives inside these cells and, even if does not replicate, it could spread in different body districts. The viral load detected in the time-course in macrophages and VERO E6 confirms our hypothesis that the virus actively replicates in permissive VERO E6, but does not replicate in macrophages.

ACE2 is expressed in multiple cell populations that can be found in the lungs, including alveolar type II pneumocytes and macrophages (Bao *et al.,* 2020), suggesting that SARS-CoV-2 can potentially infect ACE2+ macrophages. In a similar way, HCoV-229E can infect macrophages as they express the APN receptor needed for viral entry (Yeager *et al.,*1992) and bypass the endosome to enter the target cell by using TMPRSS2 (Shirato *et al.,* 2017). The endosomal pathway in macrophages is critical to identify invading pathogens, allowing HCoV-229E to infect macrophages without triggering an antiviral response, thus enhancing its pathogenesis. Human macrophages infected with HCoV-229E undergo cell death due to the lytic release of new viral particles (Collins, 2002), suggesting that HCoV-229E can infect and replicate in macrophages. SARS-CoV-1 can also infect macrophages by phagocytosis and can be detected in phagolysosomes of infected human macrophages (Ylla *et al.,* 2005). In our experiments, we observed that viral entry into VERO E6 was blocked when hyperimmune plasma from a convalescent subject and anti-S MAbs were used. On the other hand, the presence of N-protein in infected macrophages, even in presence of MAbs and/or hyperimmune plasma suggested that the main mechanisms of viral entry in macrophages was phagocytosis rather than receptor binding.

5.6. Conclusions

So far, this study provides a comprehensive analysis of SARS-CoV-2 immunological signature, starting from the characterization of NT Abs in SARS-CoV-2 natural infection and vaccination, to the quantification and analysis of cell-mediated immune response. The study dissected the immunological implication of monoclonal antibodies and vaccines, focusing on the BNT162b2 vaccine in healthcare workers and cancer patients treated with ICIs. Finally, the putative role of innate immune cells in viral spread has been suggested, by showing *in vitro* transfer of virus from macrophages and monocytes to VERO E6 cells.

In the next future, the long-term immunogenicity elicited by vaccines will be tested in both healthcare workers and cancer patients. Moreover, other patient groups will be considered, including transplanted patients and subjects with rheumatologic and autoimmune diseases. All these data will be useful for the scientific community for the design of specific vaccination

strategies, including the administration of a booster dose of vaccine in those subjects with declining immune response. Additionally, the role of B-cell memory response should be further explored and associated with the humoral and T-cell response longevity.

In conclusion, the emergence of COVID-19 pandemic has dramatically changed the field of infectious diseases, posing new issues in terms of pandemic control and prevention. Further studies are necessary, not only to better study the SARS-CoV-2 but also to deeper study and characterize the *Coronaviridae* family that showed a high pandemic potential in the last twenty years.

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