UNIVERSITÀ DEGLI STUDI DI PAVIA

FACOLTÀ DI MEDICINA E CHIRURGIA

DOTTORATO IN MEDICINA SPERIMENTALE, XXXII CICLO

STUDY OF THE CLINICAL AND MOLECULAR PHENOTYPE AND NATURAL HISTORY OF ENTEROPATHIES WITH VILLOUS ATROPHY OF UNKNOWN ORIGIN AND THERAPEUTIC POTENTIAL OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS

Tutor:
Chiar.mo Prof. Federico Biagi

Tesi di Dottorato

Dr.ssa Annalisa Schiepatti

matricola: 451337

Anno Accademico 2018-2019

“Share your knowledge. It is a way to achieve immortality”

Dalai Lama

A Umberto, ai miei genitori e a mio fratello

SUMMARY

INTRODUCTION 1

-Concise summary of the phD project 2

-Concise summary of the project on the study of natural history and development of new therapeutic strategies in complicated coeliac disease 4

1. BACKGROUND 5

1.1 Coeliac disease and its complications 5

1.2 Refractory coeliac disease 7

1.3 Enteropathies with villous atrophy and negative coeliac serology (seronegative villous atrophy) 9

1.4 Enteropathies with villous atrophy of undefined etiology 14

1.5 Cellular therapies and mesenchimal stem cells 15

-mesenchimal stem cells 15

- Rationale for using MSC in the treatment of enteropathies unrelated to gluten ingestion 15

2. PhD PROJECT 17

-Brief description on background 17

2.1 AIMS 17

-Experimental part 1- Description of natural history of enteropathies found in the context of SNVA 17

-Experimental part 2- Description of clinical and molecular phenotypes and natural history of IVA 17

-Experimental part 3- Eligibility to MSC treatment 18

-Experimental part 4- Evaluation of efficacy, safety and security of serial bone-marrow derived MSC infusions 18

2.2 PATIENTS AND METHODS 19

-Diagnostic criteria for patients affected by SNVA and IVA 19

-Collection of data on clinical, histological, molecular phenotypes and natural history of patients affected by SNVA and IVA 19

-Eligibility to MSC treatment and proposed therapeutic protocol 20

-Scheduled protocol of therapy for patients who met the enrolment criteria 21

-Evaluation of efficacy, safety and security of serial bone-marrow derived MSC infusions 22

-Criteria to evaluate clinical and histological response to MSC 22

-Procedures for safety monitoring during the trial 23

-Criteria for withdrawal of patients from the study 23

3. RESULTS

-Experimental part 1- Description of natural history of enteropathies found in the context of SNVA 24

-MORTALITY AND DIFFERENTIAL DIAGNOSES OF VILLOUS ATROPHY WITHOUT COELIAC ANTIBODIES **24**

- THE HIGH MORTALITY OF PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY AND SMALL BOWEL VILLOUS ATROPHY 36

-Experimental part 2- Description of clinical and molecular phenotypes and natural history of enteropathies with IVA 45

- CLINICAL PHENOTYPE AND MORTALITY IN PATIENTS WITH IDIOPATHIC SMALL BOWEL VILLOUS ATROPHY: A DUAL CENTRE INTERNATIONAL STUDY 45

-Results of the project on the study of natural history and development of new therapeutic strategies in complicated coeliac disease 66

- RISK OF COMPLICATIONS IN COELIAC PATIENTS DEPENDS ON AGE AT DIAGNOSIS AND TYPE OF CLINICAL PRESENTATION 66

- NON-RESPONSIVE AND REFRACTORY COELIAC DISEASE: THE LARGEST UK EXPERIENCE FROM THE NHS ENGLAND RARE DISEASES COLLABORATIVE NETWORK **75**

-Experimental parts 3 and 4-Efficacy and safety of MSC infusions in refractory coeliac disease and in non-coeliac enteropathies with villous atrophy: the Pavia experience 76

CONCLUSIONS 77

REFERENCES 79

ACKNOWLEDGEMENTS 86

INTRODUCTION

This PhD project started in 2016 and aimed firstly to define the clinical and molecular phenotype and natural history of enteropathies with VA of undefined etiology, that need to be clearly distinguished from both coeliac disease and its complications and other non-coeliac enteropathies with VA due to a known cause. Secondly, we aimed to evaluate the therapeutic potential of mesenchymal stem cells infusions for the treatment of these rare conditions.

The main clinical part of this PhD project (February 2018-August 2019) has been carried out in collaboration with the NHS England National Centre for coeliac disease and rare diseases, Sheffield, UK.

The current PhD project is part of a wider research project that started in 2011 with the aim of investigating risk factors, natural history and potential new treatments for complicated forms of coeliac disease.

In this thesis we will describe the results of the aforementioned PhD project as well as the latest results of the project on complicated forms of coeliac disease carried out over the same timeframe (October 2016-September 2019).

CONCISE SUMMARY OF THE PhD PROJECT (in collaboration with NHS England National Centre for coeliac disease and rare diseases, Sheffield, UK)

Background. Small bowel villous atrophy is mainly caused by coeliac disease, a common chronic gluten-dependent enteropathy, which is burdened by a high mortality due to its complications. Although VA is the histological hallmark of coeliac disease and its complications, this lesion is not specific to coeliac disease and can be found in other enteropathies unrelated to gluten ingestion. This is the case for autoimmune enteropathy, common variable immunodeficiency, infections, drug-related enteropathies and some forms of lymphoproliferative disorders primarily involving the small bowel.

Finally, there are some enteropathies with villous atrophy, whose etiology remains undefined despite extensive investigations. Correct diagnosis and appropriate management of these forms of enteropathies of unknown etiology are challenging. Because of the negative coeliac serology at time of diagnosis, the overlap of histological patterns and their unresponsiveness to gluten withdrawal, they are often misdiagnosed as forms of complicated or refractory coeliac disease. Moreover, similarly to complicated forms of coeliac disease, response to traditional immunosuppressive therapies is often unsatisfactory and contemporary mortality data suggest poor outcome. The identification of diagnostic criteria enabling the distinction of these enteropathies from refractory coeliac disease and the definition of their natural history is of outmost importance for clinical practice. Recently, there has been a growing interest on the therapeutic potential of bone marrow derived mesenchymal stem cells for the treatment of autoimmune enteropathy, refractory coeliac disease and other autoimmune disorders unresponsive to traditional immunosuppressive regimens.

Aims.

- To define the clinical and molecular phenotypes and natural history of enteropathies with villous atrophy of unknown etiology and to target the differential diagnosis with refractory coeliac disease

and other non-coeliac enteropathies with villous atrophy. This is a first mandatory step in order to identify patients that may be eligible to treatment with mesenchymal stem cells.

- To evaluate safety and efficacy of serial infusions of bone-marrow derived mesenchimal stem cells for the treatment of these enteropathies.

CONCISE SUMMARY OF THE PROJECT ON THE STUDY OF NATURAL HISTORY AND DEVELOPMENT OF NEW THERAPEUTIC STRATEGIES IN COMPLICATED COELIAC DISEASE

Background. Mortality in coeliac disease is higher than in the general population and it is mainly due to the development of complications, whose natural history is poorly understood. The lack of effective and standardized therapies for complicated coeliac disease and their high mortality make the early recognition of coeliac patients at risk of developing complications and the discovery of new targeted therapies an urgent need. Some encouraging results related to the immunomodulatory properties of mesenchymal stem cells on gliadin-specific T-lymphocites which drive the inflammatory process responsible for the intestinal damage in coeliac disease, may represent a promising starting point for the development of potential new treatments in refractory coeliac disease. Contemporaneously, a mandatory step is the distinction of refractory coeliac disease from other non-coeliac enteropathies with villous atrophy refractory to traditional immunosuppressants that may be alternatively treated with advanced cellular therapies.

Aims.

- To identify predictors of complications enabling clinicians to early recognize coeliac patients at higher risk of poor long-term outcomes
- To define the natural history of complicated coeliac disease
- To try to identify clinical and molecular phenotypes of non-coeliac enteropathies with villous atrophy that may often be misdiagnosed as refractory coeliac disease
- To develop targeted therapies alternative to traditional immunosuppressants for the treatment of refractory coeliac disease and other forms of complicated coeliac disease

The last two endpoints strictly link this project to the proper PhD project we previously described.

1. BACKGROUND

1.1 COELIAC DISEASE AND ITS COMPLICATIONS

Coeliac disease (CD) is a chronic gluten-dependent enteropathy occurring in HLA DQ2/DQ8 individuals and characterised by a certain degree of villous atrophy (VA) and positive tissue-transglutaminase/endomysial antibodies in the vast majority of cases [1-3]. Prevalence of CD has been reported to be as high as 1% in the general population and recent evidences suggest it is still going to increase [4-7]. CD is also burdened by a mortality twice as high as that of the general population and mainly due to the development of complications (CCD), which include refractory CD (RCD), ulcerative jejunitis (UJI), enteropathy-associated T-cell lymphoma (EATL), abdominal B-cell lymphomas (BCL) and small-bowel carcinoma (SBC) [8-18]. These conditions are mainly pre-neoplastic/neoplastic in nature and, although very rare (estimated annual incidence around 0.2% of all coeliac patients), they are burdened by a very dismal prognosis, mainly because of the lacking of effective standardised therapies [8,13].

In the last 15 years it has emerged that the mortality of coeliac patients depends on several factors, strict adherence to a gluten free diet (GFD), clinical type and age at diagnosis of CD being the most important ones [9-12, 19]. Other factors such as a long diagnostic delay and male sex were initially described as risk factors for complications [10,20], but they were not subsequently confirmed [12,19]. Finally, HLA typing was shown to correlate with the severity of clinical picture at diagnosis of CD and onset of complications [21,22].

Therefore, great attention has been dedicated either to the early identification of coeliac patients at higher risk of developing complications or the development of more effective and targeted therapies.

In the last years our Coeliac Centre in Pavia in collaboration with other Italian and European Centres for the study of CD has focused the attention on both these research fields. Not only we have studied the natural history of CCD [13], but we also tried to identify early predictors for developing complications in CD [12,19].

We conducted an Italian multicenter study to investigate the natural history of 87 patients affected by a form of CCD. We found that, although the complications of CD tend to develop just after the diagnosis of CD in most cases, they can also arise even after many years. Secondly, we demonstrated that on the basis of the initial response to a GFD it is possible to identify two different clinical forms of CCD, that are characterized by substantial differences in terms of survival (Figure 1). More precisely, in patients who do not show any remission of symptoms that led to the initial diagnosis of CD on a GFD (type A cases-primary complications), the prognosis is worse than in patients in whom the complication of CD arose after initial remission of symptoms (type B cases-secondary complications) [13].

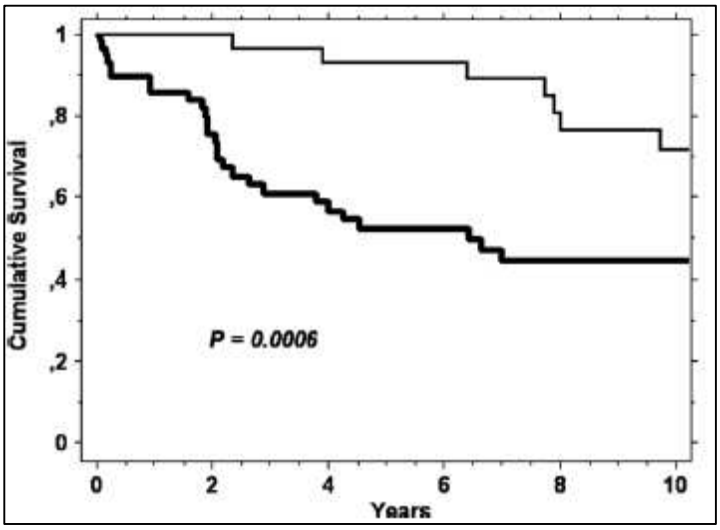


Figure 1. Kaplan Meier curve showing cumulative survival from diagnosis of complicated coeliac disease to death in 87 cases divided according to type of natural history of disease. Thin line: type B cases, who initially responded to a gluten-free diet; thick line: type A cases, who never respondedto a strict gluten-free diet. [13]

1.2 REFRACTORY COELIAC DISEASE

RCD is one of the most common complications of CD. By definition, it is characterized by lack of clinical and histological response to a GFD, strictly conducted for at least 12 months and in the absence of other complications and overt malignancies [1-3,14,18,23-25]. On the basis of the immunophenotype of intraepithelial lymphocytes (IELs), RCD is further divided into type 1 and type 2 RCD, which are characterized by different prognoses and therapeutical approaches [1-3,14,18,23-26]. Type 1 RCD is characterized by a policlonal phenotype of IELs. On the contrary, the hallmark of type 2 RCD is a phenotipically aberrant intraepithelial T-lymphocyte population CD103+, CD7+ CD3-, CD4-, CD8+ with concomitant expression of intracellular CD3ε [24, 26, 27]. This T-cellular subset displays monoclonal rearrangement for γ-chain of T-cell receptor (TCR) [24,26]. It has been shown that 60-80% of type 2 RCD patients may develop an overt EATL within 5 years from diagnosis. On the contrary, in type 1 RCD the evolution to EATL is almost anecdotal and malnutrition and infectious complications are the main causes of death in this subgroup of patients [23, 25, 28-30].

Distinction between type 1 and type 2 RCD is crucial for clinical practice and relies on the the demonstration of the clonal phenotype of IELs. A percentage >50% of CD3- CD8+ IELs on traditional immunohistochemistry, or >20% of CD103+ CD3- CD8+ by means of flow cytometry as well as the presence of monoclonal rearrangement for genes encoding the gamma-chain of TCR are the key diagnostic criteria established so far for the identification of type 2 RCD [23-27].

A combination of nutritional support and immunosuppressive treatments are usually the basis for therapy in RCD [23, 31]. However, up to now, a standardized therapy for RCD has not been found yet and the optimal treatment for each patient is based on the results of trials on small series of patients and on the clinical experience of referral centres. Apart from nutritional support, main options for treatment include systemic steroids [23, 25, 31], topical

steroids such as budesonide [32,33], traditional immunosuppressive regimens [31, 34, 35], biologics [36-38], cladribine [39,40] and autologous hematopoietic stem cells transplantation [41, 42]. However, all these therapies have provided little effect on the final outcome of the patients and they are also burdened by some serious collateral effects. Systemic steroids increase the risk of osteoporosis, diabetes and infections, which are difficult to manage in the context of severe compromised patients. It has been described that azathioprine might increase the risk of developing intestinal lymphoma in patients affected by type 2 RCD [34, 35]. Finally, although hematopoietic stem cell transplantation had allowed to improve survival in a cohort of 13 patients with type 2 RCD, this therapy was not sufficient to prevent the development of lymphoma in some of them [41, 42]. Moreover, it is a very invasive procedure for severely compromised patients.

In the last years in our Celiac Centre in Pavia, we have not only tried to identify early predictors of complications, but we have also investigated the therapeutic potential of alternative treatments in patients affected by RCD and other non-coeliac enteropathies with VA, ie.autoimmune enteropathy unresponsive to traditional immunosuppressants. More precisely, we have obtained promising results with serial infusions of autologous bone-marrow derived mesenchymal stem cells (MSC) as rescue therapy for one case of type 2 RCD and one case of autoimmune enteropathy unresponsive to traditional immunosuppressive regimens (see paragraph 1.5) [43, 44].

The early identification and treatment of patients affected by RCD is an urgent need for clinicians.

1.3 ENTEROPATHIES WITH VILLOUS ATROPHY AND NEGATIVE COELIAC SEROLOGY (SERONEGATIVE VILLOUS ATROPHY)

Villous atrophy is the histological hallmark of CD and its complications. Although the normalization of the histological lesions on a GFD is pathognomonic for both seroposive CD and the rare forms of seronegative CD, VA cannot be considered highly specific for this condition, as it can be found in other non-coeliac enteropathies [45-48]. Although a growing interest has been dedicated to non-coeliac enteropathies - particularly after the discovery of olmesartan-associated enteropathy in 2012 [49, 50]- these conditions still pose a diagnostic challenge to clinicians. Because of the negative coeliac serology at time of diagnosis, the frequent overlap of clinical and histological pattern and their unresponsiveness to gluten withdrawal, they are often misdiagnosed as forms of complicated or refractory CD. This is the case for autoimmune enteropathy [51, 52], common variable immunodeficiency [53,54], and some forms of lymphoproliferative disorders primarily involving the small bowel [55], just to name a few.

A detailed description of these enteropathies found in the context of seronegative villous atrophy (SNVA) and a practical guide to the differential diagnosis with seronegative CD/refractory CD has been provided in the following review paper [48].

Review article

Overview in the clinical management of patients with seronegative villous atrophy

Annalisa Schiepatti[a,c,d], David S. Sanders[d], Martina Zuffada[a], Ombretta Luinetti[b], Adam Iraqi[a] and Federico Biagi[a]

Differential diagnosis and management of enteropathies found in the context of seronegative villous atrophy (VA) are still a clinical challenge. Although seronegative coeliac disease may be the most frequent cause of serology-negative VA, other conditions must be taken into account in the differential diagnosis of seronegative VA. The rarity of these enteropathies with frequent overlapping of histological features may result in misclassification of such patients as affected by a seronegative or a refractory form of coeliac disease with consequent inappropriate treatments and long-term morbidity. The aim of this review is to summarize the current knowledge and to provide an evidence base and practical algorithmic approach for the investigation and management of seronegative VA. Eur J Gastroenterol Hepatol 31:409–417

Introduction

Coeliac disease (CD) is a chronic gluten-dependent enteropathy affecting about 1% of the population worldwide [1,2]. Villous atrophy (VA), crypt hyperplasia and an increased intraepithelial lymphocyte count are well known to be the histological hallmarks of untreated CD. In adult patients, IgA tissue transglutaminase (tTG) and/or endomysial antibodies (EmA) on a normal gluten-containing diet (GCD) are required to establish the diagnosis of CD [3,4]. There has been recent interest in the diagnostic role of deamidated gliadin peptides [5–7]. The vast majority of CD patients carry the HLA DQ2 and/or DQ8 molecules and other HLA molecules such as DQ7.5 may very rarely predispose to the development of CD [3,4,8]. Although not that common, CD can manifest with negative specific serology at diagnosis and is therefore referred to as seronegative coeliac disease (SCD) [9].

Although the normalization of the histological pattern on a gluten-free diet (GFD) is pathognomonic for both seropositive and SCD, VA cannot be considered highly specific for this condition as it can be found in other enteropathies unrelated to gluten ingestion that do have a wide aetiological heterogeneity (Table 1) [3,4,9–20]. Correct identification and targeted management of these other forms of enteropathy may avoid the misdiagnosis of seronegative villous atrophy (SNVA), which in turn can result in patients being placed unnecessarily on a lifelong GFD or even incorrectly classified as refractory CD and given immunosuppressive therapies [9,12–16]. Therefore, this review will provide an evidence base and practical approach for the investigation and management of patients with SNVA.

European Journal of Gastroenterology & Hepatology 2019, 31:409–417

Keywords: differential diagnosis, seronegative coeliac disease, villous atrophy

[a]Coeliac Centre/First Department of Internal Medicine, [b]Department of Pathology, Fondazione Policlinico San Matteo, [c]Department of Experimental Medicine, University of Pavia, Pavia, Italy and [d]Academic Department of Gastroenterology, Royal Hallamshire Hospital, University of Sheffield, Sheffield, UK

Correspondence to Annalisa Schiepatti, MD, First Department of Internal Medicine, Coeliac Centre, IRCCS Policlinico San Matteo Pavia, University of Pavia, Piazzale Golgi 19, 27100 Pavia, Italy

Tel: +39 038 250 2284/2559; fax: +39 038 250 2618; e-mail: annalisa.schiepatti01@universitadipavia.it

Received 5 October 2018 **Accepted** 9 November 2018

Seronegative coeliac disease

SCD is a form of CD characterized by negative IgA/IgG tTG/EmA and a certain degree of VA showing clinical and histological response to a GFD [3,4,9]. SCD may be found in early disease with lesser degree of VA [21–23], late-stage disease (with possible refractory CD or lymphoma) [24] and dermatitis herpetiformis [25]. Also, patients who are IgA tTG/EmA negative because of a concomitant immunosuppressive treatment or because they had already been started on a GFD or steroids before serological testing may have a negative serology [9,26]. Although not that common, forms of SCD have also been described in first-degree relatives of coeliac patients [27]. Finally, patients with total IgA deficiency may have negative IgA tTG or IgA EmA, but if IgG tTG or EmA were to be checked, then the serology may be positive [9].

The epidemiology of SCD is poorly defined. It is not fully elucidated whether the differences in the prevalence figures that range from 10 to 20% of all coeliac patients in earliest papers [21–24,28] and afterwards decreased to 6.5% [29] and then around 2% [15,16] reflect a true change in the prevalence of the disease or simply a consequence of better serological markers [9]. Conversely, three contemporary studies concordantly state that SCD is the most common cause of SNVA among patients of White ethnicity, accounting for up to one-third of these cases [13,14,16]. Patients with SCD are older at diagnosis and have more commonly classical features of malabsorption [10–13,19]. This may be in accordance either with a long-lasting disease or with the fact that duodenal biopsies were performed on the basis of clinical picture and regardless from the antibodies result [24]. Deposits of IgA tTG2 antibodies have been found in the small bowel mucosa of seronegative coeliac patients,

IgA and IgG TTG/EMA negative villous atrophy

Non-CD SNVA	Seronegative coeliac disease
Immunomediated and/or immunoproliferative	True SCD
Autoimmune enteropathy	Early-stage disease
Common variable immunodeficiency	Late-stage disease
Crohn's disease	Refractory CD and/or EATL
Lymphoproliferative disorders (MEITL, CD4$^+$ lymphomas, IPSID)	Dermatitis herpetiformis
Inflammatory	Family history of CD
Eosinophilic gastroenteritis	Masked SCD
Peptic duodenitis	Immunosuppressants
Collagenous sprue	GFD already started
Infectious	
Giardiasis and other parasitic infestations	
Whipple's disease	
HIV enteropathy	
Tuberculosis	
Small intestinal bacterial overgrowth	
Tropical sprue	
Helicobacter pylori-induced duodenitis	
Iatrogenic	
Drugs-related (ARB2s, NSAIDs, azathioprine, micophenolate, methotrexate)	
Graft-versus host disease	
Chemotherapy	
Radiation	
Transplanted small bowel	
Idiopathic	
Partial VA spontaneously normalizing on GCD, likely infectious	
Persisting subtotal/total VA refractory to immunosuppressants	

ARB2s, angiotensin II type 2 receptors blockers; CD, coeliac disease; EATL, enteropathy-associated T-cell lymphoma; EMA, endomysial antibodies; GCD, gluten-containing diet; GFD, gluten-free diet; IPSID, immunoproliferative small intestinal disease; MEITL, monomorphic epitheliotropic T-cell lymphoma; SCD, seronegative coeliac disease; SNVA, seronegative villous atrophy; TTG, tissue-tranglutaminase antibodies; VA, villous atrophy.

Table 1. Aetiological classification of serology negative villous atrophy

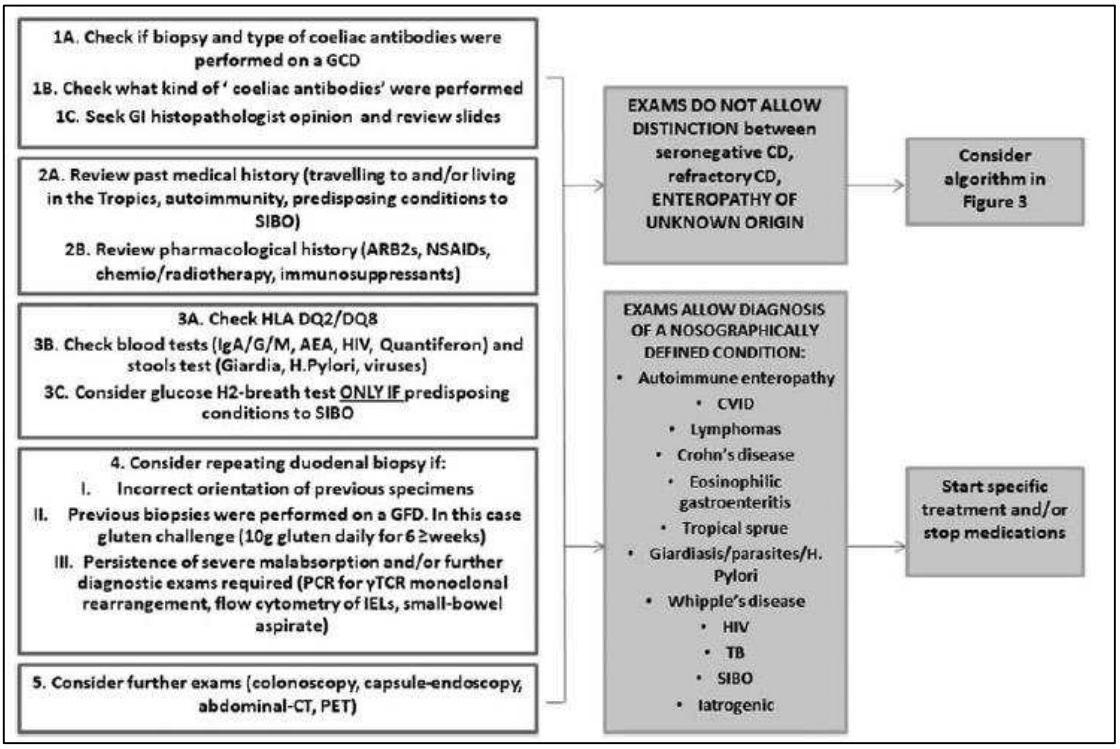


Figure 2. Stepwise algorithm to investigate seronegative villous atrophy-part 1. AEA, antienterocyte antibodies; ARB2s, angiotensin II type 2 receptors blockers;CD, coeliac disease; CVID, common variable immunodeficiency; GCD, gluten-containing diet; GFD, gluten-free diet; IELs, intraepithelial lymphocytes; SIBO, small intestinal bacterial overgrowth; TB, tuberculosis.

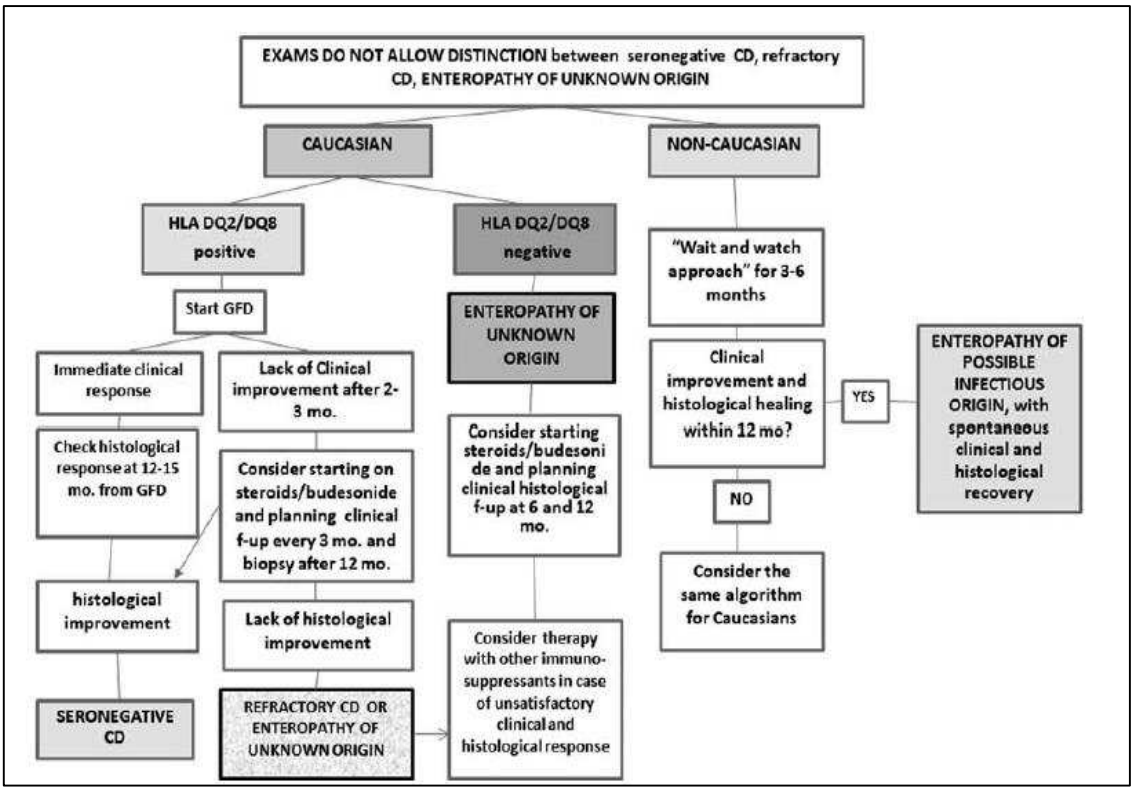


Figure 3. Stepwise algorithm to investigate seronegative villous atrophy-part 2.

CD, coeliac disease; GFD, gluten-free diet; mo, months.

In the absence of current guidelines for the diagnosis and management of SNVA, there are still some points that need to be clarified. They include, firstly, the study of natural history and long-term outcomes of enteropathies found in the context of SNVA. There is only one study by a large UK referral centre which evaluated clinical phenotypes and mortality in SNVA. In this study mortality in SNVA was higher than in conventional seropositive CD [46]. Moreover, both mortality in seronegative CD and in non-coeliac SNVA were higher than in conventional CD. Although the undoubt relevance of this paper, the Authors did not distinguish the group of non-coeliac VA according to their etiology and did not study extensively forms of SNVA of unknown origin.

Secondly, the correct diagnosis of seronegative CD is mandatory. Current knowledge suggests that, despite being very rare, seronegative CD may be the most common cause of SNVA. We have recently proposed a redefinition of the diagnostic criteria for seronegative CD (Table 2) and we have identified the main causes leading to this rare form of CD [56].

REVIEW

Seronegative coeliac disease: clearing the diagnostic dilemma

Annalisa Schiepatti[a], *David S. Sanders*[b], *and Federico Biagi*[a]

Purpose of review
Seronegative coeliac disease is a poorly defined form of coeliac disease that poses an important challenge to clinicians particularly with regards to the differential diagnosis. This is probably because of lack of a consensus on its definition and incorrect use of specific coeliac serology. Seronegative coeliac disease (SCD) is uncommon and epidemiological data are scarce and contrasting. Therefore, the aim of this review is to provide a critical summary of the most recent work on this topic and a definition of SCD.

Recent findings
SCD is rare among coeliac patients but conversely SCD remains one of the most common causes of seronegative villous atrophy. The diagnostic workup of seronegative villous atrophy (SNVA) must ensure exclusion of other enteropathies before starting patients on a lifelong gluten-free diet. This is crucial in order to ensure that patients are not given the wrong diagnosis, which in turn can have implications for their inappropriate treatment and long-term morbidity. Finally, there is some data to suggest that seronegative enteropathies have a higher mortality than conventional coeliac disease.

Summary
Seronegative coeliac disease is a rare condition that accounts for a very small percentage of cases in the large population of coeliac patients. Strict criteria for the diagnosis of this condition need to be fulfilled and prompt identification of these patients is crucial in order to ensure the appropriate intervention on a case-by-case basis.

Keywords
coeliac disease, endomysial antibodies, seronegative, tissue transglutaminase antibodies

INTRODUCTION

Coeliac disease is a chronic enteropathy characterized by a certain degree of villous atrophy and positive serology (IgA or IgG tissue transglutaminase/endomysial antibodies) while on a gluten-containing diet. Histological lesions and serology broadly show normalization on a gluten-free diet (GFD) [1,2].

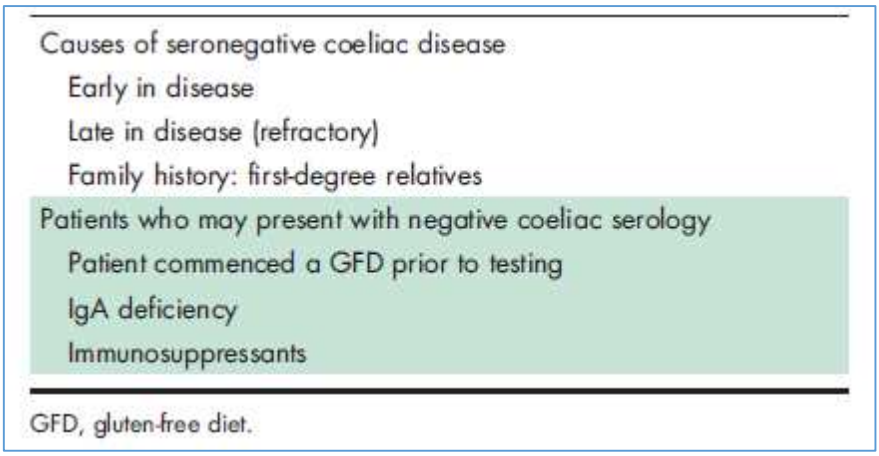
A small percentage of coeliac patients who display villous atrophy but are negative to specific serology at diagnosis do exist and they are considered to be affected by a so-called form of seronegative coeliac disease (SCD). Diagnosis of this condition strictly relies on the histological response to a GFD, after other rare forms of enteropathy unrelated to gluten ingestion have been excluded [1,2].

However, the definition of SCD has generated some confusion in the reported literature and this reflects our growing understanding of this uncommon condition [3–7]. For example, we would not classify a patient with raised intraepithelial lymphocytes only and no villous atrophy on duodenal biopsy (Marsh grade 1) as having SCD but such cases have been categorized in this way by other investigators [8–10]. Another example of confusion could be a paediatric patient with partial villous atrophy and weakly positive IgA endomysial antibodies being labelled as SCD because of negative IgA tissue transglutaminase [9]. Finally, we are not sure that patients with villous atrophy, no IgA and IgG tissue transglutaminase antibodies and no evidence of histological recovery after the beginning of a GFD are affected by SCD [8–10].

[a]Coeliac Centre/First Department of Internal Medicine, University of Pavia, Pavia, Italy and [b]Academic Department of Gastroenterology, Royal Hallamshire Hospital & University of Sheffield, UK

Correspondence to Professor David S. Sanders, Academic Department of Gastroenterology, Royal Hallamshire Hospital & University of Sheffield, Glossop Road, Sheffield S10 2JF, UK. Tel: +44 1142261179; fax: +44 114 2712692; e-mail: David.sanders@sth.nhs.uk

Curr Opin Gastroenterol 2018, 34:154–158
DOI:10.1097/MOG.0000000000000436



Causes of seronegative coeliac disease
Early in disease
Late in disease (refractory)
Family history: first-degree relatives
Patients who may present with negative coeliac serology
Patient commenced a GFD prior to testing
IgA deficiency
Immunosuppressants

GFD, gluten-free diet.

Table 2. Classification of seronegative coeliac disease

1.4 ENTEROPATHIES WITH VILLOUS ATROPHY OF UNDEFINED ETIOLOGY

Despite the current availability of refined diagnostic tools, it should be acknowledged that forms of enteropathy with VA of unknown origin do exist [18, 48]. Although patients with similar enteropathies have been reported in literature [45-47], the clinical and molecular features, genetic background and natural history of these obscure enteropathies have never been investigated systematically. Moreover, it is not yet clear whether these still unrecognized conditions represent the phenotypic expression of a single clinical entity or the final manifestation of some conditions with distinct etiology. Terms such as non-coeliac refractory sprue and undefined sprue have been used previously to label these conditions [18, 45]. However, this nomenclature is currently obsolete and we would rather prefer a definition of 'enteropathies with idiopathic VA' to be used in clinical practice. Quite often these patients do not receive a prompt diagnosis and they can be easily misdiagnosed as having a seronegative or refractory form of CD, given the negative serological tests for CD and the lack of response to a GFD [45-48,56].

In the last seventeen years in our Centre for the diagnosis and management of Coeliac Disease in Pavia we found patients affected by such forms of enteropathy unrelated to gluten ingestion and of unknown origin, which did not show response to traditional immunosuppressive therapies. Similarly, the Sheffield Centre identified patients with VA of unexplained etiology [46]. These patients are the

focus of the current PhD project. Therefore, the clinical part of the project has been carried out concomitantly in the Centres of Pavia and Sheffield in order to describe in details this kind of patients.

1.5 CELLULAR THERAPIES AND MESENCHIMAL STEM CELLS

Mesenchimal stem cells

Advanced cellular therapies based on the use of MSC have recently emerged as a promising therapeutic tool for many immune-mediated pathological conditions, given the multifaceted immunosuppressive and regenerative properties of these cells [57,58]. In this regard, bone marrow derived MSC, given their easy isolation from different sources and *ex vivo* expansion, the ability to undergo multilineage differentiation, and to home to inflamed and damaged sites, are the most commonly used [59]. MSC were also found to exert a powerful immunosuppressive and immunomodulant actions on all cells involved in the immune response, with an ultimate anti-inflammatory and pro-tolerogenic effect [60, 61]. Finally, the substantial lack of immunogenicity makes them an attractive therapeutic tool since an immunoablative conditioning regimen is not needed for their transplantation. Finally, a recent metanalysis about adverse effects related to the use of MSC in clinical trials did not identify any particular concern in terms of safety, with only transient fever thought to be treatment-related [62].

Rationale for using MSC in the treatment of enteropathies unrelated to gluten ingestion

In humans MSC have been safely and successfully employed as rescue therapy in several pathological conditions [57, 58,63,64] with impressing results in terms of complete remission of steroid-refractory acute graft-versus-host disease [65], a condition whose intestinal lesions resemble closely those of untreated CD. As far as the treatment of intestinal inflammatory conditions is concerned, in the First Department of Internal Medicine of our Centre in Pavia and together with the Cell factory of Policlinico San Matteo, Pavia, Italy, autologous MSC have been successfully employed for refractory forms of fistulizing Crohn's disease and for steroid refractory adult autoimmune enteropathy [44,66].

Worthy of note is that more recently, serial infusions of autologous bone marrow-derived MSC has been used as rescue therapy for a patient affected by type 2 RCD [43].

We think that the great immunosuppressive and regenerative properties of MSC play a crucial role in determining their therapeutic effectiveness in the above mentioned clinical settings. In fact, we observed a stable increase in the number of regulatory T-lymphocites both in serum and intestinal biopsies and complete resolution of fistulae in patients with Crohn's disease and mucosal healing in those with autoimmune enteropathy and RCD. Finally, a recent paper by our group have demonstrated the immunomodulant role of MSC on restricted gliadin T-lymphocites of coeliac patients, which are responsible for the intestinal lesions to occur during the active phase of the disease [67].

2. PHD PROJECT- in collaboration with the NHS England National Centre for coeliac disease and rare diseases, Sheffield, UK.

Brief description on background

As previously described in paragraphs 1.3 and 1.4 diagnosis of enteropathies found in the context of SNVA is challenging. These entities are rare and their natural history is poorly defined. A particular subgroup of enteropathies is that of enteropathies with VA of undefined origin, which are oftern misdiagnosed as seronegative or refractory forms of CD. For the purpose of our study we would refer to these enteropathies as forms of idiopathic villous atrophy (IVA). The current PhD project aims to:

-nvestigate the natural history of non-coeliac enteropathies found in the context of SNVA with particular emphasis on those forms of unknown etiology;

-escribe the clinical and molecular phenotypes and natural history of IVA and to evaluate the efficacy and safety of bone-marrow derived autologous MSC infusions as potential therapeutic tool for these patients.

2.1 AIMS

Experimental part 1- Description of natural history of enteropathies found in the context of SNVA

This part of the study aims to describe the natural history and mortality of patients affected by enteropathies found in the context of SNVA and compare these figure with conventional seropositive CD.

Experimental part 2- Description of clinical and molecular phenotypes and natural history of IVA

This part of the study aims to identify the clinical and molecular phenotype, histological features and HLA genetic background of patients affected by IVA. We also aim to define the natural history and long-term outcomes of IVA.

Experimental part 3- Eligibility to MSC treatment

We aim to identify patients affected by forms of enteropathy with IVA who are refractory to a GFD and to traditional immunosuppressive therapies and who might be eligible to the treatment with serial infusions of bone-marrow derived MSC.

Experimental part 4- Evaluation of efficacy, safety and security of serial bone-marrow derived MSC infusions

We aim to evaluate efficacy (clinical and histological response) and safety (any kind of adverse drug reaction) of this kind of advance cellular therapy in patients affected by IVA unresponsive to traditional immunosuppressants.

2.2 PATIENTS AND METHODS

Diagnostic criteria for patients affected by SNVA and IVA

Diagnostic approach to SNVA has been described previously [48].

Diagnosis of IVA was made in adult patients (age> 18 years old) with evidence of frank VA (at least Marsh 3a/Corazza-Villanacci grade B) on correctly oriented biopsies taken from second duodenal portion and in whom no cause for their VA was found. This means that CD, its complications and all the other known causes of VA have been thoroughly excluded following a systematic algorithmic approach [48].

Collection of data on clinical, histological, molecular phenotypes and natural history of patients affected by SNVA and IVA

In order to depict the clinical and epidemiological features of SNVA and IVA, the following data will be collected for each patient:

- age at diagnosis of villous atrophy
- gender
- presenting symptoms at time of diagnosis of VA
- personal and/or family history of CD and/or other autoimmune disorders
- date of last follow-up in clinic or date of death, cause of death
- outcome (alive/dead, date of development of complications) and length of follow-up
- HLA typing
- type and length of therapies performed
- main results of the exams performed at diagnosis and during follow-up (blood tests, endoscopies, histology, radiological exams)

For the histopathological and molecular study data about the following procedures performed on duodenal biopsies of each patient at time of diagnosis of villous atrophy and during follow-up will be collected:

- traditional hematoxilin-eosin staining and Masson's trichrome staining for sub-epithelial collagen on duodenal specimens and traditional immunohistochemistry with staining for CD3, CD8, CD4, CD5, CD56, CD30, CD 138 markers.

- analysis for TCR-γ monoclonal rearrangement by means of PCR on formaline-fixed paraffin embedded duodenal specimens.

- immunophenotipying of mucosal intraepithelial lymphocites by means of flow cytometry in order to identify aberrant CD3- CD8+ IELs [27].

Eligibility to MSC treatment and proposed therapeutic protocol

Patients affected by a form of enteropathy with SNVA may be eligible to serial infusions of MSC if the following criteria are satisfied:

- Lack any clinical (persistence of symptoms of severe malabsorption) and histological (persistence of villous atrophy, ie. at least Marsh 3a or Corazza-Villanacci B2) response to at least 12 months of a strict GFD and at least 3 months of traditional immunosuppressive regimens including systemic corticosteroids, budesonide and azathioprine
- have given written and signed informed consent
- show absence of any kind of malignancy
- have clinical and histological refractoriness to any kind of therapy, as mentioned above.

- for women of a childbearing age, they must have negative serum or urine pregnancy test. Both men and women should use appropriate contraceptive methods.

Exclusion criteria for eligibility to serial infusions of MSC include the following:

- Known history of alcohol or drug abuse in the 12 months prior to inclusion;
- Positivity for HIV, Hepatitis B or C tests, or active infections
- Presence of significant comorbidities, such as diabetes mellitus, uncontrolled hypertension, invalidating psychiatric or neurological disorders, organ failure (renal impairment defined by creatinine clearance <60 ml/min or by serum creatinine ≥2.0 mg/dl – hepatic impairment defined by total bilirubin ≥2.0 mg/dl and AST + ALT ≥2.5 × upper normal value - cardiac failure - respiratory insufficiency)
- Malignancy
- Previous haematopoietic stem cell transplantation
- Patients currently receiving, or having received within 3 months prior to enrolment into this clinical study, any investigational drug

Scheduled protocol of therapy for patients who met the enrolment criteria

For patients fulfilling the aforementioned inclusion criteria, the scheduled treatment consists of intravenous MSC infusions every 3 months at a standard dose of 2.000.000 MSC/kg body weight. The global length of the treatment is one year (total of 4 scheduled MSC infusions). Only autologous bone marrow-derived MSC preparations matching all the release criteria for clinical use, will be used for therapeutic purpose, after re-suspension in a sodium chloride solution and human albumin 20% (3/1 v/v), at a concentration of 1.0-2.0x10^6 cells/ml in a sterile syringe. The route of administration

is intravenously and the dosage is of 1.0- 2.0×10^6 MSC/kg body weight that will be infused at the velocity of 5 ml/minute and scheduled four months apart for four times. The minimum and the maximum dosage allowed is 1.0 and 2.5×10^6 MSC/kg body weight, respectively. MSC will be isolated and expanded ex-vivo from bone-marrow blood samples in accordance with criteria of *Good Manifacturing Practice.* For further details on the procedures of purification, expansion and cryopreservation of MSC we refer to the protocol we previously use for treating patients affecting by fistulising Crohn's disease at the Fondazione IRCCS Policlinico San Matteo, Pavia, Italy [66].

Evaluation of efficacy, safety and security of serial bone-marrow derived MSC infusions

After the end of the 4th MSC infusion a 6 months mandatory follow-up is scheduled. Control visits are scheduled every 2 months during the treatment period and every 3 months during the 6 months period of mandatory follow-up. This is required in order to evaluate clinical conditions and the possible development of any adverse drug reaction. Blood tests will be performed the day of each infusion and after 3 and 6 months after the end of the scheduled treatment. Follow-up duodenal biopsies will be repeated the day of the IV infusion and 6 months after the end of the scheduled treatment or in any time during the course of the treatment if bad clinical conditions would make it necessary.

Criteria to evaluate clinical and histological response to MSC

Clinical response will be evaluated in terms of increase of at least 10% in the BMI compared to baseline, without necessity of total parenteral nutrition and normalization of blood test which were altered at the baseline. Histological response will be evaluated as resolution of villous atrophy (Marsh 0.2) on correctly oriented duodenal specimens at the end of the mandatory 6 months period of follow-up. Immunophenotyping of intraepithelial lymphocytes by traditional immunohistochemistry and flow cytometry, clonality of γ T-cell receptor by means of polymerase chain reaction assay, will also be evaluated on duodenal specimens at the end of the follow-up period.

Procedures for safety monitoring during the trial

As far as the safety evaluation is regarded, the monitoring of systemic tolerance, adverse events (for example, a noxious reaction) and serious adverse events (i.e. a life-threatening condition requiring hospitalization or resulting in disability or death), will be performed from the signing of the informed consent onwards and during every visit. In particular, unexpected adverse events will be monitored and promptly registered, with particular regard to to any opportunistic infection or the appearance of signs or symptoms of malignancy

Criteria for withdrawal of patients from the study

Development of severe adverse events, death or malignancy, psychiatric or neurological disorders, unwillingness to continue the treatment.

RESULTS of the PhD project

Experimental part 1- Description of natural history of enteropathies found in the context of SNVA

Short article

Mortality and differential diagnoses of villous atrophy without coeliac antibodies

Annalisa Schiepatti[a,*], Federico Biagi[a,*], Giacomo Fraternale[a], Claudia Vattiato[a], Davide Balduzzi[a], Simona Agazzi[a], Claudia Alpini[b], Catherine Klersy[c] and Gino R. Corazza[a]

Objective Villous atrophy (VA) of the small bowel is mainly related to coeliac disease (CD), whose diagnosis is made on the basis of positive endomysial/tissue transglutaminase antibodies while on a gluten-containing diet in the vast majority of patients. However, VA can also occur in other conditions whose epidemiology is little known. Our aim was to study the epidemiology and clinical features of these rare enteropathies.
Patients and methods Clinical and laboratory data of all the patients with VA directly diagnosed in our centre in the last 15 years were collected and statistically analysed.
Results Between September 1999 and June 2015, 274 patients were diagnosed with VA. A total of 260 patients were also positive to coeliac antibodies; the other 14 had VA, but no IgA endomysial antibodies: five had common variable immunodeficiency, three had dermatitis herpetiformis, two had IgA deficiency associated with CD, one had abdominal lymphoma, one had unclassified sprue, one had olmesartan-associated enteropathy and one had seronegative CD. Mortality was 6.0 deaths per 100 person years (95% confidence interval: 2.2–16) in patients with VA but negative coeliac antibodies, whereas only 0.2 deaths per 100 person years (95% confidence interval: 0.1–0.6) occurred in coeliac patients.
Conclusion Patients with VA and negative endomysial antibodies are rare. However, these forms of VA identify specific causes that can be diagnosed. These patients are affected by a very high mortality. Eur J Gastroenterol Hepatol 29:572–576

Introduction

In the western world, small bowel villous atrophy (VA) is well known to be mainly related to coeliac disease (CD), a gluten-dependent enteropathy characterized by a certain degree of VA and positive endomysial/tissue transglutaminase antibodies (EMA/TTA) [1].

Although characteristic, VA is not specific for CD, and other enteropathies with VA unrelated to gluten ingestion, despite being very rare, do exist [2,3]. This is the case of small bowel malignancies, enteropathy associated with common variable immunodeficiency (CVID), autoimmune enteropathy, unclassified sprue, collagenous sprue, giardiasis, eosinophilic gastroenteritis and medication-related enteropathies. Finally, there is also a form of CD characterized by VA responding to a gluten-free diet (GFD), but without EMA/TTA. According to some authors, 20% of all coeliac patients are affected by such a 'seronegative form of CD' [4,5].

European Journal of Gastroenterology & Hepatology 2017, 29:572–576
Keywords: coeliac disease, endomysial antibodies, enteropathy, tissue transglutaminase, villous atrophy

[a]First Department of Internal Medicine, Coeliac Centre, Departments of [b]Clinical Biochemistry and [c]Biometry and Clinical Epidemiology, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Pavia, Italy

Correspondence to Federico Biagi, MD, First Department of Internal Medicine, Coeliac Centre, Policlinico San Matteo, Viale Golgi, 19, 27100 Pavia, Italy

Tel: +39 0382 502973; fax: +39 0382 502618; e-mail: f.biagi@smatteo.pv.it

*Annalisa Schiepatti and Federico Biagi contributed equally to the writing of this article.

Received 8 August 2016 **Accepted** 1 December 2016

Whereas the diagnosis of CD is straightforward in the vast majority of cases, being based on the demonstration of VA and positive EMA/TTA while on a gluten-containing diet (GCD) [1], the differential diagnosis of enteropathies characterized by villous atrophy with negative endomysial antibodies (VANES) is rather challenging. In everyday clinical practice, most of the patients referred to a tertiary referral centre for the suspicion of a seronegative form of CD had previously been misdiagnosed as affected by seronegative CD [6,7].

Our aim was to study the epidemiology and the clinical features of patients affected by various forms of VANES diagnosed in our outpatient clinic in the last 15 years. The data were collected using the same strategy that we used in previous epidemiological studies [8].

Patients and methods

Data collection

Between July and August 2015, the clinical notes of all the patients attending our clinic in Pavia between September 1999 and June 2015 were retrospectively analysed. Date of birth, sex, date of diagnosis, date of last contact, date of death or onset of complication and clinical and laboratory data of all the adult patients with duodenal biopsies showing frank VA while on a GCD were collected. All the patients who had not attended our outpatient clinic in the first 6 months of 2015 were contacted over the phone to ascertain that they were still alive. In the case of death, cause and date were obtained through the Italian standard certificates of death. Patients who could not be found were considered to be lost to follow-up and the last time the

0954-691X DOI: 10.1097/MEG.0000000000000836

MORTALITY AND DIFFERENTIAL DIAGNOSES OF VILLOUS ATROPHY WITHOUT COELIAC ANTIBODIES

Annalisa Schiepatti[1], MD, Federico Biagi[1], MD, Giacomo Fraternale[1], MD, Claudia Vattiato[1], MD, Davide Balduzzi[1], MD, Simona Agazzi[1], MD, Claudia Alpini[2], MD, Catherine Klersy[3], MD, MSc, Gino Roberto Corazza[1], MD

[1]*Coeliac Centre/First Department of Internal Medicine, University of Pavia*
[2]*Clinical Biochemistry,Fondazione IRCCS Policlinico San Matteo, Pavia, Italy*
[3]*Biometry and Clinical Epidemiology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy*

European Journal of Gastroenterology and Hepatology 2018;29:572-6.

Introduction

In the western world, small bowel villous atrophy (VA) is well-known to be mainly related to coeliac disease (CD), a gluten-dependent enteropathy characterized by a certain degree of VA and positive endomysial/tissue transglutaminase antibodies (EMA/TTA) [1].

Although characteristic, VA is not specific for CD, and other enteropathies with VA unrelated to gluten ingestion, despite being very rare, do exist [47, 68]. This is the case of small bowel malignancies, enteropathy associated with common variable immune-deficiency (CVID), autoimmune enteropathy, unclassified sprue, collagenous sprue, giardiasis, eosinophilic gastroenteritis, and medication-related enteropathies. Finally, there is also a form of CD characterized by VA responding to a gluten-free diet (GFD) but without EMA/TTA. According to some authors, 20% of all coeliac patients are affected by such a "seronegative form of CD" [69, 70].

Whereas the diagnosis of CD is straightforward in the vast majority of cases, being based on the demonstration of VA and positive EMA/TTA while on a gluten-containing diet (GCD) [1], the differential diagnosis of enteropathies characterized by VA and negative EMA (VANES) is rather challenging. In every-day clinical practice, most of the patients referred to a tertiary referral centre for the suspicion of a seronegative form of CD had previously been misdiagnosed as affected by seronegative CD [45, 71].

Our aim was to study the epidemiology and the clinical features of patients affected by various forms of VANES diagnosed in our out-patient clinic in the last fifteen years. The data was collected using the same strategy we used in previous epidemiological studies [12].

Patients and methods

Data collection

Between July and August 2015, the clinical notes of all the patients attending our clinic in Pavia between September 1999 and June 2015 were retrospectively analysed. Date of birth, sex, date of diagnosis, date of last contact, date of death or onset of complication, and clinical and laboratory data of all the adult patients with duodenal biopsies showing frank VA while on a GCD were collected. All the patients who had not attended our out-patient clinic in the first six months of 2015 were contacted over the phone to ascertain that they were still alive. In the case of death, cause and date were obtained through the Italian standard certificates of death. Patients who could not be found were considered to be lost to follow-up and the last time the patient was seen in the out-patient clinic was fixed as the end of the follow-up. We specify that, to avoid a selection bias, patients diagnosed elsewhere and who came to our centre for diagnostic confirmation were excluded from the study.

To guarantee good quality samples, since 1999 we have routinely taken four duodenal biopsies in the second part of the duodenum during a gastroscopy performed under mild sedation (Midazolam 0.05 mg/kg i.v.). Biopsies are always oriented on cellulose nitrate paper, formalin-fixed, and analysed with dissection microscopy to verify orientation and look for VA. Biopsies are then paraffin-embedded and cut perpendicularly with respect to the luminal surface. Haematoxylin-eosin staining and CD3 immunohistochemical staining, to facilitate intraepithelial lymphocyte count, are routinely performed. Patients undergoing duodenal biopsies at our centre are always tested for IgA ± IgG EMA and enterocyte antibodies (EA) the same morning. We specify that we have always investigated patients with alarm symptoms or symptoms suggestive of malabsorption performing duodenal

biopsies in spite of the result of coeliac antibodies. In other words, we do not perform duodenal biopsies only in patients found to be positive to coeliac antibodies.

EMA and EA are detected on monkey oesophagus/jejunum sections using an indirect immunofluorescence kit (INOVA diagnostic, San Diego, CA). We specify that we do not routinely test for TTA. In our experience, both sensitivity and specificity of EMA and TTA are very similar and satisfactory, as previously described [72]. However, for the purpose of this work, stored frozen serum samples of patients with VANES were tested for both TTA and deamidated gliadin antibodies by means of ELISA tests (EliA Celikey IgA and Celikey IgG; EliA Gliadin DP IgA and EliA Gliadin DP IgG, Phadia AB, Uppsala, Sweden). Finally, we always measure total IgA, IgG and IgM to exclude IgA deficiency and CVID.

Diagnosis of CD was based on duodenal biopsy showing frank VA (either at dissection microscopy or histology), increased intraepithelial lymphocyte count, and positive IgA EMA while on a GCD; in the case of IgA deficiency, IgG EMA are tested and taken into account; in the case of both negative IgG and IgA EMA, diagnosis of CD was based on VA regression following gluten withdrawal [1]. Diagnosis of dermatitis herpetiformis was based on pathognomonic direct immunofluorescent findings on skin biopsy. Diagnosis of enteropathy in CVID, olmesartan-associated enteropathy, autoimmune enteropathy, unclassified sprue, and complications of CD was performed as described in the literature [12, 49-51, 53, 73,74].

Statistical analysis

Data was summarized as counts and percent if categorical and as mean and standard deviation (SD) or median and 25^{th}-75^{th} percentiles if continuous. They were compared between groups with the Fisher exact test and the Student t test or the Mann Whitney U test respectively. The prevalence of VANES was reported together with its 95% exact binomial confidence interval (95%CI). Survival and event-free survival was described by means of Kaplan Meier curves and compared between groups with the logrank test. Hazard ratios (HR) and 95%CI were estimated from a Cox model. Event

rates per 100 person years and 95%CI were computed. Finally, a linear or binomial regression model was used to compute the mean difference and 95%CI in clinical and laboratory data between cases and controls, with calculation of robust standard errors while clustering on matched pairs/triplets. A 2-sided p-value<0.05 was considered statistically significant. Stata 14 (StataCorp, College Station, TX, USA) was used for computation

Ethics

Although all the patients signed informed consent before the biopsies, they could not be asked to consent specifically to this study because of its retrospective nature. After verifying the good quality of the data, the data were all irreversibly anonymized. None of the patients had signed against participation in anonymous studies. The study was approved by the ethics committee of the Fondazione IRCCS Policlinico San Matteo according to the 1975 Declaration of Helsinki (6th revision, 2008).

Results

Between September 1999 and June 2015, VA was found in the duodenal biopsies of 274 patients. Thanks to positive IgA EMA, CD was diagnosed in 260 of them. In the remaining 14 patients (5.1%, 95%CI 2.8-8.5), IgA EMA were negative and they were defined as VANES patients. Table 3 shows the epidemiological features of these patients. Finally, 16 CD patients could not be traced and were considered to be lost to follow-up for the purpose of this paper.

Table 4 shows the demographic and clinical features of the 14 VANES patients. Briefly, CD was diagnosed in six of them despite negative IgA EMA: three patients suffered from dermatitis herpetiformis (pts. 1-3 in tables 2), two patients had CD associated with total IgA deficiency and were positive to IgG TTA (pts. 4, 5), and one patient had a real form of seronegative coeliac disease (pt. 6). Five of these six patients showed a satisfactory clinical and histological improvement after starting a GFD; the sixth patient (pt. 4) died of endocarditis just four months after the diagnosis of CD. In

three of the other eight patients, CD was excluded thanks to HLA typing (pts. 7-9). In the remaining five patients (pts. 10-14), CD could be neither confirmed nor excluded: HLA typing showed either DQ2 or DQ8 in all of them; none of them responded to a GFD; three of them developed typical complications of CD (pts. 10-12). EA were negative in all 14 VANES patients and none of them suffered from autoimmune diseases. Thus, autoimmune enteropathy was excluded in all of them [51]. Patients 7, 8, 12-14 were found to be affected by common variable immunodeficiency and were already thoroughly described [74]. Similarly, patient 9, found to be suffering from olmesartan enteropathy, and patients 10 and 11, who developed EATL, were also described [12, 50]. Finally, table 4 shows that some serological tests turned out to be positive. Sincerely, we believe that 9 positive tests out of 84 do not reduce the strength of our findings.

Cumulative follow-up was 1732 person years for the 260 coeliac patients and 97 person years for the fourteen VANES patients. Four of the 260 CD patients developed complications (type 1 refractory CD, abdominal B-cell lymphoma, small bowel carcinoma, and enteropathy associated T-cell lymphoma), as compared to 4 out of 14 in the VANES group, with a complication rate of 0.2 (95%CI 0.1-0.6) and 6.3 per 100 person years (95%CI 2.4-17.0), respectively. Finally, the combined event complication and/or death was observed in 7/244 and 5/14 patients, respectively, with an event rate of 0.4 (95%CI 0.2-0.9) and 7.9 per 100 person years (95%CI 3.3-19.0); at Cox regression, the HR was computed to 17.82 (95%CI 5.56-57.11, $p<0.001$, Figure 4).

Four patients died in the CD group (3 of unrelated causes and 1 of enteropathy-associated T cell lymphoma) and 4 in the VANES group. The corresponding mortality was 0.2 (95%CI 0.1-0.6) and 6.0 deaths per 100 person years (95%CI 2.2-16), respectively (HR 25.37, 95%CI 6.15-104.63, $p<0.001$, Figure 5).

Some differences were observed when comparing the clinical and laboratory data between patients with VANES and controls (i.e. CD patients) matched for sex, age at diagnosis (±5 years), and clinical presentation according to the Oslo criteria [75] (Table 3 and 5). Although some differences emerged,

suggesting that, similarly to patients with complicated CD, VANES patients are characterised by an increased inflammatory state, we are not sure that they are clinically relevant.

Discussion

We believe that the main result obtained by our study is the observation that in the majority of patients with VANES a precise, definitive diagnosis can be made. Since VANES patients are very heterogeneous (table 4), they need to be thoroughly investigated to reach the correct diagnosis. This is necessary because our results confirm that these conditions have a very high risk of complications and a very high mortality.

When we analysed these results, we debated whether the three patients with dermatitis herpetiformis and the two with CD in IgA deficiency should be included in the study (pts. 1-5 in table 4). They have negative IgA EMA but are positive for other celiac antibodies. We decided to maintain these patients not only to objectively and thoroughly show our results, but also because they do not reduce the strength of our findings. If we excluded them, VANES patients would be even rarer and their mortality would be even worse than the one shown by the Kaplan-Meier curves in fig. 4 and 5.

When we first planned this study, we hoped we would have been able to verify the real prevalence of villous atrophy without coeliac antibodies among all the patients with villous atrophy. However, we rapidly realized that this kind of study was not feasible. Although we tried to be as accurate as possible, including only patients directly diagnosed in our unit, and thus behaving like a secondary gastroenterology referral centre, we realized that two selection biases were nevertheless inevitable. Since we are a referral centre for CD, it is possible that patients attend our clinic because they were found to be positive to coeliac antibodies elsewhere. On the other hand, it is also possible that patients found to be negative to coeliac antibodies were referred somewhere else because CD was believed to be excluded. Since the relevance of these two opposite biases cannot be estimated, we cannot know how far from the truth is the prevalence of 14/274 (5.1%, 95%CI 2.8-8.5) that we found. Nevertheless, we found less than one VANES patient every year. Only one patient (pt. 6 in table 4) was affected by

a real seronegative form of CD, which was diagnosed through a histological and clinical response while on a GFD. So, the prevalence of real seronegative CD that we found (1/274, 0.36%, 95%CI 0.00-2.02) is by far the lowest one reported in the literature. Even if we considered the three patients affected by dermatitis herpetiformis, a form of CD known to have a lower prevalence of coeliac antibodies [13], and the two patients with CD associated with IgA deficiency to be affected by seronegative CD, the prevalence of seronegative CD in our population of CD patients would nevertheless be very low (6/274, 2.2%, 95%CI 0.81-4.70) [70, 76-78].

It is obvious that De Gaetani and Pallav found a higher number of VANES patients because they are tertiary referral centres and must have taken into account all the patients attending their clinic [45, 47]. Having excluded patients diagnosed in other centres and then referred to us, is the reason why we found only 14 VANES patients in 15 years and did not directly diagnose any patients with autoimmune enteropathy, collagenous sprue, and other conditions found by others as causes of VANES [45, 47]. To explain the discrepancy between our results and those found by other groups, who claim that 20% of coeliac patients are negative to coeliac antibodies, is, on the other hand, very difficult [70]. It could be possible that unspecific minor changes of intestinal histology were taken for CD, leading to misdiagnosis. Alternatively, clinical improvement while on a GFD could be due to other conditions such as IgE mediated wheat allergy or non-coeliac gluten sensitivity.

As far as the epidemiology of VANES is concerned, our results show that male prevalence and age at diagnosis are definitely higher in VANES patients than in CD patients, resembling what we obtained in patients with complicated CD. Also the prevalence of classic symptoms (i.e. diarrhoea and weight loss) is higher in VANES patients than in coeliac ones, as shown by others and similar to coeliac patients who will then develop complications [12, 45, 47].

The results of the survival analyses showed that mortality is higher in VANES patients than in CD. Moreover, we must consider that the fourteen VANES patients we found do not definitely represent a homogenous group of patients. Together with patients affected by dermatitis herpetiformis, a form

of CD well-known to have a very good prognosis [79], there are patients affected by CVID and intestinal lymphomas, conditions with a much worse prognosis [12,53,74].

In conclusion, our data showed that in the majority of patients with VANES a precise, definitive diagnosis can be made. Since only 6 out of 14 VANES patients showed a histological response to a GFD and were thus affected by CD (pts 1-6 in table 2), we recommend that patients with VANES should not be hastily labelled as affected by seronegative CD but need to be thoroughly investigated to exclude all the other conditions characterized by VA. Since guidelines on how to deal with VANES patients are still lacking, in our opinion EA, total IgG, IgA and IgM, HIV testing and precise medical history should be included in the diagnostic workout of patients with villous atrophy but without coeliac antibodies. This is necessary because these conditions have a very high risk of complications and a very high mortality.

	CD patients	**VANES patients**	**p value**
Presentation			
Males/patients	82/260	12/14	< 0.001
Age at diagnosis	35±12 years	49±16 years	= 0.007
Classical symptoms [75]	116/260	10/14	<0.001
Follow-up			
Complications	4/244	4/14	<0.001
Deaths	4/244	4/14	< 0.001
Combined Event	7/244	5/14	< 0.001

Table 3. Epidemiological features found in the 260 coeliac patients and in the 14 patients with villous atrophy but negative endomysial antibodies. CD: coeliac disease; VANES: villous atrophy with negative endomysial antibodies.

Pt	Sex	*Age (Years)	Clinical presentation	Respon. to GFD	EMA IgA	EMA IgG	TTA IgA	TTA IgG	dGlia IgA	dGlia IgG	HLA	Diagnosis	Follow-up (months after dgn)
1	M	17	DH	yes	NEG	NEG	**POS**	NEG	NEG	NEG	X	CD+DH	lost to foll.-up (18)
2	M	57	DH	yes	NEG	NEG	NEG	NEG	**POS**	**POS**	DQ2	CD+DH	alive (172)
3	M	29	DH	yes	NEG	NEG	NEG	NEG	NEG	**POS**	X	CD+DH	alive (111)
4	M	63	WL,diarrhoea	yes	NEG	NEG	NEG	**POS**	NEG	**POS**	X	CD+ IgAd	†, endocarditis (4)
5	M	44	WL,diarrhoea	yes	NEG	**POS**	NEG	**POS**	NEG	NEG	DQ2	CD+IgAd	alive (60)
6	F	58	GORD	yes	NEG	**POS**	NEG	NEG	NEG	NEG	DQ8	SN-CD	alive (26)
7	M	30	Anaemia	no	NEG	NEG	NEG	NEG	NEG	NEG	DQX	CVID	alive (108)
8	M	45	WL,diarrhoea	no	NEG	NEG	NEG	NEG	NEG	NEG	DQX	CVID	alive (66)
9	M	56	WL,diarrhoea	no	NEG	NEG	NEG	NEG	NEG	NEG	DQX	OAE	alive (45)
10	M	71	WL,diarrhoea	no	NEG	NEG	NEG	NEG	NEG	NEG	DQ2	US	†, EATL (54)
11	M	71	WL,diarrhoea	no	NEG	NEG	NEG	NEG	X	X	DQ2	EATL	†, EATL (1)
12	M	45	WL,diarrhoea	no	NEG	**POS**	NEG	NEG	NEG	NEG	DQ2	CVID/BCL	†, BCL (2)
13	M	43	WL,diarrhoea	no	NEG	NEG	NEG	NEG	NEG	NEG	DQ2	CVID	GC (70), alive (75)
14	F	57	WL,diarrhoea	no	NEG	NEG	NEG	NEG	NEG	NEG	DQ8	CVID	alive (60)

Table 4. Clinical features found in the 14 patients affected by villous atrophy and negative coeliac antibodies. *Age at diagnosis; BCL: B-cell lymphoma; CD: celiac disease; CVID: common variable immunodeficiency; dgn: diagnosis; DH: dermatitis herpetiformis; DQX: DQ2 and DQ8 negative; EATL: enteropathy associated T-cell lymphoma; GFD: gluten-free diet; GC: gastric cancer; IgAd: IgA deficiency; OAE: olmesartan associated enteropathy; SN-CD: seronegative CD; US: unclassified sprue; WL: weight loss; X: not performed; †: dead.

	CD, N=25 (Mean ±SD)	VANES, N=14 (Mean ±SD)	Mean Difference% (95%CI)	p-value
Hb, mg/dl	12.6 ± 2.2	12.9 ± 1.9	-0.73 (-2.10 to 0.63)	0.27
MCV, fL	88.9 ± 11.1	88.1 ± 6.7	-0.84 (-6.95 to 5.37)	0.77
Albumin, g/dl	4.1 ± 0.4	3.5 ± 0.8	-0.67 (-1.28 t0 0.56)	0.035
CRP, mg/dl	0.30 (0.23-0.40)	0.56 (0.30-1.90)	1.59 (-0.26 to 3.44)	0.09
ESR, mm/h	10.0 (4.0-14.0)	15.0 (6.0-42.0)	27.47 (-5.44 to 60.37)	0.09
Iron, mcg/ml	113.3 ± 52.6	62.4 ± 39.4	-50.9 (-78.08 to -23.84)	0.001
Ferritin, ng/ml	30.3 (5.0-96.5)	94.5 (24.0-168.5)	269.59 (-248.24 to 787.41)	0.28
BMI, kg/m^2	21.9 ± 3.6	20.2 ± 3.1	-1.68 (-4.25 to 0.88)	0.18

Table 5. Clinical and laboratory findings in the 14 patients with villous atrophy but negative celiac antibodies (VANES) and matched controls (CD). N: number; Mean ± SD or Median (25th-75th percentiles)

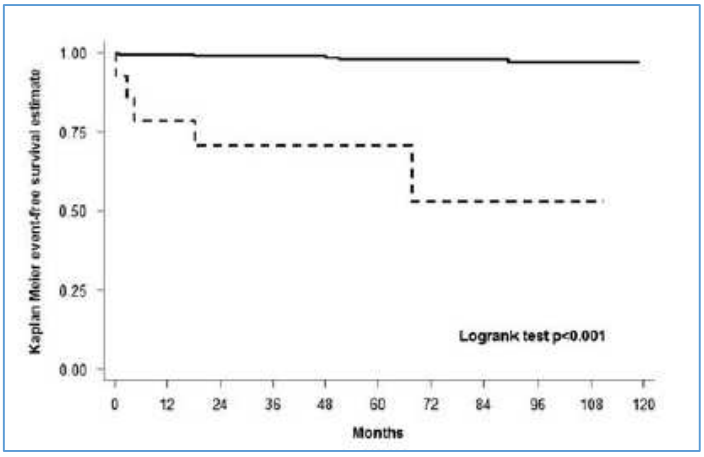


Figure 4. Kaplan–Meier curve showing that the event-free survival estimate (event =onset of complication and/or death) is statistically different between patients with villous atrophy, but negative endomysial antibodies (dashed line), and coeliac patients (continuous line).

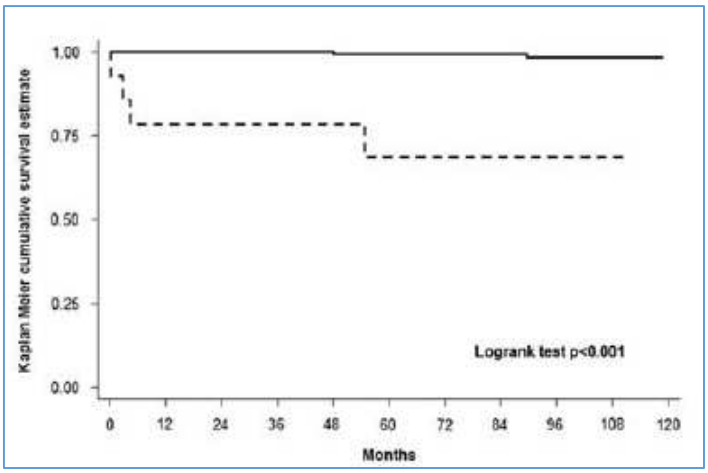


Figure 5. Kaplan–Meier curve showing that the cumulative survival estimate is statistically different between patients with villous atrophy, but negative endomysial antibodies (dashed line), and coeliac patients (continuous line).

SCANDINAVIAN JOURNAL OF GASTROENTEROLOGY
2019, VOL. 54, NO. 2, 164–168
https://doi.org/10.1080/00365521.2019.1568543

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

The high mortality of patients with common variable immunodeficiency and small bowel villous atrophy

Maria Vittoria Pensieri[a], Federica Pulvirenti[b], Annalisa Schiepatti[a], Stiliano Maimaris[a], Salvatore Lattanzio[c], Isabella Quinti[b], Catherine Klersy[d], Gino Roberto Corazza[a] and Federico Biagi[a]

[a]Coeliac Centre/First Department of Internal Medicine, Fondazione IRCCS Policlinico San Matteo University of Pavia, Pavia, Italy; [b]Department of Molecular Medicine, University of Rome Sapienza, Rome, Italy; [c]Faculty of Economics, University of Cambridge, Cambridge, UK; [d]Biometry and Clinical Epidemiology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

ABSTRACT

Objectives: Common variable immunodeficiency (CVID) is a primary humoral immunodeficiency characterised by reduced serum levels of immunoglobulins, recurrent infections, autoimmune phenomena and lymphoproliferative disorders. Gastrointestinal symptoms are very common in these patients and a coeliac-like villous atrophy was described in some of them. Since mortality in CVID is much higher than in the general population, our aim was to evaluate mortality rates and clinical predictors of survival in patients with both CVID and duodenal villous atrophy.

Patients and methods: Sex, date of diagnosis of villous atrophy, HLA genomic typing, date of death/last follow-up, type of complication were retrospectively collected from medical files. Univariate analysis for each predictor was conducted and Kaplan-Meier curves were generated to evaluate survival.

Results: Twenty-three patients were enrolled (9 females, mean age at diagnosis of villous atrophy 38 ± 13 years) and 8 of them died after a median time of 96 months (25th–75th 60–120 months) corresponding to a mortality rate of 3.9 per 100 person-years (95% CI 1.9–7.7). Mortality was higher in men compared to women (60 vs. 11/1000 person-years), although not statistically significant. Causes of death included onco-haematological disorders and infections.

Conclusions: Although based on a small cohort, our results confirm that patients with CVID and villous atrophy are burdened by a very high mortality mainly due to onco-immunological disorders and infections. Strict follow-up is required in these patients.

ARTICLE HISTORY
Received 29 October 2018
Revised 4 January 2019
Accepted 5 January 2019

KEYWORDS
Common variable immunodeficiency; villous atrophy; mortality; coeliac disease; survival

Introduction

Common variable immunodeficiency (CVID), the most common primary immunoglobulin deficiency, is a condition characterised by an elevated mortality due to infectious, neoplastic and autoimmune complications [1,2].

A significant number of patients affected by CVID complain of gastrointestinal symptoms such as diarrhoea, abdominal pain and weight loss. Although in the majority of these cases the symptoms are due to recurrent infections, particularly by Giardia Lamblia [3,4], in many patients it is also possible to find histopathological alterations, unrelated to Giardia Lamblia, such as the absence of plasma cells and an increased intra-epithelial lymphocyte count [4]. Furthermore, in a few patients, in addition to finding an increased intra-epithelial lymphocyte count, it may be possible to find intestinal lesions such as villous atrophy and crypt hypertrophy. So, the whole histological and clinical picture may closely resemble that of untreated coeliac disease [5–7]. The complexity of the problem is further increased by a recent Norwegian study showing that in CVID patients intestinal lesions are not related to the presence of GI symptoms and that patients with frank villous atrophy are very rare (1 out of 50 patients with CVID) [5].

A recent American paper found that the mortality of CVID patients is clearly increased in comparison to the general population and is related to a series of factors such as sex, age at diagnosis, the presence of complications (infectious and non-infectious) and basal immunoglobulin levels. Furthermore, this study demonstrated that patients complaining of gastrointestinal symptoms have an even greater mortality [1].

A few years ago, we described 11 CVID patients with duodenal mucosal atrophy indistinguishable from that of untreated coeliac disease [7]. In view of the high mortality of CVID patients who complain of gastrointestinal symptoms [1], we evaluated the mortality of CVID patients with severe atrophy of the duodenal mucosa. We underline that previous studies on mortality investigated the presence/absence of gastrointestinal symptoms but did not take into consideration the presence/absence of duodenal villous atrophy, likely the most severe form of intestinal involvement in CVID.

CONTACT Federico Biagi f.biagi@smatteo.pv.it Coeliac Centre/First Department of Internal Medicine, Fondazione IRCCS Policlinico San Matteo University of Pavia, Viale Golgi, 19, Pavia 27100, Italy.

THE HIGH MORTALITY OF PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY AND SMALL BOWEL VILLOUS ATROPHY

[1]Maria Vittoria Pensieri, [2]Federica Pulvirenti, [1*]**Annalisa Schiepatti**, [1]Stiliano Maimaris, [3]Salvatore Lattanzio, [2]Isabella Quinti, [4]Catherine Klersy, [1]Gino Roberto Corazza, [1]Federico Biagi

[1]Coeliac Centre/First Department of Internal Medicine, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Italy; *PhD course in Experimental Medicine, University of Pavia; [2]Centro di Riferimento per le Immunodeficienze Primitive dell'Adulto, Università di Roma "Sapienza", Policlinico Universitario Umberto I, Rome; [3]Faculty of Economics, University of Cambridge, UK; [4]Biometry and Clinical Epidemiology, Fondazione IRCCS Policlinico San Matteo

Scandinavian Journal of Gastroenterology 2019;54:164-8

Introduction

Common variable immunodeficiency (CVID), the most common primary immunoglobulin deficiency, is a condition characterised by an elevated mortality due to infectious, neoplastic and autoimmune complications [80, 81].

A significant number of patients affected by CVID complain of gastrointestinal symptoms such as diarrhoea, abdominal pain and weight loss. Although in the majority of these cases the symptoms are due to recurrent infections, particularly by Giardia Lamblia [82, 83], in many patients it is also possible to find histopathological alterations, unrelated to Giardia Lamblia, such as the absence of plasma cells and an increased intra-epithelial lymphocyte count [83]. Furthermore, in a few patients, in addition to finding an increased intra-epithelial lymphocyte count, it may be possible to find intestinal lesions such as villous atrophy and crypt hypertrophy. So, the whole histological and clinical picture may closely resemble that of untreated coeliac disease [53, 74, 84]. The complexity of the problem is further increased by a recent Norwegian study showing that in CVID patients intestinal lesions are not related to the presence of GI symptoms and that patients with frank villous atrophy are very rare (1 out of 50 patients with CVID) [84].

A recent American paper found that the mortality of CVID patients is clearly increased in comparison to the general population and is related to a series of factors such as sex, age at diagnosis, the presence of complications (infectious and non-infectious) and basal immunoglobulin levels. Furthermore, this study demonstrated that patients complaining of gastrointestinal symptoms have an even greater mortality [80].

A few years ago, we described 11 CVID patients with duodenal mucosal atrophy indistinguishable from that of untreated coeliac disease [74]. In view of the high mortality of CVID patients who complain of gastrointestinal symptoms [80], we evaluated the mortality of CVID patients with severe atrophy of the duodenal mucosa. We underline that previous studies on mortality investigated the presence/absence of gastrointestinal symptoms but did not take into consideration the presence/absence of duodenal villous atrophy, likely the most severe form of intestinal involvement in CVID.

Patients and methods

Data collection

Between October 2017 and April 2018, the clinical notes of all the patients affected by villous atrophy and CVID attending the 1st Department of Internal Medicine of the University of Pavia and the National Referral Centre for Primary Immunodeficiencies of the Adult at Policlinico Universitario Umberto I in Rome were re-evaluated. More precisely, we underline that all the patients seen at Pavia were referred by other centres for the aetiological investigation of subtotal villous atrophy not responsive to a gluten-free diet and causing a severe malabsorption syndrome. They had all considered to be affected by seronegative/refractory coeliac disease. The patients diagnosed with villous atrophy in Rome come from a series of 212 patients, who have all undergone upper GI endoscopy with gastric and duodenal biopsies, regardless of the clinical picture and the presence or absence of gastrointestinal symptoms, as previously described [81].

For enrolment in this study, the diagnosis of CVID was based on the presence of a deficiency of at least two immunoglobulin classes with values at least two standard deviations lower than the reference values and on the exclusion of other causes of primary and secondary hypogammaglobulinemia [85, 86]. Diagnosis of atrophy of the duodenal mucosa was based on at least four well-oriented endoscopic duodenal biopsies showing frank villous atrophy, crypt hypertrophy and an increased intra-epithelial lymphocyte count. Coeliac disease and other causes of atrophy of the duodenal mucosa such as autoimmune enteropathy and olmesartan enteropathy were excluded on the basis of the patient's history, the absence of specific antibodies (endomysial and enterocyte antibodies) and HLA genomic typing [1, 22, 49, 87]. Flow cytometric analysis of intraepithelial lymphocytes excluded refractory coeliac disease type 2 [27]. Finally, infection by Giardia Lamblia was excluded with fecal parasitological examination.

Sex, age at diagnosis of villous atrophy, year of diagnosis, age at last outpatient clinic access or phone contact, age at onset and type of possible complication, age at death and cause of death were recorded for each patient. We specify that, to ascertain the health status and, particularly, whether patients were alive or deceased, we contacted by telephone all patients who had not been seen in our units within the last 3 months. For those patients who we could not contact by telephone, we contacted the registry office of their place of residence. The mortality data collected from our patients were compared with both those of the Italian general population (according to Italian National Institute of Statistics) and those found in the scientific literature [80, 81]. Finally, the presence or absence of infectious and non-infectious complications, HLA genomic typing and basal immunoglobinal levels were collected for patients followed in Pavia.

Statistics

Data are described as mean and standard deviation or median and 25th-75th percentiles if continuous and as counts and percent if categorical. They are compared between centers by means of the Mann Whitney U test and the Fisher exact test, respectviely. Mortality of the disease is computed on the

whole population of patients and on subgroups by gender, age, presence of infectious or non-infectious complications, HLA typing and basal immunoglobinal levels, as the number of deaths over the sum of follow-up times and is reported as deaths per 100 person year. The median follow-up time is computed together with its 25^{th}-75^{th} percentiles with the inverse Kaplan Meier method. Survival curves are calculated according to the Kaplan-Meier methodology; they are compared with the log-rank test. Hazard ratios and 95% confidence intervals (95%CI) are derived from Cox regression models. Mortality in our series is compared to that retrieved from the literature and relative risk ratios (95%CI) are reported; p-values are obtained with the mid-P method. The observed cumulative survival in our population is plotted together with the expected survival in an Italian age and sex matched population, retrieved from the Italian vital statistics, according to Finkelstein [88]. All tests are two-sided. We use Stata 14 (StataCorp LP, College Station, TX, USA) to run our analyses.

Ethics

All patients signed informed consent before the biopsies, both for clinical and research purposes. After verifying the good quality of the data, the data were all irreversibly anonymized. None of the patients had signed against participation in anonymous studies. The study was approved by the ethics committee of the Fondazione IRCCS Policlinico San Matteo according to the 1975 Declaration of Helsinki (6th revision, 2008).

Results

Between 2001 and 2016, 12 patients with villous atrophy and CVID were diagnosed in Pavia. Eleven of them were described in a previous study [74]. Eleven patients were diagnosed in Rome from a series of 212 CVID biopsied patients, corresponding to a prevalence of villous atrophy of 5.2% (95%CI 2.6-9.1). Thus, overall 23 adult patients with villous atrophy and CVID (9 females, mean age at diagnosis of villous atrophy 38 ± 13 years) were enrolled. Table 6 summarizes the main characteristics of these patients. Genomic HLA typing showed that 6 of 11 patients from Pavia were

positive for HLA-DQ7, 5 of 11 were positive for HLA-DQ2, both were considered to be chance findings and did not affect the clinical evaluation. The two Centres were comparable except for the rate of complications with Pavia showing a higher rate of infectious complications and Rome a higher rate of non infectious complications, as defined [80]. Of note, all the patients diagnosed in Pavia had malabsorption as compared to 73% in Rome.

The median follow-up of these 23 patients was 144 months (25^{th}-75^{th} 108-168). At the time of our study, 8 patients were deceased, after a median time of 96 months (25^{th}-75^{th} 60-120 months), corresponding to a mortality rate of 3.9 per 100 person-year (95%CI 1.9.-7.7). This mortality was higher than the rate computed from the USA cohort (1.1 deaths per 100 person year, 95%CI 0-9-1.4), with a relative risk ratio of 3.42 (95%CI 1.43-7.01, p=0.002), and the rate computed from the Italian cohort (0.5 deaths per 100 person year, 95%CI 0.3-0.9), with a relative risk ratio of 7.66 (95%CI 2.35-19.93, p<0.001) [80, 81]. In addition, as shown in figure 6, the cumulative survival was lower than what expected in the general sex- and age-matched Italian Population.

Causes of death were onco-haematological in 4 patients (2 intestinal and one extra-intestinal lymphomas, one gastric cancer), pneumonias in 2 but unknown for the last 2 patients. No significant differences in mortality were elicited in any of the candidate predictors (Table 7). Nonetheless, it is worth noting that mortality rates were more than two times higher for the Pavia Centre, for male, and in the absence of infectious complications; it was lower in the presence of HLA DQ2.

Discussion

Although villous atrophy in CVID patients is rare [84], it is a condition posing important diagnostic and prognostic issues. First of all, CVID with villous atrophy poses an important problem of differential diagnosis with coeliac disease. Not only can the clinical picture be very similar, but also the histological features can be almost indistinguishable. Looking for coeliac antibodies in patients who lack immunoglobulins is useless, if not misleading [74]. Consequently, HLA typing and

histological response to a gluten-free diet remain the cornerstone for the differential diagnosis with coeliac disease and, most importantly, seronegative coeliac disease [53, 74, 89].

It is well known that CVID is characterised by a high mortality rate and our results show that in patients with CVID and villous atrophy the mortality rate is further increased. Our study is limited by a relatively small sample size and a dishomogeneous patient population, which, particularly in Pavia, is likely to have a selection bias due to the severe clinical condition affecting the patients referred to Pavia. The mortality of patients from Pavia, in whom the diagnosis of CVID was made following investigations aiming to determine the aetiology of villous atrophy and malabsorption, was indeed higher, although not statistically significant, in comparison to those from Rome in whom the diagnosis of villous atrophy was made due to endoscopic screening of patients with an established diagnosis of CVID. In addition, the differences between our findings and those present in the literature are so evident possibly due to the more severe condition (pathological lesions) in our series. Moreover, other predictors such as female gender, HLA DQ2 and infectious complications might be associated with a less severe mortality in these patients, though statistical significance was not reached, given the rarity of the condition reported in this case series.

The prevalence of villous atrophy among CVID patients from Rome confirms this to be a rare finding (11/212, 5%), although not as rare as a recent Norwegian study suggests (1/50, 2%) [84].

Although villous atrophy in CVID patients poses an important problem of differential diagnosis with coeliac disease, we found that the causes of death in these two conditions are different. While in coeliac patients it is well known that increased mortality is mostly due to the complications of coeliac disease itself [8-13], in CVID the cause of death does not appear to be always related to the intestinal involvement. It is therefore difficult to understand whether in CVID it is villous atrophy that directly increases mortality or whether villous atrophy is merely a marker of a more severe form of disease which, therefore, suffers from a higher mortality. All this could have important therapeutic consequences, potentially supporting the use of immunosuppressive drugs in addition to the current

use of immunoglobulin infusions. Only further studies by means of endoscopic and histological investigations of CVID patients will allow a better understanding of this rare, but severe condition.

Variable	*statistic*	All (N=23)	Pavia (N=12)	Rome (N=11)	p-value
Age (years)	*Mean (SD)*	**38 (13)**	39 (9)	38 (16)	0.579*
Age>39 years	*N (%)*	**11 (48%)**	6 (50%)	5 (45%)	1.000^
IgM	*Median (25^{th}-75^{th})*	**6 (4-13)**	6 (4-10)	6 (0-21)	0.551*
IgM>6	*N (%)*	**11 (50%)**	6 (50%)	5 (50%)	1.000^
Female	*N (%)*	**9 (39%)**	4 (33%)	5 (45%)	0.680^
Malabsorption	*N (%)*	**20 (87%)**	12 (100%)	8 (73%)	0.093^
Infectious complications	*N (%)*	**17 (77%)**	7 (58%)	10 (100%)	0.040^
Non infectious complications [80]	*N (%)*	**20 (91%)**	10 (83%)	10 (100%)	0.481^

Table 6. Clinical and demographic characteristics by enrolment Centre; *Mann Whitney U test; ^Fisher exact test

variable	N death	Rate per 100 pt yrs (95%CI)	HR (95%CI)	p-value
Centre				0.163
Pavia	7	5.9 (2.8-12.3)	1	
Rome	1	1.1 (9.2-8.1)	0.22 (0.03-1.83)	
Age				0.542
≤39 years	3	2.8 (0.9-8.7)	1	
>39 years	5	4.9 (2.1-11.9)	1.56 (0.37-6.60)	
Gender				0.107
Male	7	6.0 (2.8-12.5)	1	
Female	1	1.1 (0.2-7.9)	0.18 (0.02-1.45)	
Malabsorption				0.828
Absent	1	5.0 (0.7-35-5)	1	
Present	7	3.7 (1.8-7.8)	0.79 (0.09-6.54)	
Infectious complications				0.094
Absent	4	8.9 (3.3-23.7)	1	
Present	4	2.6 (1.0-6.8)	0.30 (0.08-1.33)	
Non infectious				0.792
complications [80]	1	3.7 (0.5-26.3)	1	
Absent	7	4.0 (1.9-8.4)	1.33 (0.16-11.3)	
Present				
IgM				0.851
≤6	3	3.1 (1.0-9.7)	1	
>6	4	3.7 (1.4-8.9)	1.16 (0.26-5.19)	

Table 7. Univariable Cox models for the candidate predictors of mortality

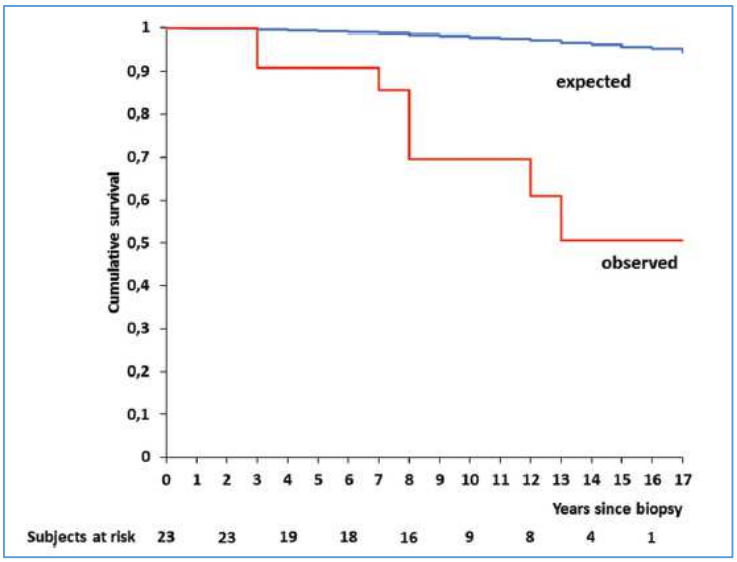


Figure 6. Kaplan–Meier curve for the observed survival of patients with common variable immunodeficiency and villous atrophy (red line) compared to the expected survival, given the Italian population_ (blue line). Number of subjects at risk are reported below the curve. _https://www.istat.it/it/popolazione-e-famiglie?dati – accessed 30 July 2018.

Experimental part 2- Description of clinical and molecular phenotypes and natural history of enteropathies with IVA

CLINICAL PHENOTYPE AND MORTALITY IN PATIENTS WITH IDIOPATHIC SMALL BOWEL VILLOUS ATROPHY: A DUAL CENTRE INTERNATIONAL STUDY

[1,2,*]Annalisa Schiepatti, MD, [2]David S Sanders, MD, PhD, [2]Imran Aziz, MD, [3]Annalisa De Silvestri, BS, [4]John Goodwin, DipRCPath, [4]Tim Key, FRCPath, [5]Lydia Quaye, PhD, [6]Paolo Giuffrida, MD, [7]Alessandro Vanoli, MD, [7]Marco Paulli, MD, [8]Simon S Cross, MD, [8]Patricia Vergani, MD, [6]Elena Betti, BSc, [6]Gregorio Maiorano, MD, [9]Richard Ellis, MD, [10]John A. Snowden, MD, [6]Antonio Di Sabatino, MD, [6]Gino R Corazza, MD, [1]Federico Biagi, MD

1 Gastroenterology Unit, IRCCS Pavia, ICS Maugeri, University of Pavia, Italy
2 Academic Unit of Gastroenterology, Royal Hallamshire Hospital, Sheffield, UK
3 Clinical Epidemiology, IRCCS San Matteo Hospital Foundation, Pavia, Italy
4 Histocompatibility and Immunogenetics Laboratory, NHS Blood and Transplant, Sheffield, UK
5 Histocompatibility and Immunogenetics, NHS Blood and Transplant, Colindale, London, UK
6 Coeliac Centre, First Department of Internal Medicine, IRCCS Policlinico San Matteo, University of Pavia, Italy
7 Department of Molecular Medicine, Unit of Anatomic Pathology, University of Pavia
8 Department of Histopathology, Royal Hallamshire Hospital, Sheffield, UK
9 Department of Gastroenterology, *Queen Alexandra Hospital, Portsmouth, UK*
10 Department of Haematology, Royal Hallamshire Hospital, Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield, UK
**PhD Course in Experimental Medicine, University of Pavia, Italy*

STATUS: SUBMITTED

INTRODUCTION

Coeliac disease (CD) is a chronic gluten-dependent enteropathy characterised by varying degrees of villous atrophy (VA) and a higher mortality than in the general population, mainly due to its complications [2,3,8,13]. Although small-bowel VA is due to CD and its complications in the vast majority of cases, these are not the only causes of VA and other non-coeliac enteropathies must be thoroughly investigated [45-48,68,87,89]. This is the case for autoimmune enteropathy (AE) [51,52], enteropathy associated with common variable immunodeficiency (CVID) [53,54], medication-related enteropathies [90-95], infections [96-100], small-bowel bacterial overgrowth [101], some

lymphoproliferative disorders primarily affecting the small bowel [55], Crohn's disease [102], tropical sprue [103] and collagenous sprue [104]. Some contemporary reports suggest that overall mortality in non-coeliac enteropathies with VA is higher than in CD [48,87,89]. However, these papers do not compare mortality in each subtype of non-coeliac enteropathy to CD [87,89] and it is likely that long-term outcomes are slightly different within the heterogeneous group of non-coeliac enteropathies [45-48,87,89,51-54,90,105]

Finally, in some instances, no definitive etiology for small bowel VA can be precisely identified [18,45-48,87,89, 106-108]. Even though patients with VA of unknown origin were described in small case series [18,45-48,87,89, 106-108], their clinical phenotypes, HLA typing, histology, natural history and therapeutic management have never been investigated systematically so far. For the purpose of the present paper, we would like to refer to these rare and still obscure enteropathies as forms of idiopathic villous atrophy (IVA).

By reporting the largest cohort of patients with small bowel IVA, the aims of this study are the following. Firstly, to retrospectively describe the clinical and histopathological phenotypes of patients with IVA (clinico-pathological study) and define their natural history and mortality in comparison to CD (follow-up and mortality study). Secondly, to study the HLA genetic profile of patients with IVA and compare it with patients affected by CD and healthy controls (genetic study), as to understand whether, similarly to CD that develops in HLA DQ2/DQ8 individuals [2,3], a specific HLA genetic background could predispose to the development of these conditions.

PATIENTS AND METHODS

The study group included patients affected by small bowel IVA who attended two referral centres (Pavia, Italy and Sheffield, UK) between January 2000 and December 2017 and were followed-up prospectively until March 2019.

Diagnostic criteria for IVA

Patients with evidence of frank VA on biopsies taken from the second duodenal portion and in whom no specific cause for their VA was identified despite thorough investigations were enrolled. For patients referred to our centres, previous histology was carefully reviewed by expert histopathologists and only those with confirmed VA were included. In all these patients, CD and its complications as well as all the known causes of serology negative VA were excluded at time of diagnosis of VA. More specifically, all the patients tested repeatedly negative to IgA endomysial antibodies (EmA), IgA tissue transglutaminase antibodies (TTG), IgA and IgG gliadin antibodies. None had evidence of clinical and histological response to a gluten-free diet (GFD) and nobody had a family history of CD or a personal history of dermatitis herpetiformis. Enterocyte antibodies were negative and serum immunoglobulin levels normal, thus ruling out AE and CVID respectively. None of the patients were taking drugs known to be responsible for VA, namely ARBs, NSAIDS, methotrexate, mycophenolate [48, 90-96]. Giardia specific stools antigens and other parasitic stool tests, tuberculosis quantiferon, HIV testing, small bowel aspirate/glucose H2-breath test, Whipple's PCR on duodenal biopsy/PAS staining on duodenal slides, H. Pylori were all negative. Traditional histology guided the exclusion of Crohn's disease, collagenous sprue, eosinophilic enteritis, lymphoprolipherative disorders and malignancies primarily involving the small bowel, including those known to be complications of CD. None of the patients had predisposing factors for small intestinal bacterial overgrowth [48]. All patients in this study were asked to continue on a gluten containg diet while these investigations were carried out [46,48].

Criteria for the classification of IVA into clinical categories

On the basis of our clinical experience and the previous literature describing some forms of IVA with spontaneous resolution [45,46,48,107,108] and others with persistence of VA [18,45-48,87,89,106], we set up criteria to retrospectively classify IVA patients into three groups (Figure 7). Patients in whom resolution of VA occurred spontaneously without any intervention (GFD,

immunosuppressants) were classified into group 1. Conversely, patients displaying persistent VA unresponsive to at least 12 months of a GFD or immunosuppressants were furtherly divided according to a combination of findings raising the suspicion of lymphoprolipherative features. These criteria included evidence of persistent gamma T-cell receptor (TCR) monoclonality on duodenal biopsies and/or presence of aberrant intraepithelial lymphocites assessed by immunohistochemistry and/or flow cytometry, and/or past medical history of extraintestinal onco-haematological disorders. Patients in group 2 did not met any of the above mentioned criteria. Conversely, if at least one of the criteria pointing at lymphoprolipherative stigmata was met, then patients were included into group 3.

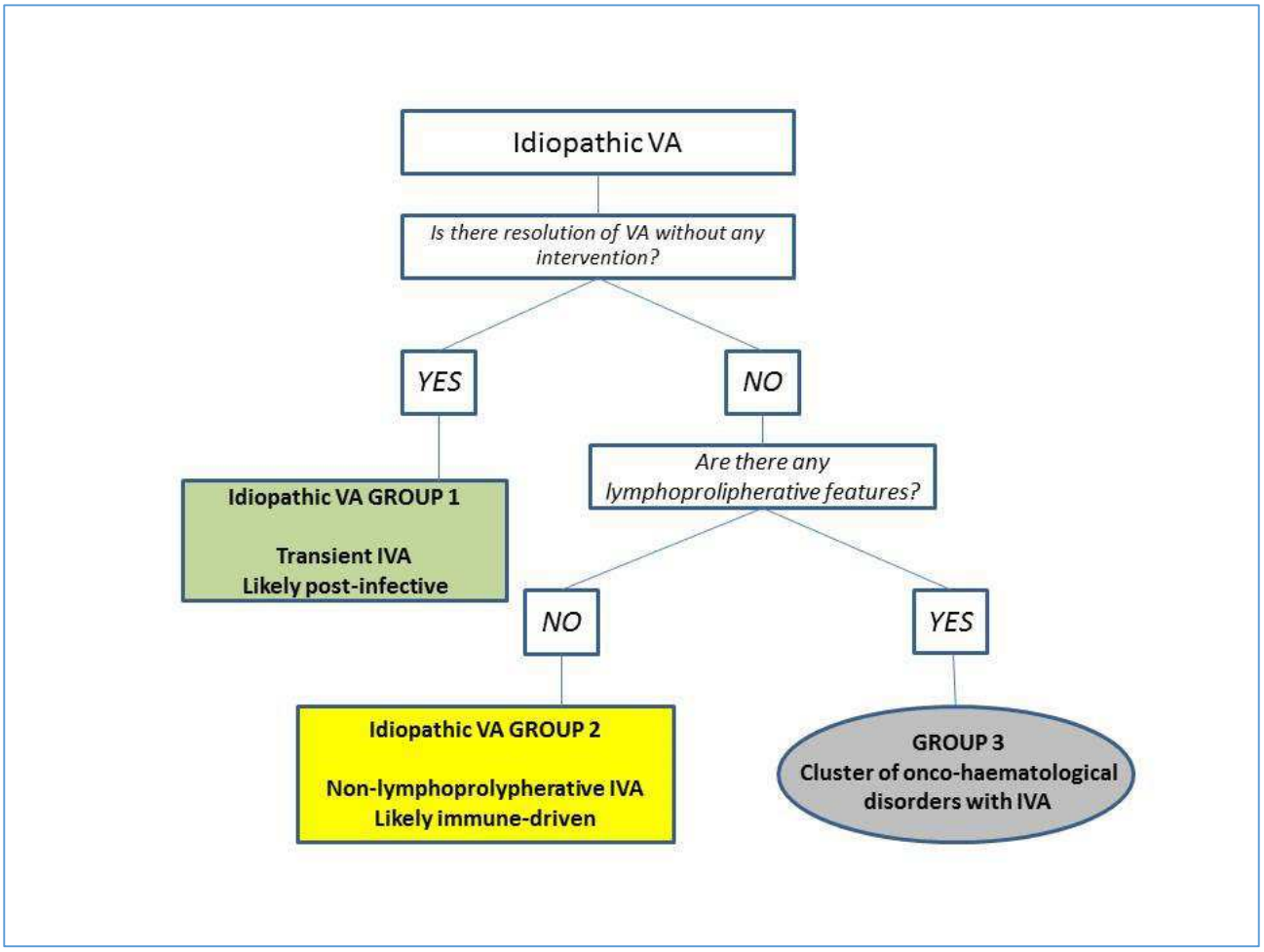


Figure 7. Criteria to classify idiopathic villous atrophy into clinical categories.VA: villous atrophy, IVA: idiopathic villous atrophy

Data collection

For the purpose of the clinico-pathological study baseline demographics, clinical and histological features of patients affected by IVA were retrospectively collected.

For the follow-up and mortality study data on clinical and histological response to therapies, onset of complications (type and date of complication), date of last follow-up in clinic/death, cause of death (when available) were recordered for IVA patients until March 2019. Histological response was defined as an improvement in the degree of VA on follow-up duodenal biopsy. Clinical response was defined as resolution of symptoms.

Survival was compared between IVA patients and 1114 coeliac patients (775 F, mean age at diagnosis 42±16 years) diagnosed in the two centres between January 2000 and December 2017 and followed-up until January 2019 (cumulative follow-up: 8758.75 persons/years). All the coeliac patients in the control group were diagnosed on the basis of VA and positive IgA EmA/TTG [2,3]. During follow-up, 25 of these coeliac patients (13F, mean age at diagnosis of CD 56±9 years) developed a complication (7 type 1 refractory CD, 4 type 2 refractory CD, 4 enteropathy associated T-cell lymphoma, 5 abdominal B-cell lymphomas, 2 small bowel carcinoma and 3 oesophageal cancer). A further survival analysis was made between this group of 25 patients with complicated CD and IVA patients. Diagnosis of the complications of CD was made as previously described [2,3,8,13,27].

Finally, for the genetic study the HLA heterodimer frequencies of patients with IVA were compared to those of ethnicity-matched adult coeliac patients and healthy controls, in the same fashion we adopted in a previous study [22]. The control groups comprised 355 patients affected by uncomplicated CD (169 Italian- 122 females, mean age 31±14 years; 187 British – 130 females, mean age 44±17 years), 44 patients affected by complicated/refractory CD (27 Italian- 17 females, mean age 50±12 years; 17 British – 12 females, mean age 49±10) and 424 healthy controls (224 Italian - 104 females, mean age 46±9 years; 200 British - 92 females, mean age 50.3±5.57). The HLA typing of healthy controls was obtained from the registry of Stem Cells Donors of Pavia, as previously described [22] and from British Bone Marrow Registry. We note that for the group of British patients

affected by refractory CD only the HLA DQB1 profile was available. We calculated the frequencies of HLA molecules encoding the heterodimers HLA-DQ2.5 (DQA1*05 DQB1*02), HLA-DQ2.2 (DQA1*02 DQB1*02), HLA-DQ7.3 (DQA1*03 DQB1*03:01), HLA-DQ7.5 (DQA1*05 DQB1*03:01) and the DQB chain encoding for HLA-DQ5 (DQB1*05), HLA-DQ6 (DQB1*06), HLA-DQ8 (DQB1*03:02) and HLA-DQ9 (DQB1*03:03).

Histology

Severity of VA was graded according to Marsh-Oberhuber classification [109] on hematoxilin and eosin stained slides from second duodenal portion. Traditional immunohistochemistry (IHC) for CD3 and CD8 lymphocyte markers was performed on formalin-fixed, paraffin-embedded duodenal specimens.

Molecular analysis for gamma-TCR gene rearrangement was performed on DNA extracted from formalin-fixed paraffin embedded duodenal specimens by means of polymerase chain reaction, in accordance to standard Euroclonality/BIOMED-2 protocol.

Aberrant intraepithelial lymphocytes, the hallmark of type 2 refractory coeliac disease, were identified as >50% of CD3+CD8- intraepithelial lymphocytes on traditional IHC or >20% CD3-CD8-CD103+CD7+ cytoplasmatic CD3+ intraepithelial T-lymphocytes by means of flow cytometry (FC) [27].

Coeliac serology

EmA and enterocyte antibodies were detected by means of indirect immunofluorescence on monkey oesophagus/jejunum slides (INOVA Diagnostics, San Diego, USA was used for the Italian patients. The Binding Site, Birmingham, UK was used for the British patients). TTG and deamidated gliadin antibodies were tested by using ELISA kits (EliA Celikey IgA and Celikey IgG, EliA Gliadin DP IgA and EliA Gliadin DP IgG; Phadi AB, Uppsala, Sweden were used for the Italian patients. Aesku Diagnostics, Wendelsheims, Germany was used for the British patients).

HLA typing

Italian patients and controls were typed for HLA class II genomic polymorphisms at the high-resolution level by means of sequences specific primers- polymerase chain reaction (PCR-SSP) and/or polymerase chain reaction utilising sequence-specific primary oligonucleotides (PCR-SSO) [110,111]. DNA was extracted from peripheral blood samples using the Wizard genomic DNA Purification kit (Maxwell 16®, Promega Instrument; Madison, WI, USA) according to the manufacturer's protocol. The polymorphism of the HLA-DQA1 and DQB1 genes was analyzed using commercial kits (Olerup SSP AB®, Stockholm, Sweden), and One Lambda Inc.; Canoga Park, CA, USA). The amplified products were visualized on 2% agarose gel, stained with 0.5 mg/mL of ethidium bromide, using the E-Gel precast Agarose Electrophoresis System (Invitrogen Life Technologies®, PA4 9RF Paisley, UK).

For British patients the HLA typing was obtained by means of a sequence-specific oligonucleotide polymerase chain reaction technique (LIFECODES HLA SSO typing – RAPID, IMMUCOR ® Georgia, USA). Genomic DNA was extracted from samples of peripheral blood using a DNA purification kit in combination with an automated DNA extraction platform (MagNA Pure Compact DNA Nucleic Acid Isolation kit and a MagNA Pure Compact instrument Roche Basel, Switzerland). For BBMR the automated DNA extraction platform used was Qiasymphony (QIAGEN®, Venlo, Netherlands). Results were analysed using a Luminex ® 100 analyser (Luminex® Corporation Texas, USA) and MATCH IT! analysis software (IMMUCOR ® Georgia, USA).

Statistical analysis

Categorical variables were described as count and percentages; quantitative variables as mean and standard deviation if normally distributed (Shapiro-Wilks test), otherwise as median and IQR. Clinical, laboratory and histopathological features were compared between IVA groups using one-way analysis of variance for normally distributed quantitative variables followed by Scheffè corrected

2x2 post-hoc comparisons. Categorical data were compared between the IVA and control groups by means of $\chi 2$ test and Fisher's exact test followed by Bonferoni corrected 2x2 post-hoc comparisons. HLA heterodimers frequencies were compared between the IVA and control groups by means of $\chi 2$ test and Fisher's exact test. For the genetic study, a correspondence analysis, which represents an explorative multivariate statistical technique, was used to provide a graphical interpretation in a bidimensional graph of the relationship between the clinical groups (three IVA groups vs. coeliac patients and healthy controls) and the genetic variants [22].

Overall survival was described through a Kaplan-Meier curve. Univariate and multivariate (including factors significantly associated with survival at univariate analysis) Cox models were fitted to study long-term mortality. Results are expressed as hazard ratio (HR) and reported with 95% CI.

P-value <0.05 was considered statistically significant. All tests were two-sided. The data analysis was performed with the STATA statistical package (release 15.1, 2017, Stata Corporation, College Station, Texas, USA).

Ethics

All the Italian patients in Pavia gave an informed consent at time of duodenal biopsy, both for clinical and research purposes. After verifying the good quality of the data, they were all irreversibly anonymised. None of the patients have signed against the participation in anonymous studies. The study was approved by the Ethics committee of the Fondazione IRCCS Policlinico San Matteo, Pavia, Italy according to the 1975 declaration of Helsinki (6th revision, 2008). Similarly, the study protocol was approved by the Yorkshire and Humber Research Ethics committee and registered with the local research and development department of Sheffield Teaching Hospital NHS Foundation Trust (REC reference 14/YH/1216).

RESULTS

Seventy-six patients affected by IVA were collected over a period of 17 years. Their baseline demographics, clinical characteristics, laboratory results and radiological findings are shown in Table 8. The most common presenting symptoms were weight loss, diarrhoea and dyspepsia. Weight loss was more frequent among patients with persistent VA, being significantly associated to group 3 (p=0.01). Prevalence of dyspepsia was higher in group 1 (p=0.003). Anaemia and hypoalbuminemia (< 3.5 g/dL) were more common in group 3 (p<0.001). Family history of autoimmune disorders was more common in group 2 (p=0.01).

	TOTAL IVA N°=76	Group 1 IVA N°=50	Group 2 IVA N°=14	Group 3 IVA N=12	Overall p-value	Group 1 vs Group 2 p-value	Group 1 vs Group 3 p-value	Group 2 vs Group 3 p-value
DEMOGRAPHICS								
Mean age±SD	48.5±17.7	49.2±18.7	43.3±13.8	51.9±13.8	0.431	-	-	-
Females, n°	38	26	7	5	0.813	-	-	-
Caucasian ethnicity	70	46	13	11	0.933	-	-	-
CLINICAL FEATURES, LABORATORY AND RADIOLOGICAL FINDINGS AT DIAGNOSIS								
Diarrhoea	46	27	9	10	0.166	-	-	-
Weight loss	47	25	11	11	**0.01**	**0.05**	**0.009**	0.356
Abdominal pain	22	13	4	5	0.561	-	-	-
Reflux	14	11	3	0	0.200	-	-	-
Dyspepsia	29	26	2	1	**0.003**	**0.01**	**0.006**	0.636
Vomiting	20	15	2	3	0.495	-	-	-
Anaemia	21	6	5	10	**<0.001**	**0.03**	**<0.001**	**0.01**
Hypoalbuminemia (<3.5g/dl)	12	0	4	8	**<0.001**	**<0.001**	**<0.001**	**0.05**
Low folate	10	1	4	5	**<0.001**	**0.001**	**<0.001**	0.484
Low B12	19	8	5	6	0.03	0.105	**0.01**	0.462
Low ferritin	17	6	9	2	**<0.001**	**<0.001**	**0.665**	0.014
Pathological VCE findings	25	5	13	7	**<0.001**	**<0.001**	**<0.001**	0.45
Pathological CT findings	7	0	1	6	**0.001**	**0.237**	**0.001**	**0.016**
PAST MEDICAL HISTORY								
Autoimmunity	14	8	2	4	0.345	-	-	-
Family history of autoimmune diseases	2	0	2	0	**0.01**	**0.007**		0.173
Travel endemics	8	6	2	0	0.420	-	-	-
Gastroenteritis	12	11	0	1	0.101	-	-	-
DUODENAL HISTOLOGY AT DIAGNOSIS								
Histology_Marsh 3a	53	49	0	4	**<0.001**	-	-	-
Histology_Marsh 3b +3c	23	1	14	8	**<0.001**	**<0.001**	**<0.001**	**0.019**
Histology_IELs	69	44	13	12	0.416	-	-	-
Histology_CH	60	36	12	12	0.08	-	-	-

Table 8. Baseline characteristics of patients with idiopathic villous atrophy. IVA: idiopathic villous atrophy; PVA: partial villous atrophy; SVA: subtotal villous atrophy; TVA: total villous atrophy; IELs: intraepithelial lymphocites; CH: cripth hyperplasia; SD: standard deviation; VCE: video capsule endoscopy; CT: computed tomography

Follow-up and mortality

Complete spontaneous histological recovery occurred in 47/50 patients in group 1 (Table 9) after a median of 10 months [IQR 5-14 months], whereas the remaining three patients declined follow-up biopsy given their persistent well-being. Four patients spontaneously started on a GFD for a few months after diagnosis of IVA, but then resumed and all were on a normal diet at time of histological follow-up. In group 2 (table 10), histological and clinical response to immunosuppressant/biologics occurred in 5/14 patients. More precisely, two responded to azathioprine, one to open-capsule budesonide treatment and two to biologics (patients 1, 6, 12-14). In other two patients (n° 2 on azathioprine and n° 8 on budesonide), despite clinical response had occurred, histological recovery was slow. Conversely, in group 3 (table 11), 10 out of 12 patients had persistence of VA on follow-up biopsy and one refused follow-up gastroscopy. Complications were found in one patient in group 2 (patients 14, Table 10) and nine patients in group 3 ($p<0.001$). Median time from diagnosis of VA to development of complications in group 3 IVA was 13 months, IQR 3.5-26.5.

Eleven out of 76 patients died (4 in group 1 IVA for causes unrelated to the enteropathy, after a median time since diagnosis of 4.5 years; 7 patients in group 3 IVA, mainly because of a lymphoprolipherative malignancy after a median of 15 months, IQR 9.5-40.5). Patients in group 2 were all alive after a median follow-up of 64 months, IQR 52-114. In the coeliac cohort 66/1114 patients (5.92%) died. Causes of death in this group included complicated CD in 13/66 (19.6%), cardiovascular causes in 9/66 (13.6%), other cancers in 8/66 (12.1%), pneumonia in 8/66 (12.1%). In 13 patients the cause of death was unknown and in the remaining 15 the causes were heterogeneous. Figure 8 shows Kaplan-Meier estimated survival in IVA and coeliac patients. Mortality in IVA was higher than in CD (Fig 8A, $p<0.001$), but with great differences within the three IVA groups (Fig 8B). Overall 5-year survival was 96% in IVA group 1, 100% in IVA group 2, 27% in IVA group 3 and 97% in the coeliac cohort. There was no difference in long-term survival between IVA group 1 and CD ($p=0.22$). Patients in group 3 IVA showed the poorest prognosis, even when compared to the 25 coeliac patients that developed a complicated form of CD (Fig 8C, $p<0.001$).

On univariate analysis age at diagnosis (HR 1.05, 95%CI 10.1-1.09, p=0.009), anaemia (HR 7.8, 95%CI 2.18-27.80, p=0.002), hypoalbuminemia (HR 16.2, 95%CI 4.68-56.58, p<0.001) and HLA DQ2.2 (HR 5.7, 95% CI 1.74-18.9, p=0.004) were statistically significant predictors of mortality in IVA patients, whereas dyspepsia was a protective factor (HR 0.12, 95%CI 0.015-0.96, p=0.046). Gender (HR 0.79, 95% CI 0.24-2.62, p=0.707), diarrhoea (HR 1.11, 95% CI 0.32-3.77, p=0.877), weight loss (HR 1.78, 95% CI 0.47-6.74, p=0.393) and HLA DQ2.5 (HR 1.49, 95% CI 0.39-5.69, p=0.560) were not significantly associated to increased mortality. On multivariate analysis only age at diagnosis (HR 1.04, 95%CI 1.00-1.07, p=0.03) and hypoalbuminaemia (HR 10.7, 95%CI 2.32-50.00, p=0.002) significantly predicted mortality.

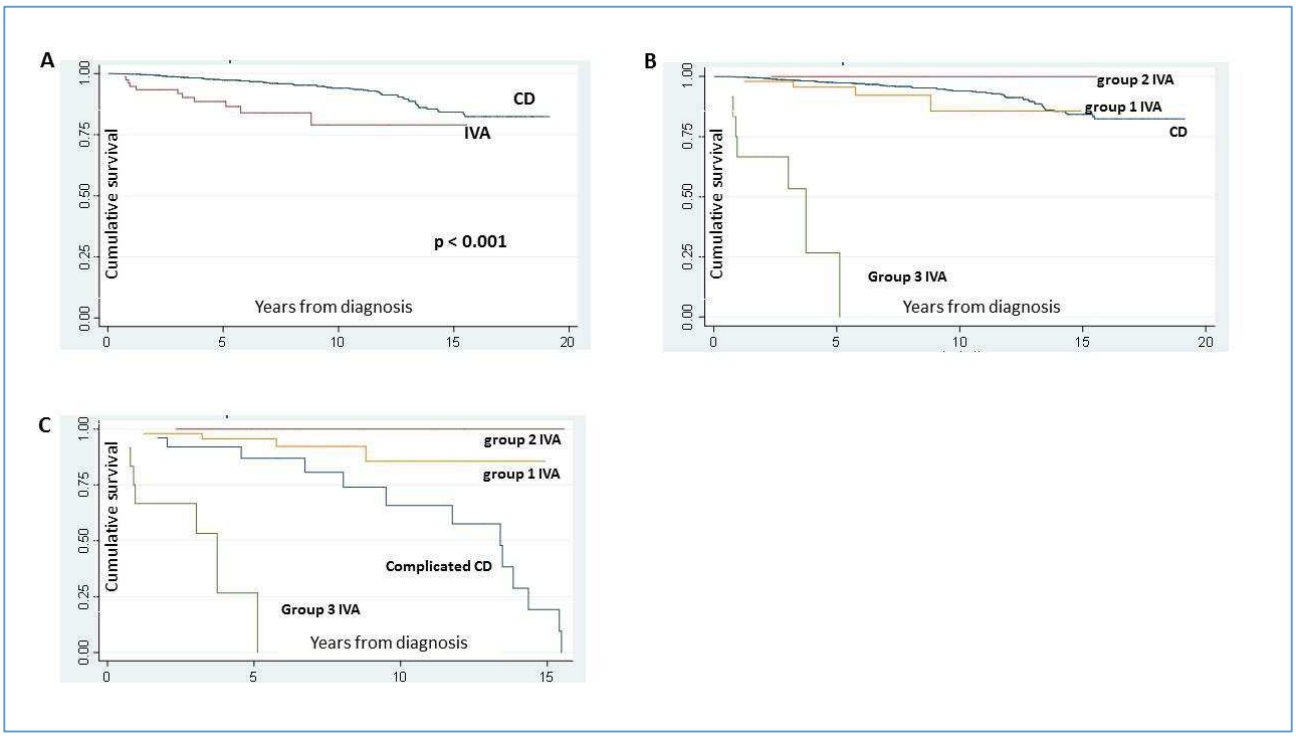


Figure 8. Kaplan Meier estimated survival curves for idiopathic villous atrophy groups compared to coeliac disease. IVA: idiopathic villous atrophy; CD: coeliac disease

Histopathological and molecular features

As shown in Table 8 partial VA was the histological hallmark of group 1 (p<0.001), while no significant differences were found for intraepithelial lymphocitosis and crypt hyperplasia. Histopathological findings for group 1 and group 2 are specified in Table 9 and Table 10. Briefly, in group 1 histology was undistinguishable from untreated CD in the vast majority of patients and only

in 6/50 histological features were in keeping with peptic duodenitis [98] (Table 9). In group 2 a coeliac-like pattern, characterized by crypt hyperplasia and intraepithelial lymphocytosis with normal CD3+ CD8+ phenotype, was evident in 11 patients (no 1-11, table 10). Only in one patient (no 4 in Table 10) histology revealed a subepithelial band of collagen, though criteria for collagenous sprue were not met [104]. A peptic duodenitis-like pattern characterised by expansion of the lamina propria by a mixed but predominantly mononuclear inflammatory infiltrate and neutrophilic cryptitis was found in 3 patients (no 12-14). Interestingly, two of them had a diagnosis of ulcerative colitis. All patients in group 2 showed regular phenotype of intraepithelia lymphocytes assessed by FC or IHC and/or polyclonal gamma-TCR both at time of diagnosis and during follow-up.

Table 11 summarises the heterogeneous histological features of group 3. An aberrant histology and/or persistent monoclonality for gamma-TCR were found in patients 1-6. Patient 7 refused follow-up, so we were not able to assess persistence or resolution of gamma-TCR monoclonality. Histology undistinguishable from conventional CD was found in patients 6 and 8-12. In patients 9 and 10 a band of subepithelial collagen was found. They both had a history of chemotherapy for extraintestinal lymphoprolipherative disorders. However, histological findings were neither sufficient to confirm a diagnosis of collagenous sprue [104], nor a possible causative effect of chemotherapy.

Genetic study

Comparison of the HLA heterodimers frequencies between IVA groups, healthy controls and coeliac patients is shown in Table 12. Of note, in group 2 HLA-DQ7.3 was carried by 33% of patients and HLA-DQ6 by nearly 60% ($p<0.001$). In group 3, HLA-DQ5 molecules were carried by 41.65% of patients and HLA-DQ2 by half of them. Group 1 IVA was genetically heterogeneous, with HLA-DQ2.5 (35%) and HLA-DQ6 (45%), being the most frequent molecules. As expected, HLA-DQ2 molecules were more commonly expressed by coeliac patients. HLA-DQ8 was poorly expressed by group 3 IVA and patients with complicated CD.

A graphical visualization of the genetic HLA diversity between the three IVA groups, coeliac patients and healthy controls is shown by the correspondence analysis on heterodimers in Figure 9. The analysis confirms that group 2 IVA is HLA genetically different from coeliac patients, being associated with HLA-DQ7.3 and HLA-DQ6 molecules. On the contrary, group 1 and 3 IVA, despite being distinct from coeliac patients, are characterized by a greater HLA genetic heterogeneity.

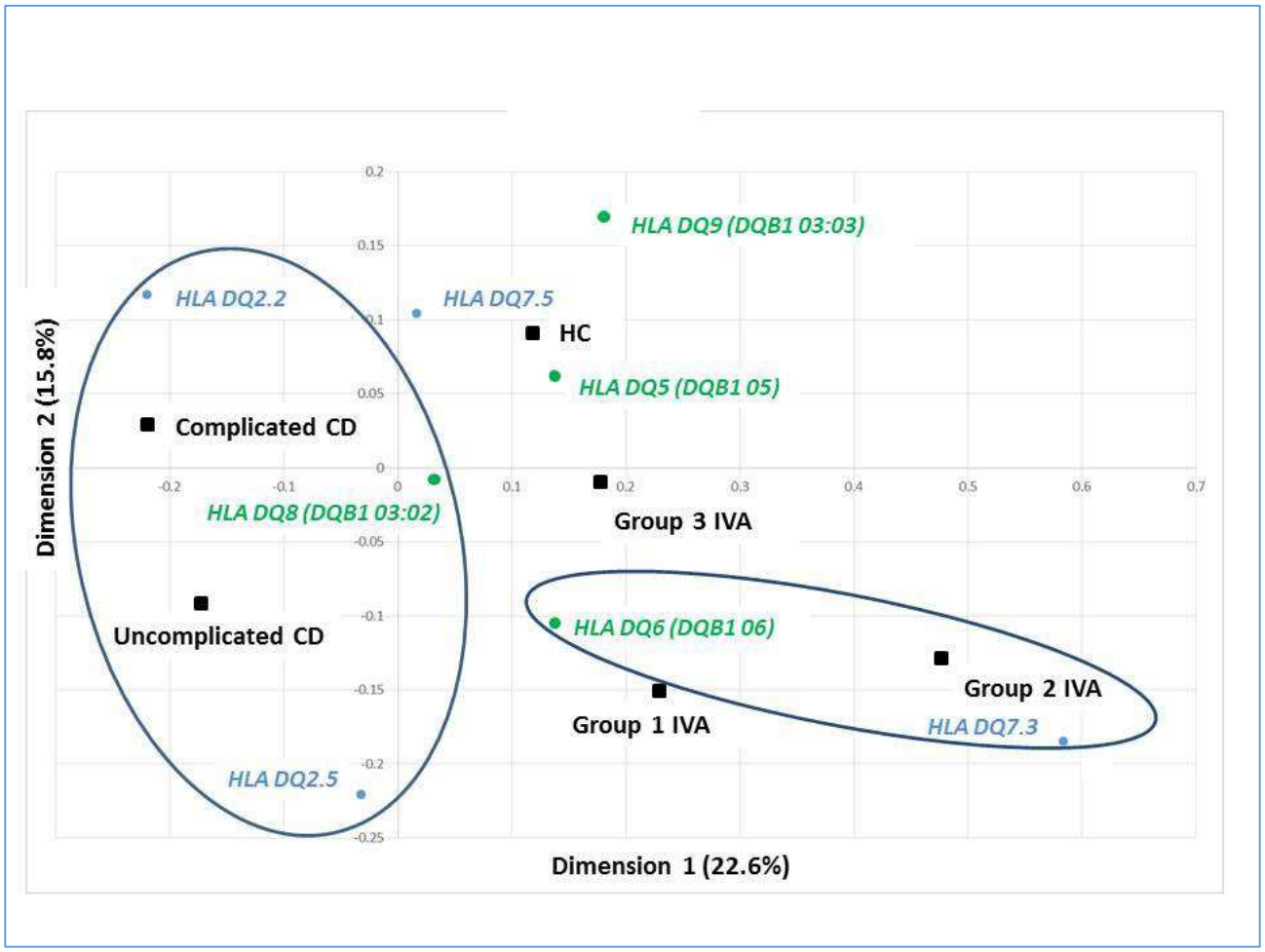


Figure 9. Genetic heterogeneity of idiopathic villous atrophy patients compared to coeliac patients and healthy controls. The correspondence analysis shows that the nearer the dots, the stronger the association. Black squares indicate the group of patients. Blue dots indicate HLA heterodimers (HLA-DQ2.5 encoded by DQA1*05 DQB1*02, HLA-DQ2.2 encoded by DQA1*02 DQB1*02, HLA-DQ7.3 encoded by DQA1*03 DQB1*03:01, HLA-DQ7.5 encoded by DQA1*05 DQB1*03:01) and green dots indicate DQB chains. CD: coeliac disease; IVA: idiopathic villous atrophy; HC: healthy controls

DISCUSSION

In this study we have described the largest cohort of patients with small bowel IVA and we have provided a clinical-based classification of these enteropathies into three groups with distinctive clinical features, HLA and natural history. We have given the most extensive description about long-term outcomes in IVA, showing for the first time that mortality in IVA is overall higher than in CD, though remarkably different between IVA subgroups. More specifically, IVA patients with lymphoprolipherative features (group 3) are burdened by the poorest prognosis and are mainly responsible for the higher mortality found in IVA than in CD. Age and hypoalbuminaemia at time of diagnosis of VA are important predictors of mortality in IVA and may be helpful to stratify patients warranting a more aggressive treatment and close follow-up. Our results on IVA add to some previous studies suggesting that overall mortality in non-coeliac enteropathies with VA is higher than in in CD [48,87,89,105].

The clinical features of patients with self-limited IVA (group 1) were already described in the past [45,46,48,107,108]. The novel finding of our study is that mortality in these patients is not higher than in CD and it is mainly due to causes unrelated to the enteropathy. This is in line with a less severe and transient enteropathy, maybe triggered by viral infectious agents, as previously hypothesised [45,46,48,107,108].

Another strength of our study is the identification of diagnostic criteria for a new form of chronic non-coeliac enteropathy characterised by persistent VA, absence of any lymphoprolipherative stigmata, specific HLA typing and long-term survival (ie. IVA group 2). Although similar patients were described in the past [18,45-48,87,89,106,112], we have now provided a more detailed description of their clinical and molecular phenotype. The first mandatory diagnostic criteria are the presence of a regular phenotype of intraepithelial lymphocites assessed by IHC and/or FC and the absence of monoclonality for gamma-TCR both at time of diagnosis and during follow-up, thus excluding type 2 refractory CD [27]. Secondly, the HLA genetic background is dominated by HLA DQ7.3 and DQ6 molecules, that are not listed among those conferring risk for developing CD [113].

Although it might be said that patients with similar features are affected by seronegative CD or refractory CD, we believe that HLA genetic background, the histological and molecular phenotypes, the absence of any sensitivity to gluten and the long-term survival are key to define a distinct clinical entity. Despite the quite high response rate to immunosuppressants and the high prevalence of family history for autoimmune disorders may support the hypothesis of an immune-driven chronic enteropathy, we think this is not sufficient to confirm a diagnosis of AE, given the negative results for enterocyte antibodies. While forms of seronegative AE do exist and are well defined in children, their existence in adults needs to be furtherly elucidated [48,51,52,112].

Interestingly, two main histological patterns were identifiable in group 2: a coeliac-like pattern in 78% of the patients and a peptic duodenitis-like pattern in the remaining 22%. All the patients with peptic duodenitis-like histology responded histologically to immunusuppressant and/or biologics. Two were concomitantly diagnosed with ulcerative colitis. Similar small bowel histology was previously described in patients with ulcerative colitis [114]. On the contrary, fewer patients with a coeliac-like pattern had histological response to traditional immunosuppressants.

Group 3 is burdened by a very high mortality, that is mainly responsible for the poorer outcome we found in IVA than in CD. Identification of these patients was made on the basis of persistent monoclonality for gamma-TCR and a previous history of extra-intestinal lymphoproliferative disorders. However, this group is the most heterogeneous in terms of histological features, with some points that are worth discussing.

First, the specificity of gamma-TCR monoclonality analysis has recently been questioned, with transient monoclonal gamma-TCR described also in coeliac patients with poor compliance to a GFD, [115,116] and even in patients with duodenal lymphocytosis associated to H. Pylori [117]. However, in our patients in group 3 monoclonality was persistent on follow-up biopsies in five out of six patients (one refused follow-up). Most importantly, three of them developed lymphoproliferative complications and 50% of them died (patients 1-6, table 11). This would enable us to identify within

group 3 a subgroup of patient with IVA, persistent monoclonality for gamma-TCR and high risk of lymphoproliferation.

The second criterion we adopted is previous history of extra-intestinal lymphoproliferative disorders. Four patients had a history of lymphoproliferative disorders pre-dating the diagnosis of VA (patients 7-10, table 11). Two of them received chemotherapy some years before and one was on treatment with idelalisib at time of diagnosis of IVA. Hence, a possible role of these medications in promoting a certain damage to the small bowel, although very remote, cannot be entirely excluded. Interestingly, forms of enterocolitis possibly related to idelalisib have been described [118].

Last but not least, two patients in this group (no 11 and 12 in Table 11) were very difficult to be classified. From one side they did not show any clear molecular lymphoproliferative features at diagnosis, so they would have matched with group 2. However, the clinical suspicion had always been that of a lymphoma in both them. During follow-up one of them deteriorated and died of angioimmunoblastic T-cell lymphoma and the other died of oesophageal cancer within less than four years from diagnosis.

Finally, although CD and its complications were thoroughly excluded at time of diagnosis of VA, we noticed that some patients in group 3 died because of conditions, that could be considered complications of CD. HLA DQ2 molecules were found in half of the patients in group 3, so we wonder whether they were seronegative coeliac patients that escaped the diagnosis before and then complicated afterwards.

Proposals for clinical management of idiopathic villous atrophy and future perspectives

Partial villous atrophy, absence of suspicious features for lymphoproliferation, and normal laboratory tests at diagnosis identify patients with a self-limited form of VA (group 1). These patients should not be prescribed a GFD and the most appropriate management may be a “watch and wait” strategy for at least six months, before assessing for histological recovery. This would avoid misdiagnoses of seronegative CD, particularly in those carrying HLA DQ2 or DQ8 molecules.

On the contrary, advanced age at diagnosis, anaemia, hypoalbuminaemia, extensive small bowel involvement on video capsule endoscopy, pathological abdominal CT and persistent monoclonality findings are important predictors to identify since the time of diagnosis patients with persistent IVA that should warrant aggressive treatments and close follow-up. In patients with persistent VA of unknown etiology we suggest to always characterise the phenotype of intraepithelial lymphocites by means of IHC and/or FC and to test for gamma-TCR clonality, since in patients with negative results for these tests prognosis is excellent (ie. Group 2 IVA). On the basis of our results we suggest prednisolone followed by azathioprine as first line therapy in these patients.

Proposals for future research may include the investigation of the molecular pathways involved in IVA, a further discrimination of enteropathies displaying lymphoproliferative features and the evaluation of the therapeutic potential of regimens alternative to traditional immunosuppressants. Depending on the nature of the enteropathy, these could include autologous and allogeneic haemopoietic cellular therapies [119-121], both as rescue therapy for patients refractory to conventional immunosuppressants or as first line therapy for those with predominantly genetic or other features suggesting poor outcomes.

Table 9. Histological features and outcomes in patients belonging to group 1. CPD: chronic peptic duodenitis; f-up: follow-up

Pt Nº	Age/sex	histology diagnosis	Other histological features	follow-up histology (months from diagnosis)	Outcome (months from diagnosis)
1	18/M	Marsh 3a	CPD	f-up refused	Alive (27)
2	48/M	Marsh 3a	CPD	Marsh 0 (105)	Alive (107)
3	40/M	Marsh 3a	Coeliac-like	Marsh 3c (7) Marsh 1 (94)	Alive (98)
4	28/F	Marsh 3a	Coeliac-like	Marsh 0 (4)	Alive (24)
5	18/M	Marsh 3a	Coeliac-like	Marsh 1,CPD (48)	Alive (73)
6	58/M	Marsh 3a	Coeliac-like	Marsh 1 (11)	Alive (77)
7	49/F	Marsh 3a	Coeliac-like	Marsh 1 (11)	Alive (81)
8	40/M	Marsh 3a	Coeliac-like	Marsh 1 (3)	Alive (147)
9	30/M	Marsh 3a	Coeliac-like	Marsh 1 (4)	Alive (156)
10	73/F	Marsh 3a	Coeliac-like	Marsh 1 (54)	Alive (68)
11	58/F	Marsh 3a	Coeliac-like	Marsh 1 (6)	Alive (146)
12	80/F	Marsh 3a	Coeliac-like	Marsh 1 (12)	Dead (105)
13	85/F	Marsh 3a	Coeliac-like	Marsh 1 (5)	Dead (14)
14	28/M	Marsh 3a	Coeliac-like	Marsh 1 (7)	Alive (67)
15	73/F	Marsh 3a	Coeliac-like	Marsh 1 (2)	Alive (60)
16	45/M	Marsh 3a	CPD	Marsh 1 (14)	Alive (60)
17	67/M	Marsh 3a	Coeliac-like	Marsh 0 (28)	Alive (80)
18	71/F	Marsh 3a	Coeliac-like	Marsh 1 (10)	Dead (38)
19	59/M	Marsh 3a	Coeliac-like	Marsh 3a (18), then lost f-up	Alive (105)
20	27/F	Marsh 3a	Coeliac-like	Marsh 3b (2) Marsh 1, (15)	Alive (111)
21	78/F	Marsh 3a	Coeliac-like	Marsh 1,CPD (6)	Dead (69)
22	68/M	Marsh 3a	Coeliac-like	Marsh 0 (5)	Alive (129)
23	30/F	Marsh 3a	Coeliac-like	Marsh 1 (15)	Alive (91)
24	31/F	Marsh 3a	Coeliac-like	Marsh 0 (5)	Alive (99)
25	65/F	Marsh 3a	Coeliac-like	Marsh 1 (14)	Alive (88)
26	42/F	Marsh 3a	Coeliac-like	Marsh 0 (20)	Alive (94)
27	66/M	Marsh 3a	Coeliac-like	Marsh 3a (5) Marsh 1 (28)	Alive (115)
28	59/M	Marsh 3a	Coeliac-like	Marsh 3a (4) Marsh 1 (16)	Alive (114)
29	51/M	Marsh 3a	CPD	Marsh 1 (6)	Alive (68)
30	48/M	Marsh 3a	Coeliac-like	Marsh 1 (3)	Alive (123)
31	59/F	Marsh 3a	Coeliac-like	Marsh 0 (14)	Alive (165)
32	46/M	Marsh 3a	Coeliac-like	Marsh 1 (1)	Alive (145)
33	56/M	Marsh 3a	Coeliac-like	Marsh 0 (5)	Alive (81)
34	37/F	Marsh 3b	Coeliac-like	Marsh 0 (4)	Alive (82)
35	62/M	Marsh 3a	Coeliac-like	Marsh 0 (9)	Alive (118)
36	23/F	Marsh 3a	Coeliac-like	Marsh 0 (8)	Alive (22)
37	64/F	Marsh 3a	Coeliac-like	Marsh 1 (6)	Alive (67)
38	62/F	Marsh 3a	Coeliac-like	Marsh 3a (5) Marsh 1 (83)	Alive (97)
39	69/F	Marsh 3a	Coeliac-like	Marsh 1 (5)	Alive (23)
40	66/F	Marsh 3a	Coeliac-like	Marsh 1 (3)	Alive (67)
41	18/F	Marsh 3a	Coeliac-like	Marsh 0 (15)	Alive (179)
42	43/M	Marsh 3a	Coeliac-like	F-up refused	Alive (63)
43	53/M	Marsh 3a	CPD	Marsh 0 (11)	Alive (22)
44	21/M	Marsh 3a	Coeliac-like	Marsh 0 (7)	Alive (19)
45	48/F	Marsh 3a	Coeliac-like	Marsh 1 (23)	Alive (33)
46	29/M	Marsh 3a	CPD	Marsh 1 (4)	Alive (57)
47	64/M	Marsh 3a	Coeliac-like	Marsh 1 (14)	Alive (26)
48	61/F	Marsh 3a	Coeliac-like	Marsh 0 (9)	Alive (61)
49	31/F	Marsh 3a	Coeliac-like	Marsh 0 (13)	Alive (68)
50	18/F	Marsh 3a	Coeliac-like	Marsh 0 (13)	Alive (49)

Table 10. Histological features and outcomes in patients belonging to group 2

LPD: lymphoprolipherative disorders; CT: computed tomography; NA: not assessed; SB: small-bowel; UC: ulcerative colitis; GFD: gluten-free diet; UJI: ulcerative jejunitis; 6-MPU: 6 mercaptopurine; AZA: azathioprine; mo: months since diagnosis

Pt N^0	Age /sex	HLA	History LPD	Abdomen CT	Histology diagnosis	γ TCR	Other histological features	Treatment	Clinical resp.	Histology f-up	VCE	Colo-noscopy	Compli-cations	Out-come (mo.)
1	29/M	DQ5/DQ7.3	-	normal	Marsh 3c	Poly-clonal	Coeliac-like pattern	GFD Prednisone+ Budesonide, AZA	AZA	Marsh 2	Mosaic mid SB	normal	-	Alive (145)
2	25/M	DQ6/DQ7.5	-	normal	Marsh 3c	Poly-clonal	Coeliac-like pattern	GFD Prednisone+ Budesonide,AZA	AZA	Marsh 3a/3b	Mosaic mid SB	normal	-	Alive (186)
3	56/F	DQ6/DQ7.3	-	normal	Marsh 3b	Poly-clonal	Coeliac-like pattern	GFD Budesonide	-	Marsh 3b	Aftous ulcerations and mosaic mid SB	normal	-	Alive (104)
4	64/F	DQ6/DQ8	-	normal	Marsh 3b	Poly-clonal	Coeliac-like pattern subepithelial collagen	GFD Budesonide	Budeso -nide	Marsh 3c	Mosaic proximal SB	normal	-	Alive (41)
5	47/M	DQ6/DQ7.3	-	NA	Marsh 3c	NA	Coeliac-like pattern	GFD Budesonide 6-MPU	6-MPU	Marsh 3c	Mosaic proximal SB	normal	-	Alive (58)
6	64/F	DQ2.5/DQ6	-	normal	Marsh 3c	Poly-clonal	Coeliac-like pattern	GFD, Budesonide	Budeso -nide	Marsh 2/3a	Mosaic mid SB	normal	-	Alive (78)
7	36/F	DQ2.5/DQ6	-	normal	Marsh 3b	Poly-clonal	Coeliac-like pattern	GFD, Budesonide, AZA	-	Marsh 3c	Mosaic proximal SB	normal	-	Alive (64)
8	56/F	DQ2.2/DQ5	-	NA	Marsh 3c	NA	Coeliac-like pattern	**GFD, Budesonide**	Budeso -nide	Marsh 3a	Mosaic mid SB	NA	-	Alive (50)
9	29/F	DQ2.5	-	NA	Marsh 3b	NA	Coeliac-like pattern	GFD	-	Marsh 3b	Mosaic proximal SB	NA	-	Alive (134)
10	33/M	DQ6/DQ9	-	Mesenteric adenopathy	Marsh 3b	Poly-clonal	Coeliac-like pattern	AZA Budesonide	-	Marsh 3c	Atrophic SB	NA	-	Alive (56)
11	57/M	DQ2.5/DQ2.5	-	NA	Marsh 3b	Poly-clonal	Coeliac-like pattern	GFD, budesonide	-	Marsh 3b	Mosaic proximal SB	normal	-	Alive (29)
12	44/F	DQ7.3/DQ7.5	-	normal	Marsh 3b	Poly-clonal	Peptic duodenitis-like pattern	Prednisone+ AZA	AZA	Marsh 1	NA	UC	-	Alive (52)
13	33/M	DQ7.5/DQ7.5	-	normal	Marsh 3c	Poly-clonal	Peptic duodenitis-like pattern	Prednisone, AZA Vedolizumab	Vedoli-zumab	Marsh 1	Atrophic SB	UC	-	Alive (70)
14	34/M	DQ5/DQ9	-	normal	Marsh 3c	NA	Peptic duodenitis-like pattern	Prednisone+ Adalimumab	Adali-mumab	Marsh 1	Ulcers and erosions mid SB	normal	UJI	Alive (47)

Table 11. Histological features and outcomes in patients belonging to group 3 LPD: lymphoprolipherative disorders; CT: computed tomography; NA: not assessed; SB: small-bowel; TCL: T-cell lymphoma; BCL: B-cell lymphoma; NHL: non Hodgkin lymphoma; GFD: gluten-free diet; TI: terminal ileum; UJI: ulcerative jejunitis; diagn: diagnosis; f-up: follow-up; LN: lymph nodes; AZA: azathioprine; MSC: mesenchymal stem cells; resp.: response

Pt N^0	Age/ sex	HLA	History LPD	Abdomen CT	Histo-logy diagn.	γTCR	Other histological features	Treatment	Clinical Resp.	Histology f-up	VCE	Colo-noscopy	complication	Outcome (mo.)
1	71/M	DQ2.2/DQ5	-	Enlarged LN, thickened ileal loops	Marsh 3c	Mono-clonal	Aberrant CD8+CD30- T-cell intraepithelial infiltrate	GFD + Prednisone	No	Marsh 3c	NA	normal	Peripheral CD8+ CD30- TCL	Dead (61)
2	38/M	DQ2.2/DQ2.2	-	NA	Marsh 3c	Mono-clonal	Aberrant CD8+CD30- T-cell intraepithelial infiltrate	GFD+ Prednisone+ single Infliximab infusion	No	Marsh 3c	NA	Previous history of Crohn's	Peripheral CD8+ CD30- TCL	Dead (10)
3	57/M	DQ2.5/DQ2.5	-	UJI	Marsh 3c	Mono-clonal	Aberrant CD3+CD8- CD2- CD5- T-cell intraepithelial lymphocites	GFD+ Prednisone+ budesonide+ 1 infusion MSC	No	Marsh 3c	UJI	normal	UJI	Dead (11)
4	37/F	DQ7.5/DQ7.5	-	normal	Marsh 3c	Mono-clonal	Aberrant CD3+CD8- CD2- CD5- T-cell intraepithelial lymphocites	GFD+steroids+ chemotherapy then MSC transplant	No	Marsh 3c	Extensive SB mosaic	normal	-	Alive (25)
5	60/F	DQ5/DQ5	-	Multiple enlarged LN	Marsh 3c	Mono-clonal	No increased IELS, increased numbers of lymphocytes and plasma cells in the lamina propria	GFD+ etoposide+ dexametason	Partial	Marsh 3a	extensive UJI	normal	HLH+UJI	Alive (42)
6	25/M	DQ6/DQ9	-	normal	Marsh 3c	Mono-clonal	Coeliac-like	GFD+AZA+ budesonide	partial	refused	Extensive SB mosaic	normal	-	Alive (28)
7	45/F	DQ7.5/DQ7.3	Indolent T lympho-blastic proliphe-ration TI	Enlarged LN, thickened TI	Marsh 3a	Mono-clonal	Chronic CD3+ CD8+ CD4+ TdT+/- T-cell infiltrate	GFD+AZA+ budesonide	No	Marsh 3a	NA	Ulcers TI	T-cell lymphoblastic lymphoma	Dead (9)
8	50/M	DQ2.5/DQ5	CLL	Multiple Enlarged LN	Marsh 3b	Poly-clonal	Coeliac-like	GFD+Idelalisib+ budesonide	Yes	Marsh 3b	Extensive SB mosaic	indolent BCL	indolent BCL colon	Alive (12)
9	44/M	DQ7.3/DQ6	NHL	Pulmonary fibrosis	Marsh 3a	Poly-clonal	Coeliac-like Full thickness biopsy: band of collagen	AZA+prednisolone	No	Marsh 1	NA	Oedema-tous colon	idiopatic sclerosing mesenteritis	Dead (9)
10	70/F	DQ2.5/DQ5	Large BCL	normal	Marsh 3a	Poly-clonal	Coeliac-like subepithelial collagen	GFD+budesonide	No	Marsh 3a	Extensive SB mosaic	normal	-	Alive (40)
11	39/F	DQ2.2/DQ2.2	-	multiple enlarged LN	Marsh 3a	Poly-clonal	Coeliac-like	GFD+budesonide	No	Marsh 3a	NA	normal	Angioimmuno-blastic TCL	Dead (36)
12	87/M	DQ5/DQ6	-	NA	Marsh 3c	NA	Coeliac-like	GFD+ Prednisolone+AZA	No	Marsh 3b	Mosaic proximal SB	normal	Oesophageal cancer	Dead (44)

Table 12. HLA heterodimers distribution in patients with idiopathic villous atrophy, coeliac disease and healthy controls. HC: healthy controls; IVA: idiopathic villous atrophy; CCD: complicated coeliac disease; UCD: uncomplicated coeliac disease

HLA	HC	GROUP 1 IVA	GROUP 2 IVA	GROUP 3 IVA	CCD	UCD	p-value
DQ2 ALL	122/424 (28.77%)	19/40 (47.50%)	3/12 (25.00%)	6/12 (50.00%)	44/44 (100%)	337/355 (94.93%)	**<0.001**
DQ2.2	67/424 (15.80%)	5/40 (12.50%)	1/12 (8.33%)	3/12 (25.00%)	15/44 (34.09)	156/355 (43.94%)	**<0.001**
DQ2.5	55/424 (12.97%)	14/40 (35.00%)	2/12 (16.67%)	3/12 (25.00%)	29/44 (65.91%)	181/355 (50.99%)	**<0.001**
DQ5	158/424 (37.26%)	11/40 (27.50%)	3/12 (25.00%)	5/12 (41.67%)	6/44 (13.64%)	44/355 (12.39%)	**<0.001**
DQ6	150/424 (35.38%)	18/40 (45.00%)	7/12 (58.33%)	3/12 (25.00%)	3/44 (6.82%)	65/355 (18.30%)	**<0.001**
DQ7 ALL	150/424 (35.38%)	12/40 (30.00%)	6/12 (50.00%)	3/12 (25.00%)	7/44 (15.91%)	63/355 (17.75%)	**<0.001**
DQ7.3	1/424 (0.24%)	5/40 (12.50%)	4/12 (33.33%)	2/12 (16.67%)	0/44 (0%)	0/355 (0%)	**<0.001**
DQ7.5	149/424 (35.14%)	8/40 (20.0%)	3/12 (25.00%)	2/12 (16.67%)	7/44 (15.91%)	63/355 (17.74%)	**<0.001**
DQ8	75/424 (17.69%)	7/40 (17.50%)	1/12 (8.33%)	0/12 (0%)	1/44 (2.27%)	44/355 (12.39%)	**0.025**
DQ9	34/424 (8.02%)	3/40 (7.50%)	2/12 (16.67%)	1/12 (8.33%)	0/44 (0%)	5/355 (1.40%)	**<0.001**

RESULTS OF THE PROJECT ON THE STUDY OF NATURAL HISTORY AND DEVELOPMENT OF NEW THERAPEUTIC STRATEGIES IN COMPLICATED COELIAC DISEASE

Digestive and Liver Disease 50 (2018) 549–552

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Alimentary Tract

Risk of complications in coeliac patients depends on age at diagnosis and type of clinical presentation

Federico Biagi[a,*,1], Annalisa Schiepatti[a,1,2], Gregorio Maiorano[a], Giacomo Fraternale[a], Simona Agazzi[a], Fabiana Zingone[b], Carolina Ciacci[b], Umberto Volta[c], Giacomo Caio[c], Raffaella Tortora[d], Catherine Klersy[e], Gino R. Corazza[a]

[a] Coeliac Centre/First Dept. of Internal Medicine, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Italy
[b] Celiac Center at Gastrointestinal Unit, AOU S. Giovanni di Dio e Ruggi d'Aragona, University of Salerno, Italy
[c] Department of Medical and Surgical Sciences, University of Bologna, Italy
[d] Department of Clinical and Experimental Medicine, Unit of Gastroenterology, University of Naples Federico II, Naples, Italy
[e] Biometry and Clinical Epidemiology, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Italy

ARTICLE INFO

Article history:
Received 13 September 2017
Received in revised form 7 November 2017
Accepted 3 December 2017
Available online 8 December 2017

Keywords:
Celiac disease/epidemiology
Gluten
Incidence
Prognosis
Risk assessment

ABSTRACT

Background: Coeliac disease is characterised by an increased mortality mostly due to its complications.
Aims: To study the risk of developing complications according to clinical presentation and age at diagnosis, a combined retrospective–prospective longitudinal study was performed in three Italian centres.
Methods: Incidence of complications and mortality rates were calculated using type and age at diagnosis of coeliac disease, sex, and centre of diagnosis as predictors. Patients referred after being found to suffer from coeliac disease elsewhere were excluded.
Results: Between 01/1999 and 06/2015, 2225 adult coeliac patients were directly diagnosed in our centres. 17 of them developed a complication and 29 died. In patients older than 60 years at diagnosis of coeliac disease, the risk of complication is 18 times higher than in patients diagnosed at 18–40 years and 9 times higher than in patients diagnosed at 40–60 years. Classical presentation increases the risk of complications by 7 times compared to non-classical presentation; in asymptomatic patients the risk of complication is virtually absent.
Conclusions: The risk of developing complications in coeliac patients is linked to age at diagnosis of coeliac disease and type of clinical presentation. Follow-up methods of coeliac patients should be tailored according to these parameters.

1. Introduction

Coeliac disease (CD) is a very common chronic gluten-induced enteropathy characterised by an increased mortality mostly due to its complications [1]. Although they are rare, these malignant conditions are burdened by a very bad prognosis [1,2]. This is the case of refractory CD (RCD, type 1 and 2), enteropathy associated T-cell lymphoma (EATL), small bowel carcinoma (SBC), B-cell lymphoma (BCL), and ulcerative jejunoileitis. While RCD type 1 has a 5-year survival rate of 80–96%, up to 50% of RCD type 2 patients can develop an overt EATL within 5 years of diagnosis. The 5-year survival rate of these last two conditions is between 40 and 58% and less than 20%, respectively [3–6]. SBC and B-cell lymphomas complicating CD also have a poor prognosis [2,7–9]. Given their malignant nature, the origin from the abdomen, the very similar clinical picture and the common underlying pathogenic mechanism of some of them [10], it is possible to consider these conditions all together as complicated forms of CD (CCD).

In the last 15 years it has emerged that the mortality of coeliac patients depends on several factors, strict adherence to a gluten-free diet, clinical type and age at diagnosis of CD being the most important ones [11]. Other factors such as a long diagnostic delay and male sex were initially described as risk factors for complications [11,12], but they were not subsequently confirmed [2,13]. Finally, HLA typing was shown to correlate with clinical types of CD and onset of complications [14].

* Corresponding author at: Coeliac Centre/1st Department of Internal Medicine, Fondazione IRCCS Policlinico San Matteo, P.le Golgi, 19, I-27100 Pavia, Italy.
E-mail address: f.biagi@smatteo.pv.it (F. Biagi).
[1] These authors contributed equally to the work.
[2] Dr. Annalisa Schiepatti is attending the PhD course in Experimental Medicine at the University of Pavia, Italy.

https://doi.org/10.1016/j.dld.2017.12.001
1590-8658/

RISK OF COMPLICATIONS IN COELIAC PATIENTS DEPENDS ON AGE AT DIAGNOSIS AND TYPE OF CLINICAL PRESENTATION

[a]Federico Biagi, [a]**Annalisa Schiepatti,** [a]Gregorio Maiorano, [a]Giacomo Fraternale, [a]Simona Agazzi, [b]Fabiana Zingone, [b]Carolina Ciacci, [c]Umberto Volta, [c]Giacomo Caio, [d]Raffaella Tortora, [e]Catherine Klersy, [a]Gino Roberto Corazza

a Coeliac Centre/First Dept. of Internal Medicine, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Italy
b Celiac Center at Gastrointestinal Unit, AOU S. Giovanni di Dio e Ruggi d'Aragona, University of Salerno, Italy
c Department of Medical and Surgical Sciences, University of Bologna, Italy
d Department of Clinical and Experimental Medicine, Unit of Gastroenterology, University of Naples Federico II, Naples, Italy
e Biometry and Clinical Epidemiology, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Italy

Digestive and Liver Disease 2018;50:549-52

Introduction

Coeliac disease (CD) is a very common chronic gluten-induced enteropathy characterised by an increased mortality mostly due to its complications [11]. Although they are rare, these malignant conditions are burdened by a very bad prognosis [11,12]. This is the case of refractory CD (RCD, type 1 and 2), enteropathy associated T-cell lymphoma (EATL), small bowel carcinoma (SBC), B-cell lymphoma (BCL), and ulcerative jejunoileitis. While RCD type 1 has a 5-year survival rate of 80–96%, up to 50% of RCD type 2 patients can develop an overt EATL within 5 years of diagnosis. The 5-year survival rate of these last two conditions is between 40 and 58% and less than 20%, respectively [15,23,25,28]. SBC and B-cell lymphomas complicating CD also have a poor prognosis [12,17, 122,123]. Given their malignant nature, the origin from the abdomen, the very similar clinical picture and the common underlying pathogenic mechanism of some of them [26], it is possible to consider these conditions all together as complicated forms of CD (CCD). In the last 15 years it has emerged that the mortality of coeliac patients depends on several factors, strict adherence to a gluten-free diet, clinical type and age at diagnosis of CD being the most important ones [10]. Other factors such as a long diagnostic delay and male sex were initially described as risk factors for complications

[10,20], but they were not subsequently confirmed [12,19]. Finally, HLA typing was shown to correlate with clinical types of CD and onset of complications [21]. The aim of the present work was to investigate whether clinical parameters already present at the time of diagnosis of CD can enable early identification of those coeliac patients at higher risk of developing complications. This would be of great help in establishing the follow-up methods of CD patients which have not been standardised so far [1,2].

Patients and methods

Data collection

Between January and December 2015 a combined retrospective and prospective design longitudinal multicentre study based on collection of clinical data was carried out in three Italian referral centres for the study of adult CD and its complications, Pavia, Bologna, and Naples-Salerno. Sex, age at diagnosis of CD, year of diagnosis, clinical type of CD, age at last outpatient clinic access or phone contact, age at onset and type of possible complication, age at death and cause of death were recorded for each adult CD patient directly diagnosed in our centres between January 1999 and June 2015. We must point out that, to avoid a selection bias, coeliac patients diagnosed elsewhere and seen in our centres to obtain a certificate entitling them to gluten-free products through the Italian National Health Service, for confirmation of the diagnosis, or for suspected CCD, were excluded. For the purpose of the statistical analysis, clinical type of CD was divided into classical, non-classical and asymptomatic according to the Oslo criteria [75] and corresponding to major, minor and silent [10]; analogously, three age groups were defined on the basis of age at diagnosis of CD (18–40 years; 41–60years; >60 years). All the patients who had not attended our centres for regular follow-up in the six months before the time of data collection were contacted over the phone to ascertain whether they were still alive and had not developed complications. For those patients who could not be reached by phone, the local council services were contacted to know whether they were still alive (in this case, information about the potential onset of a complication was not available). Causes and dates of death

were obtained through the Italian standard certificates of death. Diagnosis of CD was always based on at least four correctly oriented duodenal biopsies showing a certain degree of villous atrophy and positive IgA tissue transglutaminase/endomysial antibodies while on a normal gluten-containing diet. Seronegative patients were excluded. Diagnosis of RCD was based on persistence or recurrence of malabsorption symptoms and persistence of villous atrophy despite at least 12 months of a strict gluten-free diet [1,2,23]. Identification of aberrant CD3−CD8−CD103+CD7+cytoplasmatic CD3+ intraepithelial lymphocytes by flow cytometric analysis (>20%) and/or gamma chain T-cell monoclonal rearrangement by means of polymerase chain reaction allowed the diagnosis of RCD type 2 and the distinction from RCD type 1 [23,124]. Finally, the diagnoses of EATL, BCL, and SBC were based on histopathological criteria [15,17,122,123].

Statistics

Stata 14.2 (StataCorp, College Station, TX, USA) was used for computation. Data are reported as mean and standard deviation or as median and quartiles (25th–75th percentiles) if continuous and as counts and percent if categorical. Median follow-up (25th–75th) was computed with the inverse Kaplan–Meier methods. Clinical type of CD, sex, age at diagnosis of CD, year of diagnosis, and Centre where the diagnosis of CD was made were considered as predictors. Incidence of complications and overall mortality rate were calculated in each group. Survival and event-free survival werecompared with the Cox model between groups of patients; hazard ratios (HR) and 95% confidence intervals (95%CI) were computed.

The prognostic role of CCD was assessed with a time-dependent Cox model. Bivariable models were fitted to adjust for potential confounders in turn. The effect modification of each confounder was assessed with a test on interaction. If an interaction was found, separate models by subgroups were fitted. A p-value <0.05 was considered statistically significant. Bonferroni correction was applied for post-hoc comparisons.

Ethics

All patients signed informed consent before the biopsies, both for clinical and research purposes. After verifying the good quality of the data, the data were all irreversibly anonymized. None of the patients had signed against participation in anonymous studies. On 30th April 2014, the study was approved by the ethics committee of the Fondazione IRCCS Policlinico San Matteo according to the 1975 Declaration of Helsinki (6th revision, 2008).

Results

Between January 1999 and June 2015, 2225 adult coeliac patients (F 1660, mean age at diagnosis 36 ± 12 years) were directly diagnosed in our three centres. Between January and December 2015, 1899 of them (85.4%) were seen directly in our outpatient clinics or contacted over the phone to ascertain that they were still alive and had not developed complications. Only 326 patients (14.6%) could not be contacted or seen directly, so information on whether they were still alive or had died was provided by the respective local council services. Median follow-up was 79 months (25th–75th 37–125 months). Seventeen out of 2225 patients (11 F; mean age at diagnosis of complication 56 ±16 years) developed a complication (6 type 1 RCD, 4 EATL, 5 SBC, 2 abdominal BCL); mean age at diagnosis of CD was 52±15 years (age was >60 years in 8 of them, between 41 and 60 in 4, and <40 in 5). Median time between diagnosis of CD and diagnosis of complication was 55 months (25th–75th 29–105 months); similar results were obtained in the three different age groups (44, 58, and 48 months). Fourteen of them had a classical presentation and 3 a non-classical one. The overall incidence of complication is therefore 11 per 10,000 persons/year (95% CI 6–17 per 10,000 persons/year). Five of these 17 patients with CCD died (3 F; mean age at death 63 ± 7 years; median time between diagnosis of CD and death 85 months, 25th–75th 68–111 months). Causes of death included 3 EATL, 1 type 1 RCD and 1 SBC. Twenty-four of the 2208 patients who had not developed CCD had died (14 F; mean age at diagnosis of CD 57 ±14 years; mean age at death 64±14 years; median time between diagnosis of CD and death 91 months, 25th–75th 37–121 months). Causes of death in this group were very

heterogeneous and included 6 cardiocerebrovascular causes and 4 cancers unrelated to CD. A more precise list of these causes of death is available on request. The 326 patients for whom information was obtained from the local council services substantially had the same clinical and epidemiological characteristics of the remaining 85% of patients who were seen/contacted directly (268 F, mean age at diagnosis of CD 35 ± 11; classical presentation in 108, non-classical in 137, asymptomatic in 81). Table 12 shows the results of predictors of complications at the time of diagnosis. Age at diagnosis of CD and clinical type of CD represent strong predictors of complications. More precisely, the risk of complications progressively increases with the increase in age at diagnosis of CD. While the incidence of complications is very low in CD patients diagnosed before the age of 40 years (1/2000 persons/year), it is 1/1000 persons/year for those diagnosed with CD between 41 and 60 years, and almost 1/100 persons/year for those found to be affected by CD after the age of 60. In coeliac patients diagnosed after the age of 60 years, the risk of developing complications is 18 times higher than in patients diagnosed in the group aged 18–40 years and 9 times higher than in patients aged 41–60 years at diagnosis. The clinical type of CD is the other very important predictor of complications. Our results show that this risk is virtually non existent for asymptomatic patients. It is very low for nonclassical patients (1/2500 persons/year) but rises to more than 1/400 persons/year in classical CD patients. The risk of developing complications is 7 times higher in patients with classical presentation than in non-classical ones, and increases exponentially comparing classical and non-classical patients to asymptomatic ones. Finally, the incidence of complications does not vary according to the year of diagnosis, sex and centre where the diagnosis of CD was made. Mortality was 66 per 1000 person years (95% CI 27–158) in CCD patients and 2 per 1000 person years (95% CI 1–2.3) in CD patients without complications. Therefore, the risk of death in CCD patients is 30 times higher than in CD patients without complications ($p < 0.001$). As shown in Table 13, the effect of complications on mortality is independent of sex, centre of diagnosis, and type of clinical presentation at the bivariable time-dependent Cox model. There was no interaction between complication and any of these potential

confounders. Conversely, there was a strong modification effect by age at diagnosis of CD: we observed a decrease in the risk of dying associated with CCD with the increase of age at diagnosis.

Discussion

The most important result emerging from our study is that age at diagnosis of CD and clinical type of CD are strong predictors of complications in adult coeliac patients. These predictors are obviously already present at the time of diagnosis of CD and so they could be of great help to clinicians in addressing the follow-up of coeliac patients. Moreover, even if the evaluation of mortality was not the primary endpoint of this study, our results confirm that mortality of CD patients is primarily due to the onset of complications and that clinical type of CD and age at diagnosis influence mortality as well. Interestingly, the effect of complications on mortality is modified by the age at diagnosis of CD. While onset of complication at young age significantly affects mortality, the role of complication decreases with the increase of age, probably because other causes of death arise in these patients. Of note, given the low mortality, wide confidence intervals were obtained in the age-specific subgroup analyses. The main strengths of our study are the high number of patients enrolled, the long cumulative follow-up and the reliable information about their health status. Only 15% of our patients could not be contacted directly and information about whether they were still alive or had died was obtained from the local council services. Theoretically, some of these still alive patients could have developed a complication and could have been referred to another centre. Anyway, the clinical and epidemiological characteristics of these 326 patients were very similar to those of the 1899 patients directly seen. We therefore believe that it is very unlikely that these data can radically modify our final results. In our analysis, we could not take into account other parameters which have previously been shown to correlate with the risk of developing complications. This is the case for adherence to the gluten-free diet (GFD) and HLA typing [10,21,9,22,125]. Strict adherence to GFD is well known to be of paramount importance in preventing onset of complications [9,10,125]. However, evaluation of the strictness of GFD compliance can be made only a few months after the diagnosis of CD, while

our study specifically focused on identifying predictors of complications already present at the time of diagnosis of CD. Homozygosis for DQ2 was also shown to correlate with clinical type of CD and onset of complications [21,22]. Unfortunately, HLA typing of all 2225 patients was unfeasible. Although the incidence of complications was slightly increased in men, these data are not statistically significant, so we confirmed the previous findings that sex is not a risk factor for developing complications [12]. Again, the incidence of complications did not differ significantly between our three Centres. Since the methods of follow-up of coeliac patients do differ widely between the Centres with a specific interest in CD [1], this last point is a very important one. While in Pavia and Bologna a re-biopsy strategy is routinely performed 12–15 months after the start of a strict GFD, in the Naples-Salerno Centre it is offered only to those patients who do not present a satisfactory clinical response to a GFD [126-128]. Moreover, the clinical and histological response to a GFD have recently been suggested as parameter to stratify CD patients and specifically address their follow-up [125,126,129]. Having found the same number of complications in all our Centres, a second duodenal biopsy does not seem to be strictly required in all coeliac patients. Since complications arise dramatically more often in patients diagnosed at old age and because of classical symptoms, while they are virtually absent in patients diagnosed because of screening and at young age, a risk gradient for the development of complications could exist in coeliac patients. Given that only 17 patients developed a complication, we were only able to assess the role of each predictor in a univariable analysis. In particular, we were unable to study the combined effect of both age at diagnosis and clinical type of CD on the risk of complications. On the basis of our results, we calculated that about 6500 coeliac patients should be followed up for a median period of 80 months to observe at least 50 CCD patients. This would make it possible to fit a model to assess the combined prognostic role of age at diagnosis (3 groups) and clinical type of CD (3 groups), according to the events:predictors 1:10 rule. For a full multivariable analysis, the sample size would increase proportionally. We have already hypothesised that follow-up modalities of coeliac patients should be tailored according to their specific risk of complications [130]. Our present work identifies two clinical predictors that allow to distinguish those

individuals with the highest risk of complications and that need to receive the greatest level of support and follow-up care. So, coeliac patients diagnosed after the age of 60 and because of classical symptoms do require a careful clinical follow-up that includes upper endoscopy with duodenal biopsies. Could serial ultrasonography or CT scan be of help? A robust risk/benefit analysis is mandatory. On the contrary, asymptomatic and non-classical patients diagnosed before the age of 40 years may be followed up with biochemical exams only and referred to biopsy only in the case of an unsatisfactory clinical response. This proposal of a personalized follow-up based on clinical parameters already available at the time of diagnosis of CD represent a first attempt to standardize follow-up methods of coeliac patients. Further studies on larger series of patients are now required to help delineate the most cost-effective way of delivering follow-up care to CD patients.

	No. of pts	Incidence (95% CI) per 100 person year	Hazard ratio (95% CI)	Model p-value	Post hoc comparisons p value
Total	17	0.11 (0.06–0.17)	–		–
Sex				0.38	
F	11	0.09 (0.05–0.17)	1		
M	6	0.15 (0.06–0.37)	1.58 (0.58–4.3)		
Age at diagnosis (years)				<0.001	
18–40	5	0.05 (0.02–0.11)	1		
41–60	4	0.10 (0.04–0.27)	vs. 18–40: 2.07 (0.5–7.7)		0.83
≥61	8	0.89 (0.45–1.79)	vs. 41–60: 9 (2–38)		<0.001
			vs. 18–40: 18 (4.6–72)		0.001
Type of presentation				<0.001	
Asymptomatic	0	–	1		–
Non classical	3	0.04 (0.01–0.11)	vs. asymptomatic: 27×10^{14}		<0.001
Classical	14	0.27 (0.16–0.46)	vs non classical: 7 (1.5–33)		0.006
			vs. asymptomatic: 19×10^{15}		<0.001
Centre				0.80	
BO, PV	9	0.12 (0.06–0.22)	1		
NA-SA	8	0.10 (0.05–0.2)	0.88 (0.34–2.3)		
Year of diagnosis				0.07	
1999–2005	9	0.10 (0.05–0.20)	1		
2006–2010	3	0.05 (0.01–0.17)	0.38 (0.1–1.4)		
2011–2015	5	0.35 (0.15–0.84)	1.98 (0.6–6.8)		

Table 13. Incidence of complications×100 persons/year and hazard ratio with Bonferroni correction for the various predictors. No. of pts: number of patients with complicated coeliac disease, BO: Bologna, PV: Pavia, NA: Naples, SA: Salerno.

	Hazard ratio (95% CI)	p-Value	p interaction
CCD univariable	31.5 (11.7–84.3)	<0.001	–
CCD adjusted for sex	34.7 (12.9–93.7)	<0.001	0.61
CCD adjusted for type of presentation	21.7 (7.5–60.8)	<0.001	0.43
CCD adjusted for centre	32.6 (12.1–87.9)	<0.001	0.23
CCD adjusted for year of diagnosis	35.3 (13.0–94.4)	<0.001	0.11
CCD adjusted for age at diagnosis	6.2 (2.1–18.0)	0.001	0.001
18–40 years	127 (12–1296)	<0.001	–
41–60 years	61 (6–602)	<0.001	–
≥61 years	3.4 (0.9–13.0)	0.07	–

Table 14. Time-dependent Cox model—univariable and bivariable analysis. CCD: complicated coeliac disease.

In the NHS national centre for rare diseases, Sheffield, UK, a study has been conducted to evaluate the etiologies and long-term outcomes of coeliac patients referred to a national centre over 20 years (1998-2018) with persisting symptoms despite a GFD. This is the largest UK study about non-responsive CD and refractory CD.

More specifically, in this study all the patients were systematically investigated to establish the aetiology of their continued symptoms, including referral to a specialist coeliac dietitian to identify any lapses in GFD adherence or gluten cross-contamination.

Our preliminary data shows that 292 out of 2199 patients (67% female, mean age at diagnosis 42.8 ± 18.5) with biopsy-proven CD (13%) had persisting symptoms. The leading causes for persisting symptoms in patients without RCD were gluten contamination (22%), functional/IBS (20%), pancreatic exocrine insufficiency (7%), reflux dysmotility (5%), and microscopic colitis (5%). 74 patients had RCD; 56 had RCD I (71% female, mean age at CD diagnosis 41.8 ± 19.0) and 18 had RCD II (33% female, mean age at CD diagnosis 55.4 ± 13.3). After a median follow up of 40.5 months (IQR 21.8-73.3), mortality was 7% in the RCD I group, compared to 39% in the RCD II group ($p=0.019$). Higher age at diagnosis of CD is a predictor for having RCD in patients with persisting symptoms ($p<0.001$).This study confirms that contemporary mortality data in RCD II remains poor and advocate for novel therapies.

Experimental parts 3 and 4-Efficacy and safety of MSC infusions in refractory coeliac disease and in non-coeliac enteropathies with villous atrophy: the Pavia experience

In the period between 10/2016 and 09/2019 in our Coeliac Centre in Pavia we have treated with serial bone-marrow derived MSC infusions a total of **3 patients** affected by enteropathies with VA unresponsive to traditional immunosuppressive regimens. A brief summary of their baseline characteristics and outcomes so far are shown in table 14.

patient	diagnosis	MSC cycles, n°	Clinical response	Histological response	Adverse events	outcome
F/39	RCD 1	6	no	No	no	alive
M/57	IVA type 3*	1	no	No	no	dead
F/45	RCD 2	3	partial	To be assessed	no	alive

Table 14. Main features and outcomes of patients treated with MSC infusion in Pavia between 10/2016 and 09/2019.

***this patient was described in the manuscript on IVA, see Table 11**

More in details patient 1 (F/39) was affected by type 1 RCD and was started on MSC infusions on February 2018. At the end of the four scheduled infusions there was no evidence of either clinical or histological recovery, therefore we decided to continue the treatment for other 2 cycles (June 2018 and October 2018). This prolonged treatment provides a clinical improvement that was not, however, paralleled by a concomitant histological response (persistence of severe VA at the end of follow-up). Patient 2 (M/57) was affected by a severe malabsorption due to a form of idiopathic villous atrophy displaying lymphoprolipherative features (IVA type 3). The enteropathy was complicated by ulcerative jejunitis and the patient died two months after the first MSC infusion (patient 3 in Table 11). Finally, patient 3 (F/45) received MSC because of type 2 RCD. So far she has successfully completed three out of four scheduled MSC infusions. Clinical improvement has developed, while histological response still needs to be evaluated at the end of treatment.

These patients add to the other two we previously described [43,44].

CONCLUSIONS

Differential diagnosis and clinical management of enteropathies found in the context of SNVA are challenging. Although it has been shown that seronegative CD is likely the most common cause of SNVA [56], a variety of other conditions need to be taken into consideration. This is most commonly the case for autoimmune enteropathy, common variable immune-deficiency, parasitic infections, lymphomas and drug-related enteropathies. Other conditions, then, are listed as causes of SNVA, being anectodal though [94, 98, 99, 131,132]. Finally, also forms of enteropathies with VA of unknown origin do exist [reviewed in 48].

The results we have obtained during these years as part of the current PhD project do represent a substantial contribute to the knowledge of natural history of some of these enteropathies found in the context of SNVA.

Although both our paper and the paper by Aziz et al. concordantly report a higher mortality in SNVA than in conventional seropositive CD [46,89], we have come to the conclusion that the group of SNVA consists of heterogeneous enteropathies that do differ not only in terms of etiology, but also very likely in terms of long-term outcomes.

We think that whereas patients with dermatitis herpetiformis, olmesartan-associated enteropathy or VA due to small-intestinal bacterial overgrowth are triggered by reversible causes and usually display a very good prognosis, this is not the case for autoimmune enteropathy, common variable immunodeficiency or primary lymphomas of the small-bowel, that are burdened by a substantial risk of complications and a high mortality [46,50-52,55,89,105]. Moreover, we have demonstrated for the first time that enteropathies with VA of unknown origin encompass at least three groups of conditions with distinctive clinical and genetaic features and natural history. This is a unique finding with great implications for clinical practice. Not only we have identified patients with forms of transient VA (likely post-infective), but most importantly we have defined the diagnostic criteria for a new group of enteropathies with persistent VA unresponsive to a GFD and immunosuppressants, absence of

lymphoprolipherative features, specific HLA genetic background and long-term survival. These criteria allow the distinction with both seronegative CD and refractory CD. We have also demonstrated that mortality in these forms of VA of unknown origin is higher than in conventional seropositive CD, but mainly due to lymphoprolipherative conditions.

Another crucial point is the discovery of predictors to identify coeliac patients at higher risk of developing complications. We have identified that age at diagnosis and clinical pattern at time of diagnosis of CD can be useful predictors to identify patients at higher risk of developing complications. This can be considered in the strategy to optimize the follow-up of coeliac patients.

Finally, the results of the study on persisting symptoms in coeliac patients conducted in Sheffield, has confimed that although non-adherence to a GFD is the most common cause of peristing symptoms in CD, age at diagnosis is a risk factor for having RCD in coeliac patients with persisting symptoms despite a GFD.

The fact that contemporary mortality in some non-coeliac enteropathies remains high and closely resembles that of complicated forms of CD, clearly urge to the discovery and development of alternative strategies for the early identification and treatment of these patients.

The treatment with MSC may be considered a valid alternative treatment for patients affected by non-coeliac enteropathies who are at risk of poor long-term outcomes. However, larger studies are required on this issue.

REFERENCES

1. Ludvigsson JF, Bai JC, Biagi F, et al. *Diagnosis and management of adult coeliac disease: guidelines from the British Society of Gastroenterology.* Gut 2014;63:1210-28
2. Rubio-Tapia A, Hill ID, Kelly CP et al. *American College of Gastroenterology. ACG clinical guidelines: diagnosis and management of celiac disease.* Am J Gastroenterol 2013;108:656-76
3. Lebwohl B, Sanders DS, Green PHR. *Coeliac disease*. Lancet 2018;391:70-81.
4. Biagi F, Klersy C, Balduzzi D, et al. *Are we not over-estimating the prevalence of coeliac disease in the general population?* Ann Med 2010;42:557-61
5. Biagi F, Raiteri A, Schiepatti A, et al. *The relationship Between Child Mortality Rates and Prevalence of Celiac Disease*. J Pediatr Gastroenterol Nutr 2018:66:289-294
6. Singh P, Arora A, Strand TA et al. *Global Prevalence of Celiac Disease: Systematic Review and Meta-analysis.* Clin Gastroenterol Hepatol 2018;16:823-836.
7. Lohi S, Mustalahti K, Kaukinen K et al. *Increasing prevalence of coeliac disease over time.* Aliment Pharmacol Ther 2007;26:1217-25.
8. West J. *Celiac disease and its complications: a time traveller's perspective.* Gastroenterology 2009;136:32-4.
9. Holmes GK, Prior P, Lane MR, et al. *Malignancy in coeliac disease — effect of a gluten free diet.* Gut 1989;30:333-8
10. Corrao G, Corazza GR, Bagnardi V, et al. *Mortality in patients with coeliac disease and their relatives: a cohort study.* Lancet 2001;358:356-61
11. Biagi F, Corazza GR. *Mortality in celiac disease*. Nat Rev Gastroenterol Hepatol 2010;7:158-62
12. Biagi F, Gobbi P, Marchese A, et al. *Low incidence but poor prognosis of complicated coeliac disease: a retrospective multicentre study.* Dig Liver Dis 2014;46:227-30
13. Biagi F, Marchese A, Ferretti F, et al. *A multicentre case control study on complicated coeliac disease: two different patterns of natural history, two different prognoses.* BMC Gastroenterol 2014;14:139
14. Malamut G, Cellier C. *Is refractory celiac disease more severe in old Europe?* Am J Gastroenterol 2011;106:929-32
15. Di Sabatino A, Biagi F, Gobbi PG, et al. *How I treat enteropathy-associated T-cell lymphoma*. Blood 2012;119:2458-68
16. Catassi C, Fabiani E, Corrao G, et al. *Italian Working Group on Coeliac Disease and Non-Hodgkin's-Lymphoma. Risk of non-Hodgkin lymphoma in celiac disease*. JAMA 2002;287:1413-9
17. Raghav K, Overman MJ. *Small bowel adenocarcinomas--existing evidence and evolving paradigms.* Nat Rev Clin Oncol 2013;10:534-44
18. Biagi F, Corazza GR. *Defining gluten refractory enteropathy*. Eur J Gastroenterol Hepatol. 2001;13:561-5
19. Biagi F, Schiepatti A, Malamut G et al. *PROgnosticating COeliac patieNts SUrvivaL: the PROCONSUL score.* PLoS One 2014;9:e84163
20. Leffler DA, Dennis M, Hyett B, et al. *Etiologies and predictors of diagnosis in nonresponsive celiac disease.* Clin Gastroenterol Hepatol 2007;5:445-50
21. Al-Toma A, Goerres MS, Meijer JW et al. *Human leukocyte antigen-DQ2 homozygosity and the development of refractory celiac disease and enteropathy-associated T-cell lymphoma.* Clin Gastroenterol Hepatol 2006;4:315-9
22. Biagi F, Bianchi PI, Vattiato C et al. *Influence of HLA-DQ2 and DQ8 on severity in celiac Disease.* J Clin Gastroenterol 2012;46:46-50

23. Malamut G, Afchain P, Verkarre V, et al. *Presentation and long-term follow-up of refractory celiac disease: comparison of type I with type II.* Gastroenterology 2009;136:81-90
24. Cellier C, Patey N, Mauvieux L, et al. *Abnormal intestinal intraepithelial lymphocytes in refractory sprue.* Gastroenterology 1998;114:471-81
25. Rubio-Tapia A, Kelly DG, Lahr BD, et al. *Clinical staging and survival in refractory celiac disease: a single center experience.* Gastroenterology 2009;136:99-107
26. Cellier C, Delabesse E, Helmer C, et al. *Refractory sprue, coeliac disease, and enteropathy-associated T-cell lymphoma. French Coeliac Disease Study Group.* Lancet 2000;356:203-8
27. Verbeek WH, Goerres MS, von Blomberg BM et al. *Flow cytometric determination of aberrant intra-epithelial lymphocytes predicts T-cell lymphoma development more accurately than T-cell clonality analysis in Refractory Celiac Disease*. Clin Immunol. 2008;126:48-56
28. Al-Toma A, Verbeek WH, Hadithi M, et al. *Survival in refractory coeliac disease and enteropathy-associated T-cell lymphoma: retrospective evaluation of single-centre experience.* Gut 2007;56:1373-8
29. Daum S, Ipczynski R, Schumann M, et al. *High rates of complications and substantial mortality in both types of refractory sprue.* Eur J Gastroenterol Hepatol 2009;21:66-70
30. Gale J, Simmonds PD, Mead GM, et al. *Enteropathy-type intestinal T-cell lymphoma: clinical features and treatment of 31 patients in a single center.* J Clin Oncol 2000;18:795-803
31. Rubio-Tapia A, Murray JA. *Classification and management of refractory coeliac disease.* Gut 2010;59:547-57
32. Brar P, Lee S, Lewis S et al. *Budesonide in the treatment of refractory celiac disease.* Am J Gastroenterol 2007;102:2265-9
33. Mukewar SS, Sharma A, Rubio-Tapia A, et al. *Open-Capsule Budesonide for Refractory Celiac Disease.* Am J Gastroenterol 2017;112:959-967
34. Goerres MS, Meijer JW, Wahab PJ, et al. *Azathioprine and prednisone combination therapy in refractory coeliac disease.* Aliment Pharmacol Ther 2003;18:487-94
35. Maurino E, Niveloni S, Cherñavsky A, et al. *Azathioprine in refractory sprue: results from a prospective, open-label study.* Am J Gastroenterol 2002;97:2595-2602
36. Gillett HR, Arnott ID, McIntyre M et al. *Successful infliximab treatment for steroid-refractory celiac disease: a case report.* Gastroenterology 2002;122:800-5
37. Costantino G, della Torre A, Lo Presti MA et al. *Treatment of life-threatening type I refractory coeliac disease with long-term infliximab.* Dig Liver Dis 2008;40:74-7
38. Vivas S, Ruiz de Morales JM, Ramos F, et al. *Alentuzumab for refractory celiac disease in a patient at risk for enteropathy-associated T-cell lymphoma.* N Engl J Med 2006;354:2514-2415
39. Al-Toma A, Goerres MS, Meijer JW et al. *Cladribine therapy in refractory celiac disease with aberrant T cells.* Clin Gastroenterol Hepatol 2006;4:1322-7
40. Tack GJ, Verbeek WH, Al-Toma A, et al. *Evaluation of cladribine treatment in refractory celiac disease type II.* World J Gastroenterol 2011;17:506-513
41. Al-Toma A, Visser OJ, van Roessel HM, et al. *Autologous hematopoietic stem cell transplantation in refractory celiac disease with aberrant T cells.* Blood 2007;109:2243-2249
42. Tack GJ, Wondergem MJ, Al-Toma A, et al. *Auto-SCT in refractory celiac disease type II patients unresponsive to cladribine therapy.* Bone Marrow Transplant. 2011;46:840-6
43. Ciccocioppo R, Gallia A, Avanzini MA, et al. *A Refractory Celiac Patient Successfully Treated With Mesenchymal Stem Cell Infusions.* Mayo Clin Proc. 2016;91:812-9
44. Ciccocioppo R, Russo ML, Bernardo ME, et al. *Mesenchymal stromal cell infusions as rescue therapy for corticosteroid-refractory adult autoimmune enteropathy.* Mayo Clin Proc. 2012;87:909-14

45. Pallav K, Leffler DA, Tariq S, et al. *Noncoeliac enteropathy: the differential diagnosis of villous atrophy in contemporary clinical practice.* Aliment Pharmacol Ther 2012;35:380-90
46. Aziz I, Peerally MF, Barnes JH, et al. *The clinical and phenotypical assessment of seronegative villous atrophy; a prospective UK centre experience evaluating 200 adult cases over a 15-year period (2000-2015).* Gut 2017;66:1563-72
47. DeGaetani M, Tennyson CA, Lebwohl B, et al. *Villous atrophy and negative celiac serology: a diagnostic and therapeutic dilemma.* Am J Gastroenterol 2013;108:647- 53
48. Schiepatti A, Sanders DS, Zuffada M, Luinetti O, Iraqi A, Biagi F. *Overview in the clinical management of patients with seronegative villous atrophy.* Eur J Gastroenterol Hepatol 2019;31:409-17
49. Rubio-Tapia A, Herman ML, Ludvigsson JF, et al. *Severe spruelike enteropathy associated with olmesartan.* Mayo Clin Proc 2012;87:732–8
50. Schiepatti A, Biagi F, Cumetti D, et al. *Olmesartan-associated enteropathy: new insights on the natural history? Report of two cases.* Scand J Gastroenterol 2016;51:152-6
51. Corazza GR, Biagi F, Volta U, et al. *Autoimmune enteropathy and villous atrophy in adults.* Lancet 1997;350:106-9
52. Akram S, Murray JA, Pardi DS, et al. *Adult autoimmune enteropathy: Mayo Clinic Rochester experience.* Clin Gastroenterol Hepatol 2007;5:1282-90
53. Malamut G, Verkarre V, Suarez F, et al. *The enteropathy associated with common variable immunodeficiency: the delineated frontiers with celiac disease.* Am J Gastroenterol 2010;105:2262-2275
54. Biagi F, Bianchi PI, Zilli A, et al. *The significance of duodenal mucosal atrophy in patients with common variable immunodeficiency: a clinical and histopathologic study.* Am J Clin Pathol 2012;138:185-9
55. Foukas PG, de Leval L. *Recent advances in intestinal lymphomas.* Histopathology 2015;66:112-36
56. Schiepatti A, Sanders DS, Biagi F. *Seronegative coeliac disease: clearing the diagnostic dilemma.* Curr Opin Gastroenterol 2018;34:154-8
57. Pistoia V, Raffaghello L. *Mesenchymal stromal cells and autoimmunity.* Int Immunol 2017;29:49-58
58. Ciccocioppo R, Cangemi GC, Kruzliak P et al. *Concise Review: Cellular Therapies: The Potential to Regenerate and Restore Tolerance in Immune-Mediated Intestinal Diseases.* Stem Cells 2016;34:1474-86.
59. Bernardo ME, Locatelli F, Fibbe WE. *Mesenchymal stromal cells.* Ann. NY Accad Sci 2009;1176:101-117
60. De Miguel MP, Fuentes-Julián S, Blázquez-Martínez A, et al. *Immunosuppressive properties of mesenchymal stem cells: advances and applications.* Curr Mol Med 2012;12:574-591
61. Burr SP, Dazzi F, Garden OA. *Mesenchymal stromal cells and regulatory T cells: the Yin and Yang of peripheral tolerance?* Immunol Cell Biol 2013;9:12-18
62. Lalu MM, McIntyre L, Pugliese C, et al. *Safety of Cell Therapy with Mesenchymal Stromal Cells (SafeCell): A Systematic Review and Meta-Analysis of Clinical Trials.* Plos ONE 2012;7:e47559
63. Ansboro S, Roelofs AJ, De Bari C. *Mesenchymal stem cells for the management of rheumatoid arthritis: immune modulation, repair or both?* Curr Opin Rheumatol. 2017;29:201-207
64. Gharibi T, Ahmadi M, Seyfizadeh N, et al. *Immunomodulatory characteristics of mesenchymal stem cells and their role in the treatment of multiple sclerosis.* Cell Immunol. 2015;293:113-21
65. Le Blanc K, Frassoni F, Ball L, et al. *Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study.* Lancet 2008;371:1579-1586

66. Ciccocioppo R, Bernardo ME, Sgarella A, et al. *Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease.* Gut 2011;60:788-798
67. Ciccocioppo R, Camarca A, Cangemi GC, et al. *Tolerogenic effect of mesenchymal stromal cells on gliadin-specific T lymphocytes in celiac disease.* Cytotherapy 2014;16:1080-91
68. Katz AJ, Grand RJ. *All that flattens is not "sprue".* Gastroenterology 1979;76:375-7
69. Abrams JA, Diamond B, Rotterdam H et al. *Seronegative celiac disease: increased prevalence with lesser degrees of villous atrophy.* Dig Dis Sci 2004;49:546-50
70. Dickey W, Hughes DF, McMillan SA. *Reliance on serum endomysial antibody testing underestimates the true prevalence of coeliac disease by one fifth.* Scand J Gastroenterol 2000;35:181-3
71. Biagi F, Bianchi PI, Campanella J et al. *The impact of misdiagnosing celiac disease at a referral centre.* Can J Gastroenterol 2009;23:543-5
72. Biagi F, Pezzimenti D, Campanella J et al. *Endomysial and tissue transglutaminase antibodies in coeliac sera. A comparison not influenced by previous serological testing.* Scand J Gastroenterol 2001;36:955-8
73. Fry L. *Dermatitis herpetiformis*. Baillieres Clin Gastroenterol 1995;9:371-93
74. Biagi F, Bianchi PI, Zilli A et al. *The significance of duodenal mucosal atrophy in patients with common variable immunodeficiency: a clinical and histopathologic study.* Am J Clin Pathol 2012;138:185-9
75. Ludvigsson JF, Leffler DA, Bai J et al. *The Oslo definitions for coeliac disease and related terms.* Gut 2013;62:43-52
76. Rashtak S, Ettore MW, Homburger HA, Murray JA. *Comparative usefulness of deamidated gliadin antibodies in the diagnosis of celiac disease.* Clin Gastroenterol Hepatol 2008;6:426-32
77. Collin P, Kaukinen K, Vogelsang H et al. *Antiendomysial and antihuman recombinant tissue transglutaminase antibodies in the diagnosis of coeliac disease: a biopsy-proven European multicentre study.* Eur J Gastroenterol Hepatol 2005;17:85-91
78. Hopper AD, Cross SS, Hurlstone DP et al. *Pre-endoscopy serological testing for coeliac disease: evaluation of a clinical decision tool.* BMJ 2007;334:729
79. Viljamaa M, Kaukinen K, Pukkala E et al. *Malignancies and mortality in patients with coeliac disease and dermatitis herpetiformis: 30-year population-based study.* Dig Liver Dis 2006;38:374-80
80. Resnick ES, Moshier EL, Godbold JH, et al. *Morbidity and mortality in common variable immune deficiency over 4 decades.* Blood 2012;119:1650-57
81. Quinti I, Soresina A, Spadaro G, et al. *Long-term follow-up and outcome of a large cohort of patients with common variable immunodeficiency.* J Clin Immunol 2007;27:308-16
82. Kalha I, Sellin JH. *Common variable immunodeficiency and the gastrointestinal tract.* Curr Gastroenterol Rep 2004;6:377-83
83. Daniels JA, Lederman HM, Maitra A, et al. *Gastrointestinal tract pathology in patients with common variable immunodeficiency (CVID): a clinicopathologic study and review.* Am J Surg Pathol 2007;31:1800-12
84. Jørgensen SF, Reims HM, Frydenlund D, et al. *A cross-sectional study of the prevalence of gastrointestinal symptoms and pathology in patients with common variable immunodeficiency.* Am J Gastroenterol 2016;111:1467-75
85. Wood PM. *Primary antibody deficiency syndromes.* Curr Opin Hematol 2010;17:356-61
86. Park MA, Hagan JB. *Common variable immunodeficiency: a new look at an old disease.* Lancet 2008;372:489-02
87. Volta U, Caio G, Boschetti E, et al. *Seronegative celiac disease: shedding light on an obscure clinical entity.* Dig Liver Dis 2016;48:1018-22
88. Finkelstein DM, Muzikansky A, Schoenfeld DA. *Comparing survival of a sample to that of a standard population.* J Natl Cancer Inst 2003;95:1434-9

89. Schiepatti A, Biagi F, Fraternale G, et al. *Mortality and differential diagnoses of villous atrophy without coeliac antibodies.* Eur J Gastroenterol Hepatol 2017;29:572-6
90. Marthey L, Cadiot G, Seksik P et al. *Olmesartan-associated enteropathy: results of a national survey.* Aliment Pharmacol Ther 2014;40:1103–9
91. Ziegler TR, Fernández-Estívariz C, Gu LH et al. *Severe villus atrophy and chronic malabsorption induced by azathioprine.* Gastroenterology 2003;124:1950-7
92. Boscá MM, Añón R, Mayordomo E et al. *Methotrexate induced sprue-like syndrome.* World J Gastroenterol 2008;14:7009-11
93. Kamar N, Faure P, Dupuis E et al. *Villous atrophy induced by mycophenolate mofetil in renal-transplant patients.* Transpl Int 2004;17:463-7
94. Smale S, Tibble J, Sigthorsson G et al. *Epidemiology and differential diagnosis of NSAID-induced injury to the mucosa of the small intestine.* Best Pract Res Clin Gastroenterol 2001;15:723-38
95. Kaosombatwattana U, Limsrivilai J, Pongpaibul A et al. *Severe enteropathy with villous atrophy in prolonged mefenamic acid users - a currently under-recognized in previously well-recognized complication: Case report and review of literature.* Medicine (Baltimore) 2017;96:e8445
96. Levinson JD, Nastro LJ. *Giardiasis with total villous atrophy.* Gastroenterology 1978;74:271-5
97. Kapembwa MS, Batman PA, Fleming SC et al. *HIV enteropathy*. Lancet 1989-30;2:1521-2
98. Madsen JE, Vetvik K, Aase S. *Helicobacter-associated duodenitis and gastric metaplasia in duodenal ulcer patients.* APMIS 1991;99:997-1000.
99. Voutilainen M, Juhola M, Färkkilä M et al. *Gastric metaplasia and chronic inflammation at the duodenal bulb mucosa.* Dig Liver Dis 2003;35:94-8
100. Fung WP, Tan KK, Yu SF et al. *Malabsorption and subtotal villous atrophy secondary to pulmonary and intestinal tuberculosis.* Gut 1970;11:212-6
101. Lappinga PJ, Abraham SC, Murray JA et al. *Small intestinal bacterial overgrowth: histopathologic features and clinical correlates in an underrecognized entity.* Arch Pathol Lab Med 2010;134:264-70
102. Culliford A, Markowitz D, Rotterdam H et al. *Scalloping of duodenal mucosa in Crohn's disease.* Inflamm Bowel Dis 2004;10:270-3
103. Brown IS, Bettington A, Bettington M et al. *Tropical sprue: revisiting an underrecognized disease.* Am J Surg Pathol 2014;38:666-72
104. Rubio-Tapia A, Talley NJ, Gurudu SR et al. *Gluten-free diet and steroid treatment are effective therapy for most patients with collagenous sprue.* Clin Gastroenterol Hepatol 2010;8:344-34
105. Pensieri MV, Pulvirenti F, Schiepatti A et al. *The high mortality of patients with common variable immunodeficiency and small bowel villous atrophy.* Scand J Gastroenterol 2019;54:164-168
106. Daum S, Weiss D, Hummel M et al. *Intestinal Lymphoma Study Group. Frequency of clonal intraepithelial T lymphocyte proliferations in enteropathy-type intestinal T cell lymphoma, coeliac disease, and refractory sprue.* Gut 2001;49:804-12
107. Brown IS, Bettington A, Bettington M et al. *Self-limited coeliac-like enteropathy: a series of 18 cases highlighting another coeliac disease mimic.* Histopathology 2016;68:254-61
108. Goldstein NS. *Non-gluten sensitivity-related small bowel villous flattening with increased intraepithelial lymphocytes: not all that flattens is celiac sprue.* Am J Clin Pathol 2004;121:546-50
109. Oberhuber G, Granditsch G, Vogelsang H. *The histopathology of coeliac disease: time for a standardized report scheme for pathologists.* Eur J Gastroenterol Hepatol 1999;11:1185–94.
110. Olerup O, Zetterquist H. *HLA-DRB1*01 subtyping by allele-specific PCR amplification: a sensitive, specific and rapid technique.* Tissue Antigens 1991;37:197-204.

111. Jordan F, McWhinnie AJ, Turner S et al. *Comparison of HLA-DRB1 typing by DNA-RFLP, PCR-SSO and PCR-SSP methods and their application in providing matched unrelated donors for bone marrow transplantation.* Tissue Antigens 1995;45:103-10
112. Masia R, Peyton S, Lauwers GY et al. *Gastrointestinal biopsy findings of autoimmune enteropathy: a review of 25 cases.* Am J Surg Pathol 2014;38:1319-29
113. Megiorni F, Pizzuti A. *HLA-DQA1 and HLA-DQB1 in Celiac disease predisposition: practical implications of the HLA molecular typing.* J Biomed Sci 2012;19:88
114. Lin J, McKenna BJ, Appelman HD. *Morphologic findings in upper gastrointestinal biopsies of patients with ulcerative colitis: a controlled study.* Am J Surg Pathol 2010;34:1672-7
115. Liu H, Brais R, Lavergne-Slove A et al. *Continual monitoring of intraepithelial lymphocyte immunophenotype and clonality is more important than snapshot analysis in the surveillance of refractory coeliac disease.* Gut 2010;59:452-60
116. Hussein S, Gindin T, Lagana SM et al. *Clonal T cell receptor gene rearrangements in coeliac disease: implications for diagnosing refractory coeliac disease.* J Clin Pathol 2018;71:825-831
117. Celli R, Hui P, Triscott H et al. *Clinical Insignficance of Monoclonal T-Cell Populations and Duodenal Intraepithelial T-Cell Phenotypes in Celiac and Nonceliac Patients.* Am J Surg Pathol 2019;43:151-160
118. Louie CY, Di Maio MA, Matsukuma KE et al. *Idelalisib-associated enterocolitis: clinicopathologic features and distinction from other enterocolitides.* Am J Surg Pathol 2015;39:1653-60
119. Ciccocioppo R, Corazza GR. *Mesenchymal stromal cells: an opportunity to treat chronic inflammatory intestinal conditions.* Cytotherapy 2018;20:1223-26
120. Ahmed Z, Imdad A, Connelly JA et al. *Autoimmune enteropathy: an updated review with special focus on stem sell transplant therapy.* Dig Dis Sci 2019;64:643-654
121. Duarte RF, Labopin M, Bader P, et al. *European Society for Blood and Marrow transplantation (EBMT). Indications for haematopoietic stem cell transplantation for haematological diseases, solid tumours and immune disorders: current practice in Europe, 2019.* Bone Marrow Transplant 2019 Apr 5. doi: 10.1038/s41409-019-0516-2. [Epub ahead of print]
122. Howdle PD, Jalal PK, Holmes GK, et al. *Primary small-bowel malignancy in the UK and its association with coeliac disease.* Quarterly Journal of Medicine 2003;96:345–53
123. Leslie LA, Lebwohl B, Neugut AI et al. *Incidence of lymphoproliferative disorders in patients with celiac disease.* Am J Hematol 2012;87:754-9
124. van Wanrooij RL, Müller DM, Neefjes-Borst EA et al. *Optimal strategies to identify aberrant intra-epithelial lymphocytes in refractory coeliac disease.* J Clin Immunol 2014;34:828-35
125. Kurien M, Trott N, Sanders DS. *Long-term care for patients with coeliac disease in the UK: a review of the literature and future directions.* J Hum Nutr Diet 2016;29:617-23
126. Biagi F, Vattiato C, Agazzi S et al. *A second duodenal biopsy is necessary in the follow-up of adult coeliac patients.* Ann Med 2014;46:430-3
127. Volta U, Caio G, Stanghellini V et al. *The changing clinical profile of celiac disease: a 15-year experience (1998-2012) in an Italian referral center.* BMC Gastroenterol 2014;14:194
128. Ciacci C, Cirillo M, Cavallaro R et al. *Long-term follow-up of celiac adults on gluten-free diet: prevalence and correlates of intestinal damage.* Digestion 2002;66:178-85
129. Lebwohl B, Granath F, Ekbom A et al. *Mucosal healing and risk for lymphoproliferative malignancy in celiac disease: a population-based cohort study.* Ann Intern Med 2013;159:169-75
130. Biagi F, Corazza GR. *Do different patients with coeliac disease have different mortality rates?* Gut 2015;64:1187-8
131. Biagi F, Balduzzi D, Delvino P, Schiepatti A et al. *Prevalence of Whipple's disease in north-western Italy.* Eur J Clin Microbiol Infect Dis 2015;34:1347-8

132. Biagi F, Schiepatti A, Badulli C et al.. *-295 T-to-C promoter region IL-16 gene polymorphism is associated with Whipple's disease.* Eur J Clin Microbiol Infect Dis 2015;34:1919-21
133. Schiepatti A, Bellani V, Perlato M et al. *Inadvertent and minimal gluten intake has a negligible role in the onset of symptoms in patients with coeliac disease on a gluten-free diet.* Br J Nutr 2018;11:1-23.

ACKNOWLEDGEMENTS

I would like to express my most sincere and deep gratitude to Professor David Sanders and Professor Federico Biagi, my two mentors, for their wise guidance, their enthusiastic encouragement and their precious pieces of suggestion in every tricky moment troughout all my PhD period.

I also would like to thank all my colleagueas and friends in Pavia and in Sheffield for their advice, personal support and all the good time spent together.

My grateful thanks are also extended to Collegio Ghislieri, Pavia, Italy that funded the present phD project in 2018 (“assegno di ricerca annuale per giovani ricercatori”).

Finally, I wish to thank Umberto, my parents and my brother Alessandro for their constant support and encouragement throughout my study. You are my strenght.