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TITLE

Potential celiac disease: natural history and predictive biomarkers

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Potential celiac disease: natural history and predictive biomarkers

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Chapter 1: General introduction on potential celiac disease

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Celiac disease (CD) is an immune-mediated systemic disorder elicited by gluten and related prolamines in genetically susceptible individuals, characterized by the presence of a variable combination of clinical manifestations, CD-specific antibodies, HLA-DQ2 or HLA-DQ8 haplotypes, and enteropathy (1). Despite enteropathy has historically been described as the most prominent clinical feature, it is now widely accepted that the degree of intestinal damage can range from total villous atrophy, typical of the overt disease, to minor or absent histological alterations (2). Quantitative histology and computerized image analysis have shown that these features occur in a continuum, with flat mucosa at one end of the spectrum and normal villous architecture, with or without an abnormally higher count of intraepithelial lymphocytes, at the other end (2). The structural changes (type 1 infiltrative, type 2 hyperplastic, type 3 destructive, described by Marsh (2) were attributed to lamina propria T cell activation and the degree of damage dependent on antigen dose (amount of dietary gluten).

The term latent CD was introduced in the 90s for patients who display a normal jejunal biopsy while consuming a normal diet and, at some other time, before or since, have had a flat mucosa recovering on a gluten free diet (GFD) (3). These subjects, despite a normal mucosa, may still express subtle immunological alterations similar to those present in untreated coeliac disease; however, this definition is rarely fulfilled, only by subjects who received two duodenal biopsies: one with normal intestinal architecture and the other with villous atrophy (4). At that same time, the term potential CD (PCD) was proposed for subjects who show a normal mucosa with no or mild infiltration of intra-epithelial lymphocytes and immunological alterations specific of active CD, such as high TcR-γδ intraepithelial lymphocytes (5) or coeliac-like intestinal antibody pattern (6). Subsequently, with the Oslo definitions (7), while the use of the term latent CD was discouraged, the term potential CD was attributed to a subset of patients with normal intestinal architecture, with or without high number of intraepithelial lymphocytes, who are at risk of developing CD as indicated by positive celiac serology

(increased serum levels of anti-tissue transglutaminase (anti-TG2) and anti-endomysium antibodies (EMA). The European Society of Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) gave a similar definition in its 2012 guidelines for the diagnosis of CD (1), adding that PCD patients may present or not gluten-related clinical symptoms, and may develop or not villous atrophy over time. Anti-TG2 IgA antibodies titres are usually lower in these subjects compared to untreated CD patients with villous atrophy (8). Interestingly, during follow-up, fluctuating titres or disappearance of antibodies over time are noted (9). From a genetic standpoint, patients with potential celiac disease (PCD) more frequently show HLA haplotypes related to low or moderate risk of CD (10)(11) and a different asset of non-HLA polymorphisms (11). These clinical, genetic and immunological evidences, altogether suggest that PCD patients show different features compared to active CD, and also among PCD patients, a certain degree of heterogeneity exists.

Clinical management of PCD is still widely debated since short and long-term risks of this condition have not been sufficiently explored so far. Is PCD already a pathological condition or only a pre-disease state? Do all patients require a GFD regardless of the presence of symptoms? Despite an increasing interest in the scientific community in recent years, many of these issues remain unanswered. Certainly, PCD represents an incomparably useful model for the study of early molecular and immunological mechanisms involved in CD, with relevant implications in the implementation of prevention strategies.

In the present chapter we aim at summarizing the most recent evidences about this emerging condition both from a pathogenetic and a clinical prospective.

1.1 Pathogenesis

The mucosal immune response in active CD

The presence of intestinal gluten-specific CD4 T cells plays a key role in the development of CD (12). Gliadins, glutenins, hordeins and secalins have a high content in proline and glutamine that make them resistant to digestion by gastrointestinal enzymes (13). This results into long undigested fragments in the intestinal lumen, that interact with the intestinal epithelium and reach the lamina propria, either actively through trans-epithelial transport (14), or passively by paracellular flux. In the lamina propria or in the gut lumen (15), these peptides are an excellent substrate for the tissue transglutaminase (TG2) because of their high content in glutamines. The resulting deamidated peptides, with their negatively charged residues, have a considerably higher affinity for the HLA-DQ2 and HLA-DQ8 molecules and can finally activate a gluten-specific T cell adaptive response that

drives the disease (16) (17). The structural requirements that generate effective binding of gluten peptides have been elucidated (18) (19) (20), as well as the biased use of the T cell receptor genes (21) (22).

Pathogenic gluten-specific T cells, HLA DQ2 and/or HLA DQ8 restricted, have a Th1 phenotype characterized by the production of gamma interferon (IFN-g) (23). The pattern of cytokines produced by gluten-specific CD4 T cells includes IFN-g and IL-21 (24) (25), but not IL-17 (26). IL-21 is thought to play an important role in the development of tissue damage and particularly in the crosstalk between the gluten-specific adaptive response in the lamina propria and the intestinal epithelium. Synergistically with IL-15, IL-21 has been shown to increase cytolytic properties in CD8 cytotoxic T cells expressing natural killer like receptors (NKRs) (27).

IL-15 is a key cytokine in the pathogenesis of CD. It is significantly upregulated in both the small intestinal lamina propria and in the epithelium of CD patients (28). IL-15 seems to be critical in the development of the gluten-specific Th1 response, but also in enabling effector cytotoxic CD8 T cells to kill stressed tissue. In fact, IL15, promoting the expression of activating NK receptors by intraepithelial lymphocytes, enables them to kill epithelial cells following the recognition of stress signals (29)(30). Finally, IL-15 could contribute to the mucosal damage by impairing the suppressive function of regulatory T cell (Tregs) (31)(32). Type 1 interferons (type-1 IFN) are also upregulated in the mucosa of CD patients (33). They have been shown to contribute to the downregulation of a Treg response to dietary antigens (33). Whether their upregulation result from viral infections or other pathways remains to be confirmed.

The gluten-specific CD4 T cells is also responsible for the production of the specific autoantibodies found in CD patients sera: anti-deamidated gliadin antibodies and anti-TG2 antibodies. The model proposed is that TG2-specific B cells internalize TG2 complexed with gliadin peptides and present gluten derived peptides to specific T cells that provide help to naïve B cells resulting in activate plasma cells producing anti-deamidated gliadin antibodies and anti-TG2 antibodies (34)(34). Supported by observations in animal models (35), this model explains the gluten dependence of anti-TG2 antibodies production.

The activation of lamina propria gluten specific T cell response alone is not sufficient to induce villous atrophy. The effector phase leading to tissue damage requires the activation of cytotoxic T cells that infiltrate and progressively destroy the target organ. Under physiological conditions CD8+TcR□□□ cytotoxic T cells express the NKG2A inhibitory receptor and low levels of the activating NKG2D receptor (36) (37) (38). In active CD, intraepithelial lymphocytes downregulate the expression of the CD94/NKG2A complex and upregulate the activating CD94/NKG2C and NKG2D receptors (29)

(30). At the same time, intestinal epithelial cells upregulate MIC-A/B and HLA-E (29), ligands for NKG2D and CD94/NKG2C, respectively (39) (40) (41). In fact, in overt CD, intraepithelial lymphocytes recognize stress signals on enterocytes, such as MIC-A/B or HLA-E. These molecules are not expressed by healthy small-bowel intestinal epithelial cells, but can be induced in the context of stress, inflammation, and infection. Evidence increasingly suggests that activated CD8+ cytotoxic T cells do not induce tissue damage when they infiltrate a non-stressed/inflamed tissue, even if the appropriate antigen is expressed. In conclusion, the event that leads to enterocyte destruction and overt CD is the 'licensing' of intraepithelial lymphocytes to kill enterocytes.

The mucosal immune response in PCD

If the model proposed to explain the gluten dependence of anti-TG2 production is true (34), the presence of anti-TG2 antibodies indicates that gluten specific T cells are present in the gut of PCD patients. The lower levels of IgA antiTG2 antibodies observed in such patients (8) likely reflect a smaller amplitude of the CD4 T cell response as compared to active CD. The clinical observation that autoantibodies may disappear in a subset of PCD patients during follow-up (9) suggests that the loss of tolerance to gluten may be still reversible in some patients. This seems to be the case especially in younger children with PCD followed-up prospectively, a significant part of whom tend to become serologically negative over time (42) (43). The comprehension of the mechanisms behind this observation could be particularly relevant for the implementation of preventive strategies.

As mentioned before, in PCD patients a reduced number of inflammatory cells infiltrate lamina propria and the epithelium. However, not only the magnitude, but also the nature of the adaptive gluten-specific T cell response is different in PCD compared to overt CD. As a matter of fact, a different cytokine profile has been identified: an increased density of IL4-producing T cells with a T helper 2 (Th2) phenotype was found in PCD (44) (45), suggesting that Th2 cells could in part contrast the shift toward a IFN-γ producing Th1 response, and eventually contribute to prevention of tissue damage. In line with this concept, IFN-γ transcripts increase in parallel with the degree of mucosal alterations: IFN-γ RNA expression levels are lower in PCD patients with Marsh 0 lesion than in those with Marsh 1 lesion and in the latter significantly lower than in active CD (46). Recent findings indicate that in the process of transition to villous atrophy the almost disappearance of IL4-producing cells is accompanied by an increased expansion of TcRγδ+ intraepithelial lymphocytes (45).

Importantly, IL-15, an innate cytokine expressed in the small intestinal epithelium in the context of tissue damage and playing an important role in CD through the induction of a killing phenotype in

IELs is not hyper-expressed in PCD at the same levels as it is in active CD (46). Even more strikingly, IL-21 is downregulated in PCD (11) (25). As mentioned above, IL-15 and IL-21 synergize to promote cytolytic properties of intraepithelial CD8 T cells responsible for tissue damage (27).

The possible role of regulatory T cells (Treg) is disputed in CD, including the possibility that in PCD they could contribute to maintain mucosal integrity despite the presence of gluten-specific Th1 cells. The IL-10 / IFN-γ ratio was shown to be higher in PCD compared to active CD patients (46), and among PCD patients, in Marsh 1 compared to Marsh 0 (46). This suggests that immunoregulatory pathways may act in the intestinal mucosa of PCD patients preventing the progression toward mucosal damage. In line with this hypothesis, both the percentage and the absolute number of Foxp3+ T cells in the lamina propria of PCD patients are higher compared to controls. However, they are lower in PCD compared to active CD patients, implicating that although the immune system attempts to downregulate the ongoing inflammation through a rapid redistribution of Foxp3+ Tregs from the circulation to the tissue, yet, tissue damage occurs when immunoregulatory pathways no more counterbalance inflammatory mechanisms. Importantly, IL-15 seems to play a key role in this process. In fact, while intestinal T-regs from PCD patients are able to suppress T responder cells, regardless of the presence of IL 15 (46), in patients with villous atrophy IL-15 is able to inhibit T regulatory cells (31).

In conclusion, PCD patients miss the contextual presence of an anti-gluten CD4 T cell immunity and an upregulation of NKRs that render CD8 intraepithelial lymphocytes able to kill epithelial cells based on the recognition of stress signals. In line with this, the absence of villous atrophy in PCD is associated with the lack of (I) a fully activated NKR phenotype in CD8 cytotoxic T cells and (II) of increased levels of epithelial stress markers, including IL-15.

Nevertheless, the epithelial compartment in PCD is not completely normal. The expression of all junction proteins has been found already decreased in early stage CD when compared with non-celiac controls (47), possibly on a constitutive basis. In fact, junction protein expression correlated positively with mucosal villus morphology and negatively with the number of intraepithelial lymphocytes, the intensity of small-intestinal anti-TG2 deposits, and serum anti-TG2 antibodies. These findings show that the mucosal epithelial integrity is disrupted already in early stage CD before the disorder progresses to full-blown enteropathy.

Although the immunological events leading to the villous atrophy have not been fully elucidated yet, PCD represents an early disease stage and thus a unique opportunity to dissect those events involved in the progression toward disruption of tissue architecture and overt CD.

Evidence from animal models

Animal models are critical to understand the pathogenesis of diseases and test new therapeutic strategies. The very first experiments conducted to create an animal model of CD showed the lack of intestinal lesions in mice after systemic immunisation with gliadin and subsequent gluten feeding (48). Once the critical role of HLA emerged, mice were engineered to express HLA-DQ8 (49) and subsequently DQ2.5 (50) to recapitulate the antigen presentation occurring in human patients. Transgenic mice expressing HLA-DR3-DQ2 and human CD4, one with a NOD background and another TCR transgenic having over 90% of CD4(+) T cells specific for the DQ2-alpha-II epitope with a Th1 phenotype, did not show any sign of mucosal damage when consuming gluten. These humanized mouse models indicate that gluten ingestion can be tolerated without intestinal pathology even when HLA-DQ2-restricted CD4(+) T cell immunity to gluten is established, confirming that the presence of an adaptive immune response to gluten not sufficient to induce enteropathy.

As mentioned before, the majority of CD patients show a significant upregulation of IL-15 both in epithelium and in the lamina propria of the duodenal mucosa (51). To test the hypothesis that both an adaptive immune response and innate epithelial stress markers are necessary to induce a full activation of cytotoxic IELs, mice expressing the human HLA-DQ8 were engineered to overexpress IL-15 in both the lamina propria and the intestinal epithelium. Interestingly, neither mice expressing high IL-15 levels exclusively in haematopoietic cells mimicking what happens in the lamina propria of CD patients (DQ8-Dd IL-15 transgenic mice), nor those expressing high IL-15 exclusively in the epithelium (DQ8-villin-IL15 transgenic mice) developed villous atrophy (52). The first model resembles PCD patients, where a Th1 response to gluten is not enough to cause mucosal destruction.

Only triple transgenic mice overexpressing IL-15 in both the epithelial and the lamina propria compartments, developed villous atrophy when fed gluten for several weeks; interestingly, the damage was blocked by anti-TG2 inhibitors and reverted upon gluten withdrawal (52). This model, despite not perfect, significantly helped to dissect the events required for CD development and represents a unique model to test potential therapeutics for CD.

Analogies with type 1 diabetes

The condition featuring a specific CD4 T cell response and circulating autoantibodies without tissue damage is not unique of PCD. Also, in prediabetes, pancreatic beta islets are infiltrated by

autoreactive CD4+ T cells and autoantibodies are measurable in the serum even in the absence of beta cells destruction (53). Indeed, similar pathogenic mechanisms are shared between the two disorders. Like in CD, in NOD model of T1D, NKG2D was expressed on autoreactive intra-pancreatic CD8+ cells and the use of an anti-NKG2D prevented the progression the overt disease (54). Equally, IL-15 was found to be increased in T1D beta islet cells and its inhibition delays the evolution from prediabetes to diabetes (53). Finally, in both diseases, stress molecules like MHC-like molecules are expressed in the target tissue and tissue destruction is mediated by CD8+ T cells that destroy the target tissue in an antigen independent manner. Like in PCD, also in prediabetes IL-21 is downregulated and it becomes overexpressed only when tissue damage occurs (53). The notion that cytotoxic T cells need to be licensed in tissues to become effective killer cells and that licensing depends on IL-15 is supported by the observation in NOD mice that inhibition of IL-15 signaling at the prediabetic stage delayed diabetes development (55)

The presence of a pre-disease stage offers a unique opportunity to test preventive strategies to block or postpone overt-disease onset. Interestingly, Harold et al. recently demonstrated that a one course administration of Teplizumab, an Fc receptor–nonbinding anti-CD3 monoclonal antibody, delays the development from prediabetes to diabetes of about 3 years (56). Given the similarities discussed above PCD could be a candidate to such approach.

1.2 Clinical aspects

Diagnosis of PCD

The diagnosis of PCD is established when a positive CD serology (anti-TG2 and EMA) is associated to a normal duodenal architecture (Marsh 0 or Marsh 1 lesion) (57). Different aspects must be carefully considered before diagnosing PCD in order to reduce the risk of an incorrect diagnosis.

First, a positive anti-TG2 IgA value must be confirmed in a second blood sample together with EMA detection. Anti-TG2 IgA measurement is regarded as the most appropriate primary test in the diagnostic work up for CD by the major international diagnostic guidelines from the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) (57), the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition (NASPGHAN)

(58), and the American College of Gastroenterology (ACG) (59). Anti-TG2 IgA sensitivity for the diagnosis of CD ranges from 74% to 100%, but their specificity is lower particularly when they are marginally elevated as it is often the case of PCD (60). For this reason, before a diagnosis of PCD is

established, the presence of EMA antibodies must be confirmed in a second blood sample. In fact EMA show a higher specificity for the diagnosis of CD (60) (61). One may fear that, since biopsy is necessary to assess the presence of a normal intestinal architecture and thus diagnose PCD, the "biopsy sparing approach" allowed in children by latest ESPGHAN guidelines could miss this diagnosis in some cases. This appears to be unlikely as different multicentric studies realized in recent years have demonstrated that for high levels of anti-TG2 (>10 times the upper limit of normality) the probability to have a concomitant villous atrophy at intestinal biopsy is virtually 100% (62) (63).

The second necessary contribution is a reliable histological evaluation based on a careful morphometrical analysis and performed by an experienced pathologist. In fact, despite intestinal biopsy remains the goal standard for CD diagnosis, both a "false" villous atrophy or, vice versa, a falsely normal duodenal architecture, can be the consequence of a not proper orientation of the biopsy sample. To correctly analyze biopsies, it is essential that the plane of the section is perpendicular to the luminal surface, as judged by the fact that the crypts of Lieberkuhn are cut longitudinally and not in cross section (64). The difficulty in obtaining a correct histological assessment is demonstrated by the low interobserver agreement between pathologists (65) (66). This is particularly true for intermediate degrees of intestinal lesions. Mean kappa values ranging from 0.28 to 0.40 (low agreement), with an overall agreement kappa of 0.35 have been reported (65) and confirmed by others (67). Together with qualitative histology (Marsh-Oberhuber classification) it is then important to perform quantitative ratio between villi and crypts, as also suggested by latest ESPGHAN guidelines (57). To make a diagnosis of PCD a normal duodenal architecture must be documented: this is defined by a villous/crypt ratio higher than 2.

To overcome pitfalls in the analysis of duodenal biopsies, it has been recently proposed a "molecular morphometry" using PAXgene fixed paraffin-embedded biopsies: the mRNA expression ratio of villous epithelium-specific gene APOA4 to crypt proliferation gene Ki67 showed a similar significant distinction between paired baseline and post-gluten challenge biopsies as quantitative histomorphometry (68)(69). In the future, the mRNA analyses from duodenal biopsies could serve as a molecular surrogate for the morphometrical villous/crypt ratio, but at present in clinical settings the correct histological evaluation of duodenal biopsy samples remains the gold standard for diagnosis.

Finally, it is crucial that, before confirming diagnosis of PCD, the dietary gluten amount is checked to make sure that the normal villous/crypt architecture is not the consequence of a low gluten intake.

Epidemiology

Screening strategies of general population and at risk groups have determined an increase of both CD and PCD diagnosis. In a recently performed systematic review and meta-analysis, the prevalence of CD based on serologic test results was 1.4% and based on biopsy results is 0.7%(70). Less data are available on PCD prevalence worldwide: globally it has been estimated to be of around 10% of the total CD diagnosis, but the range is quite wide ranging from 6% to 30% (Table 1). Paparo et al. in 2005 estimated the prevalence of PCD in Italian children of 6.2% of the total CD cases (71), while Kurppa et al. estimated its prevalence up to 28% (72) and 33% (73) of CD diagnosis respectively in children and ìn adults in Finland. Other studies find a prevalence of PCD included in this range (Table 1). Just like CD, PCD is more frequent in first degree relatives of CD patients and patients with other concomitant autoimmune disorders compared to general population. Although some patients are diagnosed for the presence of clinical symptoms, most of PCD diagnosis are the result of screening of first-degree relatives of CD patients or subjects with associated autoimmune conditions. Interestingly, an increased prevalence of PCD in patients biopsied because of positive CD autoimmunity has been found in patients with type 1 diabetes (T1D) in comparison to non-T1D subjects (12% vs 7%) (74) suggesting in T1D patients with CD autoimmunity a lower intensity of mucosal T cell reaction to gluten, possibly on a genetic basis.

Genetics in PCD

CD has an important hereditary component: approximately 10% of first-degree relatives are affected by the disease (75) with concordance rates of 75–80% in monozygotic and 10% in dizygotic twins (76). The strongest and best characterized genetic susceptibility factors in CD are human leukocyte antigen (HLA) class II genes known as HLA-DQ2 and HLA-DQ8, whose role is to present antigens to immune cells. HLA-DQ is composed of a αβ heterodimer encoded by HLA-DQA1 and HLA-DQB1 genes respectively: the number, type and configuration of the DQA1 and DQB1 alleles determine disease risk (77). However, even if HLA-DQ2 or DQ8 are necessary for disease to develop, they are not sufficient implicating that other genes or environmental factors are involved. Recent large-scale genetics studies, called genome-wide association studies (GWAS), have identified a number of common non-HLA genetic factors (many in genes involved in immunity) associated with CD which, on their own, contribute a small amount to overall risk but have great potential in discovering important and novel pathways involved in disease pathogenesis. To date, non-HLA genetic loci harbouring 115 genes have been associated with CD using GWAS (78) (79), many of them shared with several others immune-related disorders like type 1 diabetes, rheumatoid arthritis and Crohn's disease suggesting common genetic backgrounds.

As discussed, the presence of DQ2 and or DQ8 HLA molecules is pathogenetically essential to produce anti TG2 antibodies, thus it is not surprising that PCD patients, just like CD patients, bear one of these molecules. However, patients with PCD more frequently show low-to-moderate HLA-related risk and in particular an increased frequency of DQB1*0302 and a reduced frequency of DQB1*02 homozygosity (11). This "gene-dosage" effect could be responsible for a less intense mucosal T cell reaction to gluten.

Also a subset of non HLA genes seems to differ in PCD patients compared to CD patients. Sperandeo et al. demonstrated that one marker of the KIAA1109/IL-2/IL-21 candidate region differentiated potentials from celiac (rs4374642: $x2 = 7.17$, p value = 0.01). Overall it seems that at least 6 different non-HLA polimorphims are differently represented in PCD and CD patients and the presence of some of them can help predicting evolution to villous atrophy in PCD patients (11). In terms of expression the same authors showed that the expression of IL-21 was completely suppressed in potentials compared to celiacs (p value = 0.02) and to controls (p value = 0.02). In contrast IL-2, KIAA1109 and c-REL expression were over-expressed (11).

Clinical symptoms and response to GFD

The presence or not of clinical symptoms does not affect the diagnosis of PCD that only requires a positive CD serology with a preserved intestinal architecture, but it is essential to establish the most adequate clinical management. In fact, despite clinical management of asymptomatic patients is still not clear, symptomatic patients with PCD are usually addressed to a GFD trial (9).

Percentage of symptomatic cases among PCD patients, as well as symptoms described varies considerably among studies. In general, it seems that in children most of PCD patients are asymptomatic while symptoms increase in adult population (Table 1). It should be considered however that screening of at risk subjects, such as first degree relatives is most frequently performed by paediatricians and this could partially explain such a discordance. In a cohort of 340 PCD children, only 60 (17%) started a gluten-free diet (GFD) because of the presence of symptoms (9). Similar proportion were reported in older studies ranging from 12% (80) to 19% (81). The figure was recently confirmed by the same group: Mandile et al (82) reported 14% of symptomatic PCD patients: most frequently reported symptoms were failure to thrive, abdominal pain and diarrhoea. The CELIPREV study also demonstrated that the majority of PCD children are asymptomatic (42). However, this result could be underestimated because of the study design: the CELIPREV study in fact investigates the incidence of PCD in a prospective cohort of children with CD-predisposing HLA genes followed-

up since birth. Vice versa, always in children, the Finnish study group reported that in 96% of the cases PCD patients presented clinical symptoms (mainly abdominal pain and signs of malabsorption) and only in 1% of their cohort they were asymptomatic and screened because of first degree relative (72). More recently the same group also reported that mor subtle signs of malabsorption already significantly present in PCD children compared to controls. They reported that the development of anemia and iron deficiency in CD is a continuum and may already be present in children with normal intestinal architecture, suggesting an early diagnosis and possible dietary treatment of these patients (83).

As mentioned before, in adult population, symptoms in PCD seems to be more common and do not differ significantly from CD patients with villous atrophy**.** Volta et al (84) demonstrated that 79% of PCD patients were symptomatic at diagnosis: among these symptomatic cases, 16% had the classic phenotype characterized by diarrhoea and weight loss, whereas the other 51 symptomatic PCD patients showed the occurrence of iron deficiency anaemia (more frequently than folic acid deficiency anaemia), osteopenia, aphthous stomatitis, irritable bowel syndrome. Similarly, in a study conducted by Biagi et al (85) 44% presented diarrhoea and/or weight loss and 44% anaemia or minor symptoms of malabsorption. More recently Newton et al reported that the vast majority (around 92%) of PCD adult patients were symptomatic at diagnosis. Most of them were screened because of gastrointestinal symptoms while extraintestinal manifestation and haematinic deficiencies were less common in PCD compared to CD patients (86).

Volta (84) and Biagi (85), both showed a response rate to GFD of 100% while in the group of Newton et al one third of patients did not improved after GFD was commenced (86). To attribute a symptom to CD it is important to demonstrate that symptoms are dependent on the presence of gluten in the diet. Also in this case, there is considerable heterogeneity between children and adults, with the latter that seem to take a greater benefit from the GFD. In the paediatric study cohort of Mandile et al (82) the percentage of responding and non-responding patients to GFD was similar, even though anti-TG2 serum level always declined upon antigen elimination, confirming the adherence to the diet. In particular, about half of the patients showed a positive clinical response in the first 12 months while the other half showed a partial or absent clinical response. Moreover, not all symptoms showed the same tendency to disappear after GFD. For instance, abdominal pain and diarrhoea were more prone to improve than failure to thrive. Furthermore, it was clear that not all symptoms were gluten dependent. In the same study, authors also analysed intestinal changes in duodenal biopsies at the time of PCD diagnosis and after at least one year of GFD: the mild intestinal inflammation and infiltration were not significantly affected by GFD. Based on these data, the authors concluded that

caution is necessary in paediatric PCD before attributing all abnormalities to gluten, as often symptoms and inflammatory markers of intestinal mucosa do not improve even after a prolonged period of GFD. A different experience has been reported in Finland: in a paediatric cohort all the patients put on a GFD resolved their symptoms and viceversa, in all symptomatic patients left on a gluten-containing diet (GCD) symptoms persisted (72). In adult Newton et al investigated more deeply the group of patients with PCD that did not respond to GFD (30% of the entire cohort) and a non-CD cause of the symptoms (including inflammatory bowel disease, irritable bowel syndrome and pancreatic insufficiency) was identified in 50% of the cases (86).

Unfortunately there is paucity of randomised studies to assess the effect of GFD in PCD. In a study conducted by Kurppa et al (73) 23 Marsh I–II patients were randomized either to continue on a GCD diet or start a GFD. After 1 year, clinical, serologic, and histologic evaluations were repeated. A total of 47 participants had small-bowel mucosal lesions compatible with CD (Marsh III), and these served as disease controls. In subjects with Marsh I–II lesion left on GCD the small-bowel mucosal villous architecture deteriorated in all participants, and the symptoms and abnormal antibody titres persisted. In contrast, in the GFD group (Marsh I–II) the symptoms were alleviated, antibody titres decreased, and mucosal inflammation diminished with a trend similar to patients with Marsh III lesion. When the trial was completed, all participants chose to continue on a life-long GFD. Another prospective clinical trial published one year later by the same study group (87) moved the knowledge one step forward, by evaluating the self-assessed gastrointestinal symptoms, quality of life and bone mineral density of subjects with positive EMA but only mild enteropathy. 27 PCD and 46 CD patients were evaluated at baseline and after 1 year of GFD. The total Gastrointestinal Symptoms Rating Scale (GSRS) score significantly decreased only in celiac group, but indigestion decreased significantly in the mild enteropathy group. Equally the self perceived well being assessed by PWBG total score significantly increased only in celiac groups, but after one year of GFD depression significantly improved in PCD patients. Most of the mild enteropathy patients also had increased bone mineral density after the GFD was commenced, prompting the authors to state that “there was a trend towards improved bone mineral density after the treatment”. However, it must be specified that this trend did not reach the statistical significance. In conclusion, the authors demonstrated that also asymptomatic adult PCD, who were randomized to a GFD, despite the fact that all subjects prior to the study described themselves as “asymptomatic”, improved significantly on the GSRS. The authors thus concluded that even apparently asymptomatic patients with positive antibodies benefit from a GFD.

Natural history

Studies conducted in prospective paediatric cohorts have clearly shown that in PCD progression to villous atrophy is not the rule. More than 20 years ago we started to prospectively follow up PCD children from diagnosis with periodical clinical, serological and histological evaluations. Patients with clinical symptoms were addressed to a GFD trial whereas asymptomatic patients were suggested to continue on a gluten GCD unless different parental choice. The first published study (81) in 2011, highlighted a cumulative incidence of villous atrophy of 30% in three years, and the subsequent two studies on the same cohort reported similar rates (33% at 9 years and 43% at 12 years) (80) (9), with most of the "events" (progression to villous atrophy) occurring in the first years of follow up. The prospective study also demonstrated how a relevant proportion of patients, around one third of them, even stopped producing autoantibodies (anti-TG2 and EMA) in a permanent way, meaning that in some cases the autoimmune process may be reversible. The disappearance of CD autoantibodies was particularly frequent in individuals diagnosed in young age. It is possible that infants, especially those under 3 years of age, have a more "plastic" immune system, and, in some cases, their oral tolerance to gluten may be regained. Other prospective studies on children, shown similar results, with even lower progression rates. Results from the CELIPREV study group, designed to prospectively follow up children with CD-predisposing HLA genes since birth, have revealed a progression rate of 13% at 10 years. In this case, even 83% of the subjects permanently stopped producing antibodies (42).

Similarly in adults, when patients are monitored on a GCD, progression rates to villous atrophy are comparable. Biagi et al (85) between years 1999 and 2011 prospectively followed up a small cohort of PCD adults: 24 patients maintained a GCD and started a follow-up: 14 of them underwent at least one histological reevaluation that revealed villous atrophy in 5 of them (time between diagnostic and follow-up biopsy: 12 ± 8 months) while the remaining nine patients still had a normal mucosal architecture after a mean follow-up time of 30 ± 29 months. Volta et al (84) few years later prospectively followed another cohort of adult PCD patients: sixteen of the 77 PCD patients (21%) were put on a GCD and remained asymptomatic. All of them were followed up from 1 to 10 years (mean 3-year follow-up). Only 1 of the 16 PCD patients on gluten containing diet became symptomatic after 2-year follow-up and duodenal biopsy confirmed the concomitant development of villous atrophy. CD positive associated serology disappeared in 4 patients in the first 6 months. More recently Newton et al reported that only one third of PCD patients that continue to manifest clinical symptoms on a gluten containing diet developed VA, with a mean progression time of two years (range 1-72 months). Vice versa, in Finnish prospective study cohort, both in adult and children a more consistent incidence of CD in patients previously diagnosed with PCD has been found, respectively of 87% and 96%, prompting the authors to suggest a GFD to all PCD patients (73) (72)

Predictive biomarkers

Since evolution to an overt form of CD is not certain, the appropriateness of a lifelong indication to GFD appears to be questionable, especially where symptoms are absent. It is then important to predict who has more chances to evolve to villous atrophy. Risk factors associated to the development of villous atrophy have been carefully analysed in different retrospective and prospective studies with the aim of identifying since diagnosis patients more prone to develop villous atrophy in future. These patients could be proposed to immediately start a GFD regardless of symptoms, avoiding serological and histological repeated medical checks. It has been hypothesized that deposition of anti-TG2 IgA antibodies along the epithelial basement membrane precedes the development of villous atrophy (88) (89) (90) but their role has recently been reconsidered, especially when the impact of anti-TG2 deposits is compared to other risk factors present at the same time. Also an infiltrated intestinal mucosa in the first duodenal biopsy performed, the presence of a high risk HLA haplotype, a set of non-HLA genes and a young age at diagnosis seem to predispose to an increased risk to develop villous atrophy (9).

A discriminant analysis was conducted to explore the capacity of each variable to discriminate between children who remained potential on follow-up from those who developed flat mucosa. It emerged that intraepithelial gamma delta lymphocytes were the best discriminators, followed by the age at diagnosis and genetic profile of the individual. For each case, the model obtained by the discriminant equation allowed the computation of an individual discriminant score able to predict the chances of an individual to belong to the group of those who will develop villous atrophy or not. Overall, the discriminant analysis model allows to correctly predict 80% of children to the group where they actually belong (9).

Risks

There is still a scarce knowledge about possible nutritional and immunological long term risks developing in asymptomatic individuals maintained on a gluten containing diet. Imperatore et al (91) have demonstrated that patients kept on GCD have a higher risk of developing immune-mediated disorder than those following a GFD (61% vs 18%, P = 0.03, OR = 3.3), with no difference between patients with Marsh 0 or Marsh 1 lesion. This result however has never been confirmed in other studies with acceptable sample sizes and the hypothesis that continuous gluten exposure can itself increase the risk of developing other concomitant autoimmune disorders remains an open issue in

PCD. Equally, because the lack of long term studies and their randomisation, it is still unknown whether and to what extent subtle signs of malabsorption can appear years after the diagnosis in PCD individual regardless the presence of clinical symptoms. Interestingly, Dickey et al.(92) measured bone mineral density in 31 endomysial antibody-positive adult patients who were excluded for CD (i.e., classified as having Marsh 0 or Marsh 1 lesions). They found osteopenia to be present in 30% and osteoporosis in 10% of these patients, and the degree of bone disease did not differ from that found in patients diagnosed with overt CD. No similar studies have been performed in children, where the possible alterations e.g. bone density are still reversible and in general malnutrition risks are higher because of the growth process.

Conclusions

In conclusion, PCD is a very heterogeneous condition, both from a clinical point of view as it that include symptomatic and asymptomatic individuals, and in their natural history as subjects may both progress to villous atrophy or, on the contrary, even stop producing autoantibodies in a permanent way. The mechanisms that regulate these phenomena are not known, nor environmental factors that may precipitate full blown CD. Risk factors at diagnosis associated to future evolution have been carefully analysed and include among others increased number of IELs in the intestinal epithelium, age at diagnosis, HLA and non-HLA genes. This can guide the clinician in the delicate choice of starting or not a GFD regardless of the presence of symptoms. Even when symptoms are present however, their gluten dependency must be verified. Further studies are needed to address the issue of whether a continuous gluten exposure can increase long-term immunological and nutrition risks PCD patients kept on a gluten containing diet.

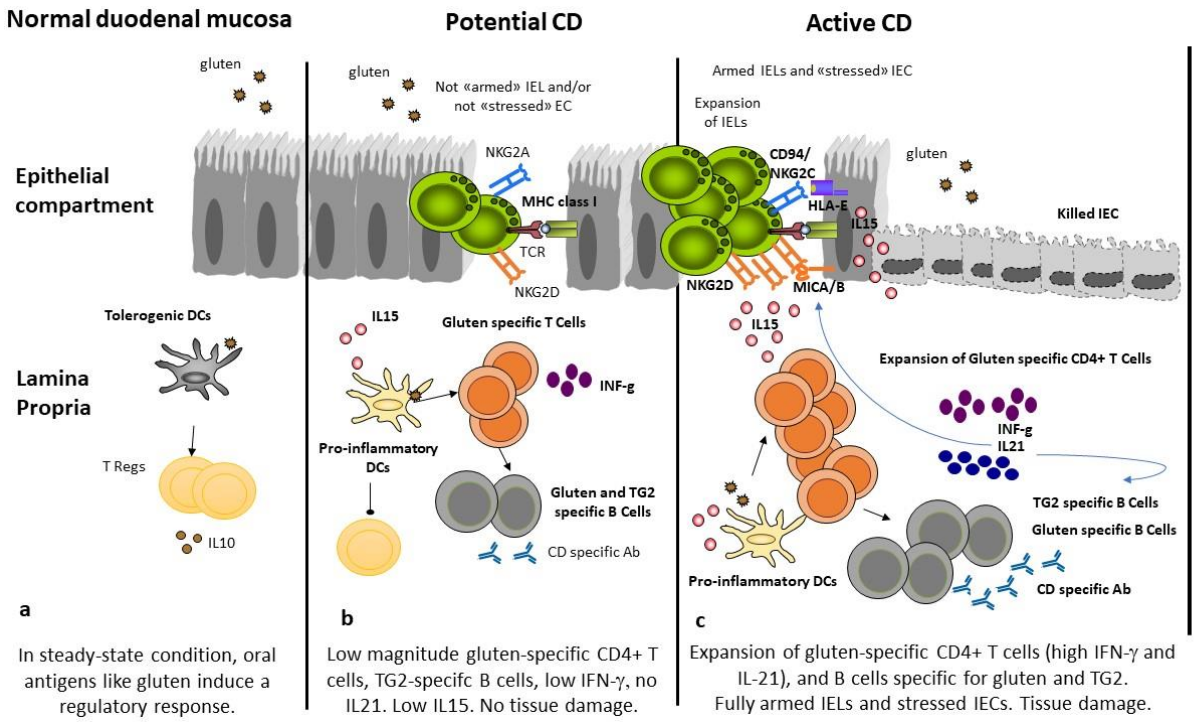

Figure 1. Schematic on the immunopathogenic events leading to villous atrophy. At steady-state, in the absence of a pro-inflammatory environment in the small intestinal mucosa, gluten prompts a regulatory response as a consequence of its presentation via dendritic cells (DC) with a tolerogenic phenotype (left panel). In potential celiac disease (CD), a certain degree of IL-15 upregulation in the lamina propria leads to pro-inflammatory DC, that present deamidated gluten peptides to naïve T cells leading to the rise of a gluten-specific CD4+ T cell response, featuring the secretion of IFN-g. Those T cells in turns activate a B cell response characterized by the production of antibodies specific for tissue transglutaminase 2 (TG2) and deamidated gliadin peptides. The magnitude of the T cell-INF-γ response is low, and not sufficient to determine a full activation of intraepithelial lymphocytes (IELs). Due to this, and to the lack of epithelial IL-15 and IL-21 in the lamina propria, in potential CD, intraepithelial lymphocytes (IELs) lack a fully activated phenotype and thus fail to kill intestinal epithelial cells (IECs). In fact, Potential CD patients typically lack tissue damage (middle panel). In active CD, a higher magnitude of gluten-specific T cell leads to higher INF-γ and IL-21 levels. High levels of IL-15 are also found both in the epithelium and lamina propria. More cytotoxic IELs are recruited, expressing lower levels of inhibiting natural killer receptors (NKR) such as NKG2A and high levels of activating receptors like NKG2D, that bind stress signal molecules (MICA/B) on IECs, this asset on both IELs and IECs leads to the final development of villous atrophy (c).

	Population	Prevalence (%)	Symptomatic (%)	Response to GFD (%)	Follow-up (years)	Evolution to VA (%)
Volta et al 2016	Adults	79/735 (10%)	61/79 (79%)	100%	10	1/10 (10%)
Biagi et al 2013	Adults	47/187 (23%)	23/47 (69%)	100%	7	4/14 (36%)
Imperatore et al 2017	Adults	56/452 (12%)	43/56 (77%)	p<0,05	6	6/13 (46&)
Kurppa et al 2009	Adults	23/70 (32%)	20/23 (87%)	100%	1	8/10 (80%)
Newton et al 2023	Adults	84/2775 (3%)	77/84 (92%)	29/41 (71%)	6	5/15 (30%)
Kondala et al 2016	Adults and children	n.e.	29/57 (49%)	n.e.	1	5/57 (9%)
Tampowpong et al 2012	Children	62/320 (19%)	n.e.	n.e.	n.e.	n.e.
Tosco et al 2011	Children	n.e.	20/106 (19%)	11/20 (55%)	3	10/86 (11%)
Auricchio et al 2014	Children	n.e.	26/210 (12%)	n.e.	9	23/175 (13%)
Auricchio et al 2019	Children	n.e.	44/340 (13%)	n.e.	12	42/280 (15%)
Lionetti et al 2012	Children	24/96 (25%)	1/24 (4%)	n.e.	2	1/21 (5%)
Lionetti et al 2019	Children	26/553 (5%)	0	n.e.	10	3/23 (13%)
Kurppa et al 2010	Children	17/59 (29%)	16/17 (94%)	n.e.	2	7/8 (87%)
Mandile et al 2017	Children	n.e.	44/330 (19%)	19/35 (54%)	3	n.e.

Table 1. Summarising table on studies regarding Potential Celiac Disease

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Chapter 2. Aim and outline of the thesis.

Potential celiac disease (PCD) it's a clinical condition characterised by the presence of a positive CD associated serology without intestinal damage. Its clinical management is extremely heterogeneous among different centres and Countries due to the lack of sufficient knowledge about its natural history. We already know that, especially if children are considered, that development of full blown disease is not the rule, with evolution rates that vary between 5 and 87% among different studies. Indeed, the aim of this thesis is the better characterization of long term natural history of potential celiac disease in children, the identification of biomarkers able to predict future evolution to villous atrophy and the immunological aspects that sustain this condition.

Chapter 3 describes the whole cohort of PCD children we prospectively follow up since diagnosis on, analysing clinical, immunological and histological aspects. We will describe the clinical strategies for monitoring them with a scheduled follow up and the long-term outcome of PCD patients maintained on a gluten containing diet. As we will show, only around one third of them will develop symptoms or villous atrophy, while the vast majority will remain asymptomatic without developing intestinal damage.

In *chapter 4* we thus investigated whether, in those PCD children that persistently remain clinically asymptomatic and histologically healthy, growth, nutritional and autoimmune parameters can be somehow altered, even in a subclinical way, by chronic gluten consumption. We performed a case control study where we compared different parameters in the same patient at diagnosis and on the occasion of last follow up. We will demonstrate that these patients do not tend to develop complications and gluten consumption seems to be safe for PCD children that do not present clinical symptoms or intestinal damage.

Of course, it would be very interesting from a pathological point of view and useful from a clinical point of view to identify since diagnosis those patients committed to develop the classical form of the disease. In this context, in *chapter 5* we study how to possibly identify since diagnosis, PCD patients committed to develop villous atrophy in future. Our group had already demonstrated in a previously published work that the combining of clinical, histological and genetical features was able to predict future evolution with an accuracy of prediction of around 80%, somewhat uncertain to take such a life-long clinical decision. Moreover, the traditional multivariate model we proposed required stringent assumptions that may not be answered in the clinical setting. Starting from the dataset available for PCD, we propose the application of Machine Learning (ML) methodologies to

extend the analysis on available clinical data and to detect most influent features predicting the outcome. The best model, optimized Boosted Trees, is able to classify PCD starting from the selected 19 features with an accuracy of 0.80, sensitivity of 0.58 and specificity of 0.84. (Chapter 5.1). In the same chapter (chapter 5.2) we also analyse how the different concentration of 92 serum inflammatory proteins is able to correctly differentiate since diagnosis PCD children destined to develop villous atrophy with an even higher precision (around 95%). This study also highlights how specific changes in abundance inflammatory proteins in circulation are associated with small intestinal damage.

The correct study of the intestinal mucosa is fundamental for CD diagnosis, and even more for PCD diagnosis, where, in presence of a positive CD associated serology, villous atrophy must be excluded. In *chapter 7* we analyse other aspects of the deep relation between celiac disease and the intestinal mucosa. In the first study we aimed at investigating histological and immunohistochemical features in CD patients on a long-term gluten free diet (GFD) and to correlate them to the GFD duration. We will demonstrate that villi to crypt ratio as well as the level of inflammation in lamina propria (expressed by the number of CD25 positive cells) normalise after GFD while other parameters, such as γδ positive cells, decreases but never normalise, representing a hallmark of the condition regardless of the stage of disease. In the second work we aimed at reporting distribution, clinical, and immunohistochemical features of patients with a biopsy proved intestinal atrophy in absence of a positive CD associated serology. We will show that this condition is not rare representing up to 5% of the cases of VA but the entity of seronegative celiac disease is virtually absent in the paediatric age. Immunohistochemical analysis may be helpful in excluding CD, whereas the finding of mucosal anti-TG2, particularly with a weak staining, shows no absolute specificity for CD.

Chapter 3. Prospective cohort of PCD children

INTRODUCTION

PCD patients may be asymptomatic and may or may not evolve to complete VA. For this reason, the clinical management of this condition remains debated (1). Concerning symptomatic PCD patients, the scientific community is eager to suggest a trial with a gluten-free diet (GFD). Nonetheless, it was recently demonstrated that only approximately half of these patients actually improve (2). By contrast, the clinical management of asymptomatic PCD patients is more intricate. Some consider PCD as the first step of the overt disease (3-5), thus, a GFD is indistinctly prescribed to everyone. However, in a recent study, we demonstrated that only approximately 40% of these patients will evolve to VA in 12 years[3]. Additionally, another one-third will stop producing autoantibodies. Considering these findings, prescribing a GFD to everyone could appear as overtreatment. It seems to be clear that PCD is a heterogeneous condition, and patients would require personalized management. Unfortunately, until now, there is no way to precisely differentiate from the beginning patients who will develop VA from those who will not, despite our group recently identified a subset of risk factors.

The aim of this work is to better characterize clinical, immunological and histological features of PCD paediatric patients and to study their natural history on the longer follow up possible.

PATIENTS AND METHODS

Study design

From 2000 on, we enrol all PCD children that are diagnosed in our tertiary care centre and we prospectively follow them with a standardized protocol. Patients enter the study, after a small bowel biopsy, if they showed at least two consecutive tests positive for anti-TG2 antibodies, confirmed by positive EMA IgA antibodies, total serum IgA in the normal range, HLA DQ2- or DQ8 -positive haplotypes and a normal duodenal architecture (Marsh stages 0– 1) in all the 5 biopsies analysed. The intestinal status is deeply characterized performing both histomorphometry and immunohistochemistry analysis. Children with symptoms suggestive of CD immediately start a gluten-free diet. All the remaining asymptomatic patients remain on a gluten-containing diet. They represent the prospective study cohort. Every 6 months, antibodies and clinical conditions are

checked, and a small bowel biopsy is taken every 2 years, if the occurrence of symptoms does not require it earlier. The study was approved by the University Federico II ethical committee.

Antibodies

To measure serum anti-TG2 antibodies, an enzyme-linked immunosorbent assay kit was used, based on a human recombinant antigen (Eu-tTg IgA, Eurospital, Trieste, Italy). The cut off -point for positivity was ≥ 7 IU. Serum IgA EMA was measured by indirect immunofluorescence on 7- μ m-thick frozen sections of human umbilical cord as the source of antigen. Samples were considered positive if a thin fluorescent network appeared around the smooth muscle fibers.

Genotyping.

H LA typing was performed using six single-nucleotide polymorphisms (SNPs) to identify DQ2.2, DQ2.5, DQ7, and DQ8 risk variants based on strong linkage disequilibrium at the HLA loci.

SNPs rs2187668, rs2395182, rs4713586, rs7775228, rs4639334, and rs7454108 were genotyped with TaqMan assays. (Applied Biosystems, Foster City, CA) on a 7800HT Fast Real-Time PCR system (Applied Biosystems). HLA-DQ risk types were predicted using the method described by Monsuur et al.6 and validated in several populations of European origin. All markers had a genotyping success rate >99 % and did not deviate ($P>0.05$) from the Hardy– Weinberg equilibrium, with the exception of SNP rs4713586, which failed to produce clear allele clusters in the TaqMan assay and was therefore excluded from further analyses. The genotype at the rs4713586 locus allows for discrimination between DQ2.2 and the rare DQ4 type and is therefore needed to predict DQ2 risk carriage in DQ7 / DQ2.2 individuals.

Duodenal biopsy and immunohistochemical analysis.

In each patient, esophagogastroduodenoscopy with five biopsies, one from the bulb and four from the distal duodenum was carried out. According to our protocol, four of five fragments, including one from the bulb were fixed in 10% formalin, embedded in paraffin, and then stained with hematoxylin. The histological and morphometrical analysis by light microscopy was performed by two experienced pathologists. A villous height: crypt depth ratio ≥ 3 was considered normal7. Among biopsies with a normal villous height: crypts depth ratio, Marsh 0 was defined by the presence of less than 25 intraepithelial lymphocytes(IELs) per 100 enterocytes and Marsh 1 by the presence of more than 25 intraepithelial lymphocytes (IELs) per 100 enterocytes. The Marsh score was given on the basis of the score of the fragment with the worst picture.

The evaluation of these four fragments was made blinded to any serology results. One fragment (not from the bulb) was put in an optimal cutting temperature compound (Killik, Bio-Optica, Milan, Italy), stored at −80° C , and used for immunohistochemical staining for CD3+, TCRγδ+, and CD25+cells, as previously reported8. The number of stained cells per millimeter of epithelium determined the density of cells expressing CD3 and TCRγδ in the intraepithelial compartment. Cutoff values for CD3+ and TCRγδ+ cells were 34 mm and 3.4 mm per epithelium, respectively. On the other hand, the number of cells expressing CD25 in the lamina propria was evaluated within a total area of 1 mm2. The usual cutoff value for CD25+ cells is 4 mm2 lamina propria. To determine the cutoff values to be used, 100 children with untreated CD and 50 non-CD control children were studied. Percentiles were obtained using the SPSS software (IBM, Chicago, IL). Cutoff values represented the 90th percentile of non-CD patients.

RESULTS

Patients baseline clinical and histological features

We have overall enrolled 515 PCD children with a mean age at diagnosis of 6 years (range 1-18); 66% are female. Reasons that prompted the screening were familiarity for autoimmune disorders in 55% of the cases (28% for CD), other autoimmune concomitant disorders in 11% of the cases (mainly thyroiditis and type 1 diabetes) and symptoms in 34% of the cases. Most frequent symptoms were abdominal pain, failure to thrive and diarrhoea (respectively 25%, 24% and 13%). However, after an appropriate medical evaluation, in only 72/515(14%) patients' symptoms were considered as possibly gluten related and patients were addressed to a gluten free diet (GFD). So our prospective cohort included 443 patients. All patients were anti-tissue transglutaminase positive and EMA positive at diagnosis (37% showed a weak EMA positivity) and all received a duodenal biopsy that assessed a normal duodenal architecture both by Marsh classification and by histomorphometry (mean villi/crypts ratio 3.1 range 2-5.2)

Long term outcome

Mean follow-up time was of 45 months (maximum 17 years). 108 on 515 (21%) are still on a active follow up, with regular annually visits. 119/443 abandoned the cohort because they permanently stopped producing antibodies despite being on a GCD: before abandoning the prospective follow up, they must have presented at least 4 determinations of negative antiTG2 antibodies every 6 months. 124/443 (28%) abandoned the prospective cohort because they started a gluten free diet (GFD). In

15% of the cases the reason was the development of VA (15%), in 13% of the cases they started a gluten free diet because of the development of relevant clinical symptoms but they refused duodenal biopsy.

Risk factors for evolution to symptomatic or atrophic CD

Among clinical factors, there was no difference in sex, familiarity for CD or coexistence of other autoimmune disorders between children that remained potential compared to those who developed the disease (p value >0.05). Vice versa, we demonstrated that an older age at diagnosis was associated to an increased risk to develop symptoms or VA (p value < 0.05, Fig 1). Although all potential celiac cases are at risk for HLA, surprisingly, children bearing an HLA at low or medium risk appear not to have more chances to remain potential than those with double DQ2 (p value >0.05). PCD patients destined to evolve to disease have at time of diagnosis a duodenal mucosa that is architecturally comparable to those who will not evolve, as demonstrated by the fact that there is no difference in villi to crypts ratio (p value >0.05, Fig 2d). Equally, signs of non-specific inflammation in lamina propria do not differ, as demonstrated by a comparable number of CD25 positive cells in lamina propria in the two groups (p value >0.05, Fig2 c). Viceversa, the infiltration of the intestinal epithelium is significantly increased in patients that will evolve to disease compared to those who will not, as demonstrated by the number of CD3 positive cells and γδ positive cells (p value < 0.05, Fig 2 a,b).

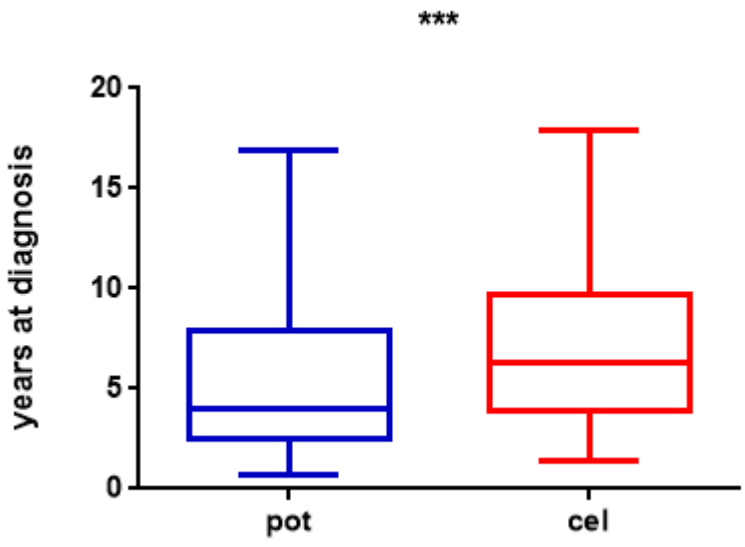

Figure 1. Age at diagnosis in patients that remain potential is significantly lower compared to those to evolve to full blown disease

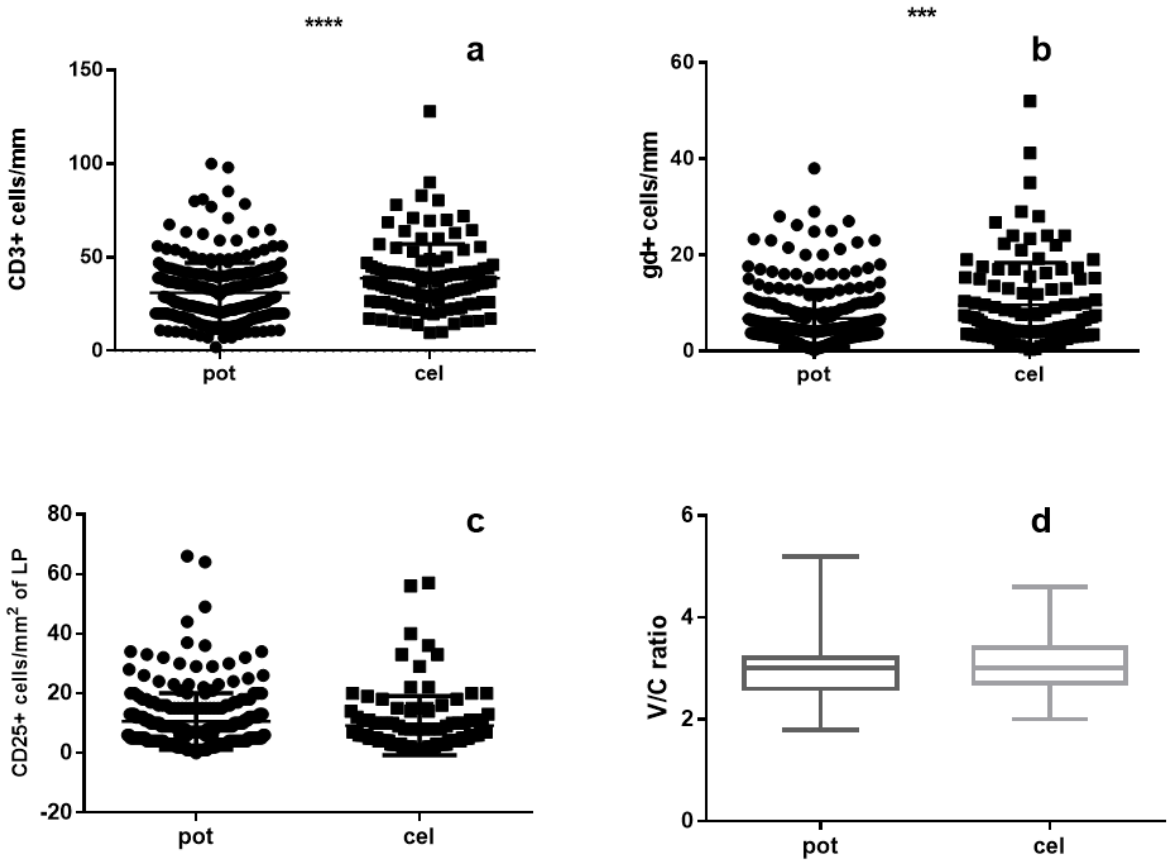

Figure 2. CD3 + cells and γδ + cells are significantly increased in PCD patients that will evolve to villous atrophy compared to those who will remain potential (a,b). No significant difference in CD25 positive cells and villi to crypts ratio (c, d).

DISCUSSION AND CONCLUSIONS

Children who produce anti-TG2 antibodies and have a normal small intestinal architecture (patients with potential celiac disease) are no longer rare because they represent approximately 10% to 20% of large case series of celiac disease centres and because of the implementation of case finding strategies (4) Unfortunately, the management of these patients is not universally agreed on. Recent studies have demonstrated that not necessarily all patients with potential celiac disease will progress up to villous atrophy: many of them remain "potential" after long-term follow-up and others even stop producing antibodies over time, indicating, in some cases, a reversibility of the process. In this study, we provide

more solid data on potential celiac disease natural history (3,5-7). We analysed prospectively, for the longest follow-up (up to 17 years), the largest cohort (515 patients) ever studied. In contrast to the observations in adults, most of our patients had no symptoms and/or signs of disease: PCD in our cohort was often diagnosed in asymptomatic children screened because of familiarity. Monitoring asymptomatic patients on a gluten containing diet according to a scheduled follow up program, we demonstrated patients can potentially evolve into two opposite scenarios: in around one third of the cases they will stop producing antibodies despite eating gluten while in another third of cases they will evolve to CD. The remaining third will keep on producing low titres of autoantibodies without developing symptoms or intestinal damage. In an univariate analysis, most important risk factors to discriminate at diagnosis patients destined to evolve to disease are age at diagnosis and the infiltration of the intestinal epithelium. Younger children are more prone not to evolve suggesting that they could have a more "plastic" immune system, and, in some cases, their oral tolerance to gluten is not completely broken. Equally, despite the intestinal architectural structure of patients destined to evolve to disease is comparable to patients that remain potential, as well as the degree of non-specific inflammation in lamina propria, patients that will develop disease show at diagnosis an increased number of intraepithelial lymphocytes, especially with a gamma delta phenotype. This reinforces the hypothesis, already speculated in literature, that the intestinal epithelium presents some constitutively altered hallmark of CD since the very early stages of the disease.

In conclusion, we confirmed that potential celiac disease is a very heterogeneous condition and is not necessarily the first step of overt disease; thus, GFD should not be prescribed indistinctly to all patients. Individual features, such as age, degree of mucosal infiltration and a specific subgroup of intraepithelial lymphocytes, may help to differentiate a subgroup of patients who will develop villous atrophy or symptoms over time.

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Chapter 4. Potential celiac disease in children: health status on a long-term gluten-containing diet

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INTRODUCTION

Celiac disease (CD) has been defined by ESPGHAN as a systemic immune-mediated disorder elicited by gluten and related prolamines in genetically susceptible individuals [1]. It is characterised by the presence of a variable combination of clinical manifestations, CD-specific autoantibodies, HLA compatible haplotype and enteropathy. This histological alteration can range from a completely altered mucosa to one with normal architecture, with preserved villous to crypt ratio, with or without an increased number of intraepithelial lymphocytes (IELs). The latter condition is defined as Potential Celiac Disease (PCD) [2]; it is characterised by a positive CD-associated serology without intestinal damage [3]. The implementation of screening strategies in at-risk groups as well as an increased attention to subtle signs of the disease, has led in recent years to a more liberal use of serological diagnostic tools and to an increased detection of these subjects. Globally, potential CD (PCD) is estimated to be of around 10% of the total CD diagnosis, but the range is quite wide going from 6% to 30% [4]. Its clinical management is variable due to the lack of sufficient knowledge of its natural history. Some recent studies, mostly performed on adult patients, suggest that gluten-dependent symptoms may be present even without villous atrophy (VA). They also show that most patients evolve to overt CD when continuing on a gluten-containing diet (GCD) [5-9]. On the contrary, there are more and more evidences that in children PCD is often an asymptomatic condition and evolution to VA is not the rule [10-13]. Some patients even stop producing autoantibodies despite being on a GCD, suggesting, in these cases, a reversibility of the autoimmune process [12]. More in detail, a recent work from our group demonstrated that the cumulative incidence of progression to villous atrophy was 43% over a 12-year period, suggesting that more than half of the patients remained

asymptomatic and histologically healthy over a long term follow-up on a gluten containing diet [12]. Based on these data, the scientific community, supported by the latest European Society of Paediatric Gastroenterology Haepatology and Nutrition (ESPGHAN) guidelines on CD follow-up in children, suggests a gluten containing diet to asymptomatic PCD patients, reserving gluten free diet (GFD) to the symptomatic ones [3].Unfortunately, the issues of whether a chronic gluten consumption may induce over time clinical or even subclinical complications on children nutritional status, as well as the appearance of other concomitant autoimmune diseases is very poorly studied in literature.

Taking advantage of the opportunity to study different aspects of PCD natural history in our prospective cohort of PCD children [12], we designed a retrospective study with the primary aim of understanding whether, in the group of PCD patients that remained asymptomatic during follow-up, a long-term chronic gluten exposure could somehow alter, even in a subclinical way, their growth or nutritional status. The secondary aim was to assess an eventual increase in other autoimmune disorders during the follow-up on a gluten-containing diet.

MATERIALS AND METHODS

We performed an observational study selecting from the whole cohort of PCD children those persistently asymptomatic on a gluten containing diet.

Patients were classified as Potential Celiac because of twice positive anti-transglutaminase (anti-TG2) antibodies, confirmed by anti-endomysial antibodies (EMA), and a normal or just infiltrated intestinal mucosa (Marsh stages 0 or 1). All had HLA-DQ2 and/or -DQ8 haplotypes. Total serum IgA antibodies were within the normal range.

Our whole PCD cohort includes 515 patients enrolled since 2000: 72 immediately started a GFD because of the presence of symptoms, 443 were prospectively followed-up: mean follow-up time was of 45 months (maximum 17 years). During follow-up, clinical visits were scheduled and blood samples were obtained every 6-12 months while a duodenal biopsy was performed every 2 years, or earlier in case of symptoms. The amount of gluten intake was evaluated by a dedicated nutritionist based on a 3 day food records and expressed as grams of gluten taken per day. 124/443 (28%), started a gluten free diet (GFD) both because the development of VA (15%) or relevant symptoms without a new duodenal biopsy.

Among 319 patients with PCD that did not develop symptoms or VA, we were able to retrospectively collect detailed clinical nutritional and autoimmune data for 171 (53,6%) of them (flow chart reported in Figure 1).

For these patients we had the following data: growth parameters (height, weight, body mass index and relative percentiles matched for age and sex), serum levels of anti-TG2, albumin, iron metabolism (haemoglobin, ferritin, serum iron), bone metabolism (calcium, phosphorus, alkaline phosphatase, vitamin D, parathormone), glucose metabolism (fasting glycaemia and HbA1C), lipid profile (LDL, HDL, triglycerides), autoantibodies for thyroid (anti-TG and anti-TPO), type 1 diabetes (antiIAA, anti-IA2, antiZn-T8, anti-GAD) and ANA.

We compared parameters at time of diagnosis (when the first duodenal biopsy was performed) and after a long-term gluten containing diet, in occasion of their last medical check. We excluded from the analysis patients that were supplemented with micronutrients such as iron or vitamin D. Since the cohort included also patients that had permanently stopped producing antibodies over time (repeatedly negative determinations in serum for at least 2 years, N=37) we run a second analysis excluding them, to ascertain if a possible nutrition impairment could have been limited to patients who were persistently producing anti-TG2. Similarly, to avoid the possible bias related to a relatively short follow-up, we run another sub-analysis only considering patients with a follow-up longer than 3 years (N=75). Parameters were compared using a paired t-test for normally distributed variables and contingency table with Chi square test for the qualitative ones. Statistical analysis was performed using SPSS software ver. 27.1. The study was approved by the ethical committee of University Federico II.

RESULTS

Whole cohort

Of 171 PCD patients 60% were female, the mean age at diagnosis was 6 years (range 1.6-17 years). 36% reported in their history familiarity for CD. All were EMA positive, Marsh 0 and Marsh 1 lesions were present in 50/50% respectively. Patients were followed up for a mean time of 3 years (range 0.35-15.3 years) on a gluten containing diet (Table 1). No one received iron supplementation and 6/171 (3.5%) received vitamin D supplementation since diagnosis: of course they were excluded from the analysis aimed to evaluate vitamin D changes over time since it would have been biased by the exogenous somministration. No significant difference was noted between the amount of gluten per

die at diagnosis and at the last follow-up (mean 23 gr gluten/day SD 11 at diagnosis vs 21 gr/day SD 10.8 at last follow-up, p= 0.3). Their anti-TG2 antibodies significantly decreased from 3.7 time the upper limit of normality at diagnosis to 1.8 at last follow-up. A significant increase of BMI (mean at time 0 vs time at last follow-up: 16.4 kg/m2 SD 2.3 vs 17.89 kg/m2 SD 3.5 p<0.0001), of BMI percentile (48.8 centile SD 32 vs 53.0 centile SD 33, p 0.04) and of weight percentile (46.8 centile SD 31.3 vs 51.3 centile SD 30.1, p 0.01) were observed. We also noted a significant increase of haemoglobin (12.8 gr/dl SD 0.9 vs 13.3 gr/dl SD 1, p<0.0001), of serum iron (81.0 µg/dl SD 31.9 vs 88.9 µg/dl SD 38.3, p 0.03), of HDL (54.2 mg/dl SD12.1 vs 56.4 mg/dl SD 12.4, p 0.02); of basal glucose (67.1 mg/dl SD 9.8 vs 69.7 mg/dl SD 8.6, p 0.003) and HbA1C (5.21% SD 0.25 vs 5.32% SD 0.27, p 0.01). All parameters remained within the limit of normality at any time. Equally, we noted a significant decrease in the levels of calcium (9.84 mg/dl SD 0.40 vs 9.74 mg/dl SD 0.42, p 0.03) and phosphorus (4.8 mg/dl SD 0.61 mg/dl vs 4.5 mg/dl SD 0.64, p 0.0002). Also in this case all parameters remained in the limit of normality and vitamin D, parathormone and alkaline phosphatase did not change significantly. All other parameters analysed did not significantly change. We scrutinized individual patients for variables indicative of the nutritional status. 3/171 (1.75%) had abnormal haemoglobin values for age and sex at the end of their follow-up on a gluten containing diet: one had a known thalassemia tract, in one haemoglobin levels anyway improved compared to the time of diagnosis and in one remained comparable (with an improved ferritin value). 18/171 (10%) patients had values of BMI <10 cent for age and sex, in 10 of them it remained stable of even improve at the end of follow-up, in 8 of them (4.6% of total) BMI decreased. Hypoalbuminemia never developed (Figure 2).

There was not a statistically significant change in the number of patients that presented at least one positive autoantibody for thyroiditis (anti-TG and anti-TPO, p 0.06) or for type 1 diabetes (anti-IAA, anti-IA2, antiZn-T8, anti-GAD, p 0.15). ANA were detected in 23 patients at the end of the period of follow-up, in no case at diagnosis.

Subgroup analysis: excluding patients that permanently stopped producing antibodies during follow-up and excluding patients with a follow-up < 3 years.

We run a second and a third analysis with the same procedures excluding respectively patients that permanently stopped producing antibodies during follow-up and patients with a follow-up < 3 years. Results overlapped with those obtained in the whole cohort. Detailed results are reported in Table 2.

DISCUSSION

Despite the numerous efforts recently spent in the aim of finding alternatives strategies, GFD remains the cornerstone of CD therapy. It is safe and effective both in solving clinical symptoms and restoring a normal intestinal architecture, the latter being the final goal of CD therapy [14]. Anyway, it must be considered that only one randomised clinical trial has clearly demonstrated benefits of the GFD when adopted in asymptomatic patients with an atrophic mucosa [15]. It is even less certain the role of gluten free diet in a condition like PCD, where intestinal mucosa is already architecturally healthy in presence of a positive CD associated serology. Besides, prescribing an inappropriate GFD is not a safe option as it can itself induce nutritional deficiencies, body weight gain and an important social and psychological impairment [16]. Current ESPGHAN guidelines suggest to start a trial with GFD in symptomatic patients, despite it has been shown that gastrointestinal symptoms in PCD are not always gluten dependent and may not improve with the diet [17]. Asymptomatic PCD patients are even more challenging. Indeed, they include a heterogeneous group of patients, that can eventually develop VA or clinical symptoms later in time, but more frequently just keep on producing autoantibodies without any intestinal damage or even, permanently stop producing them. Many studies have tried to identify risk factors able to predict, since the time of diagnosis, future evolution of PCD patients. We previously found that an older age at diagnosis, an increased number of IELs (especially those expressing on their surface γδ receptors) together with an individual genetic predisposition (that includes both HLA and non-HLA genes) are sufficient to correctly predict at entry, 80% of the children who will not develop a flat mucosa over follow-up and approximately 69% of those who will develop flat mucosa [12]. These data however, cannot represent more than a guide for the clinician and ESPGHAN recommendation currently do not suggest GFD in asymptomatic patients whatever the risk class is, even if they encourage to refer PCD patients follow-up in an experienced tertiary care centre. The same guidelines highlight in the conclusive section the necessity to study long-term risks eventually developing in asymptomatic PCD patients maintained on gluten-containing diet [14].

In this context, we took advantage of our consistent cohort of PCD patients prospectively monitored on a gluten containing diet, to assess if, in the subgroup of patients clinically asymptomatic and without intestinal damage, chronic gluten exposure could impact on the nutritional status or on the development of other autoimmune disorders. We designed a retrospective observational study to compare in each patient different parameters at diagnosis and after a long period of GCD.

It is well known that in atrophic CD patients, the maintenance of a gluten containing diet easily induces a progressive weight loss and biochemical signs of malnutrition such as anaemia and in more serious cases hypoalbuminemia linked to the concomitant enteropathy.

Vice versa, we could not find a worsening in the growth, iron metabolism, glycaemic metabolism and nutritional status in our cohort of PCD patients. We found some parameters even improved (like BMI, haemoglobin, serum iron basal glycaemia and HbA1C), anyway always remaining in the limit of normality. We surprisingly found a significant reduction in the levels of serum calcium and phosphorus, but the stability of alkaline phosphatase levels, parathormone levels and vitamin D levels during the follow-up did not support the hypothesis of a concomitant bone remodelling. This can be explained by the fact that we collected data from a very big cohort of patients and data show a Gaussian distribution. In this data set, even very small changes reach statistically significant relevance, but this does not mean they have a concomitant clinical relevance. Also performing a detailed analysis on single patients for the most important variables, we could not identify patients that became anaemic, hypoalbuminemic during follow-up. Since our cohort includes also patients that permanently stopped producing anti-TG2 antibodies, we wondered whether nutritional complications could happen only in the subgroup of patients that keep on producing autoantibodies during follow-up. This does not seem the case, as in this group, we had just overlapping results compared to the whole cohort. We also evaluated how circulating autoantibodies, markers of other autoimmune disorders known to be associated to CD, increased during a follow-up on a gluten containing diet. Very few data in literature is available on this topic: only one study on adult PCD in fact seems to suggest that PCD patients kept on a GCD have an increased risk to develop other concomitant autoimmune disorders [18]. This was not our case as we could not find an increase in autoantibodies for thyroiditis or type one diabetes during follow-up, which are the most important autoimmune pathologies known to be associated to CD. Moreover, we are aware that these antibodies without clinical symptoms only represent a marker of autoimmune predisposition in patients that are genetically predisposed to autoimmunity and the causative role of gluten cannot be elucidated in a study design like ours.

This study presents some limitations. The best way to verify the effect of GFD on PCD patients would be a double blind randomised clinical trial with a sufficiently long follow-up time. In this context, quality of life should also be investigated through standardised questionnaires. In our case we performed a retrospective observational study, not randomised and without a real control group of asymptomatic PCD patients addressed to a GFD. We also tried to overcome the bias of follow-up duration, as nutritional and autoimmune complications need time to become detectable: we run an

analysis only on the subgroup of patients with a follow-up longer than 3 years, and also in this case we found overlapping results compared to the whole cohort. Finally, one could argue that we excluded from the analysis just those patients that went worse developing symptoms or VA. We should consider that in these cases, based on current recommendations, patients would anyway start a GFD, whereas we wanted to answer the question of what happens to those patients that we normally follow-up on a gluten containing diet, to be sure not to cause them a damage, even if subclinical.

In conclusion our pilot study provides reassuring results on the maintenance of a gluten containing diet in the subgroup of PCD patients that are clinically asymptomatic during follow-up as it seems that their nutritional and autoimmune status is not impaired, even when a long follow-up is considered. Further studies are requested to validate these data on larger cohort of individuals ideally designing and performing a double blind randomised clinical trial. This could also help to correctly investigate the role of gluten in the induction of other concomitant autoimmune disorders in individuals with a predisposing genetic background.

	N	**sex**	**Age at diagnosis (years)**	**Follow-up duration (years)**	**Familiarity for CD**	**Marsh**
Whole cohort	171	F 60%	6 (1.6-17)	3 (0.35-15.3)	Yes 35%	0 50% 1 50%
Only anti-Tg2 persistently + patients	134	F 57%	5.9 (1.6-17)	3.19 (0.35-15.3)	Yes 36%	0 46% 1 54%
Only patients with a follow-up > 3 years	75	F 65%	5.7 (1.5-13.3)	5.7 (3.01-15.3)	Yes 36%	0 43% 1 57%

Table 1. Epidemiologic features of the study cohort.

		Only anti-Tg2 persistently seropositive patients (N=134, 78%)			**Only patients with a follow-up > 3 years (N=75, 44%)**		
		Mean value T0	Mean value Tx	p-value	Mean value T0	Mean value Tx	p-value
Anti-Tg2 and gluten intake	Anti-Tg (xULN)	*3.9*	*2.2*	*<0.005*	*4.2*	*1.9*	*<0.005*
	Gluten (g/die)	23	21	0.2	22.8	21.5	0.47
Growth	Height (ct)	50.5	48.6	0.25	54.08	49.2	0.05
	Weight (ct)	47.2	50.6	0.09	50.01	55.7	0.11
	BMI (ct)	49.7	51.3	0.49	*49.7*	*59.2*	*0.01*
Iron metabolism	Hb (mg/dl)	*12.8*	*13.3*	*<0.005*	*12.8*	*13.4*	*<0.005*
	Blood iron (µg/dl)	*81.6*	*90.8*	*0.03*	*81.7*	*95.6*	*0.02*
	Ferritin (ng/ml)	34.4	35	0.8	36.8	37.8	0.76
Glycemic metabolism	HbA1C (%)	5.27	5.3	0.08	*5.2*	*5.3*	*<0.005*
	Glycaemia (mg/dl)	*67.3*	*70*	*0.008*	68.3	70.9	0.07
Nutritional status	Albumin (g/dl)	4.57	4.59	0.43	4.6	4.6	0.14
	Cholesterol (mg/dl)	154.92	154.99	0.97	162.6	157.7	0.15
	HDL (mg/dl)	*54.3*	*57.7*	*0.01*	53.3	55.1	0.46
Bone metabolism	Calcium (mg/dl)	9.8	9.7	0.06	*9.9*	*9.65*	*<0.005*
	Phosphorus (mg/dl)	*4.8*	*4.5*	*<0.005*	*4.9*	*4.5*	*<0.005*
	ALP (U/L)	229	218.6	0.16	226.3	215.3	0.44
	PTH (pg/ml)	41.9	45.7	0.23	42.4	46.8	0.17
	Vitamin D (ng/ml)	24.6	25.4	0.57	19.3	21.6	0.07
Thyroid autoantibodies	Anti-TG and Anti-TPO	9 positive (7%)	11 positive (8.2%)	Ns Ns	7 positive (9%)	11 positive (14%)	Ns Ns
Type 1 diabetes autoantibodies	Anti-IAA			Ns			Ns
	Anti-IA2	5 positive	4 positive	Ns	8 positive	4 positive	Ns
	AntiZn-T8	(4%)	(3%)	Ns	(10%)	(5%)	Ns
	Anti-GAD			Ns			Ns

Table 2. Summarizing results in subgroup analysis. ALP: Alkaline phosphatase, PTH: parathormone. Ns. Not significate. ULN: upper limit of normality

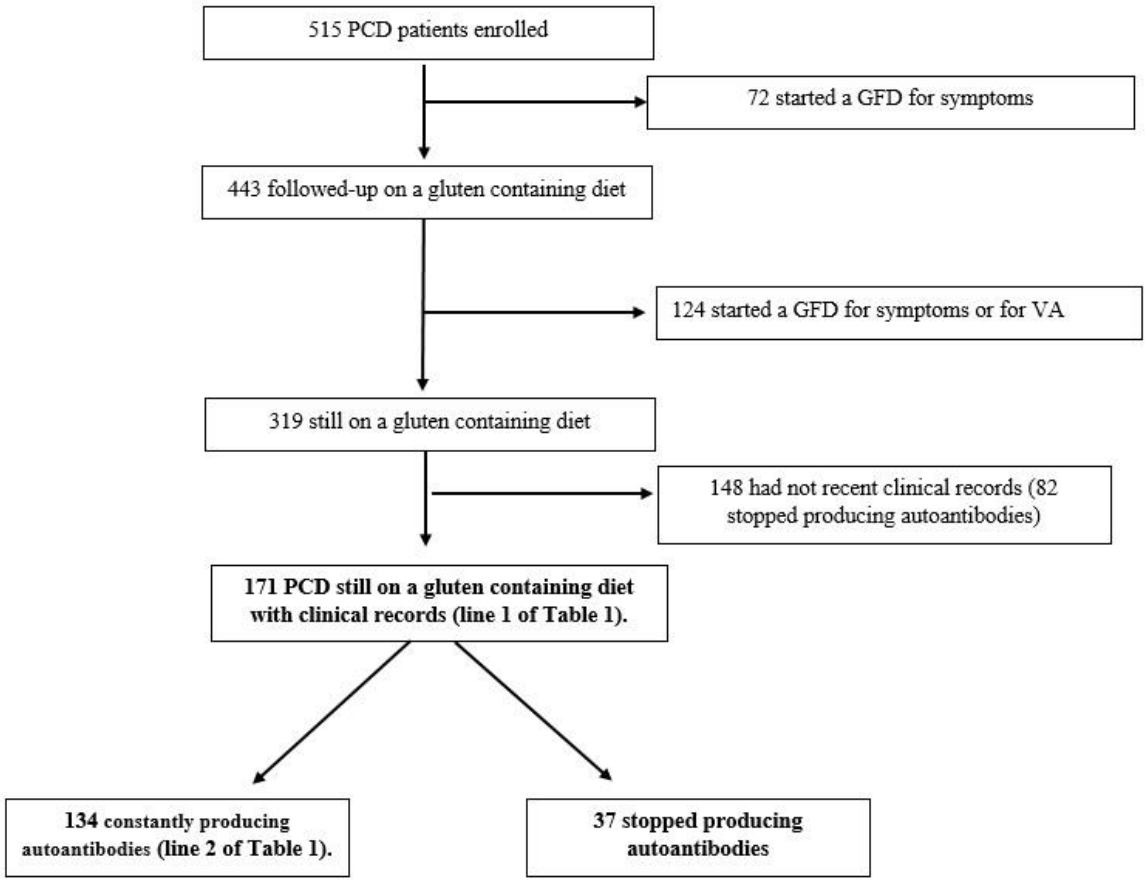
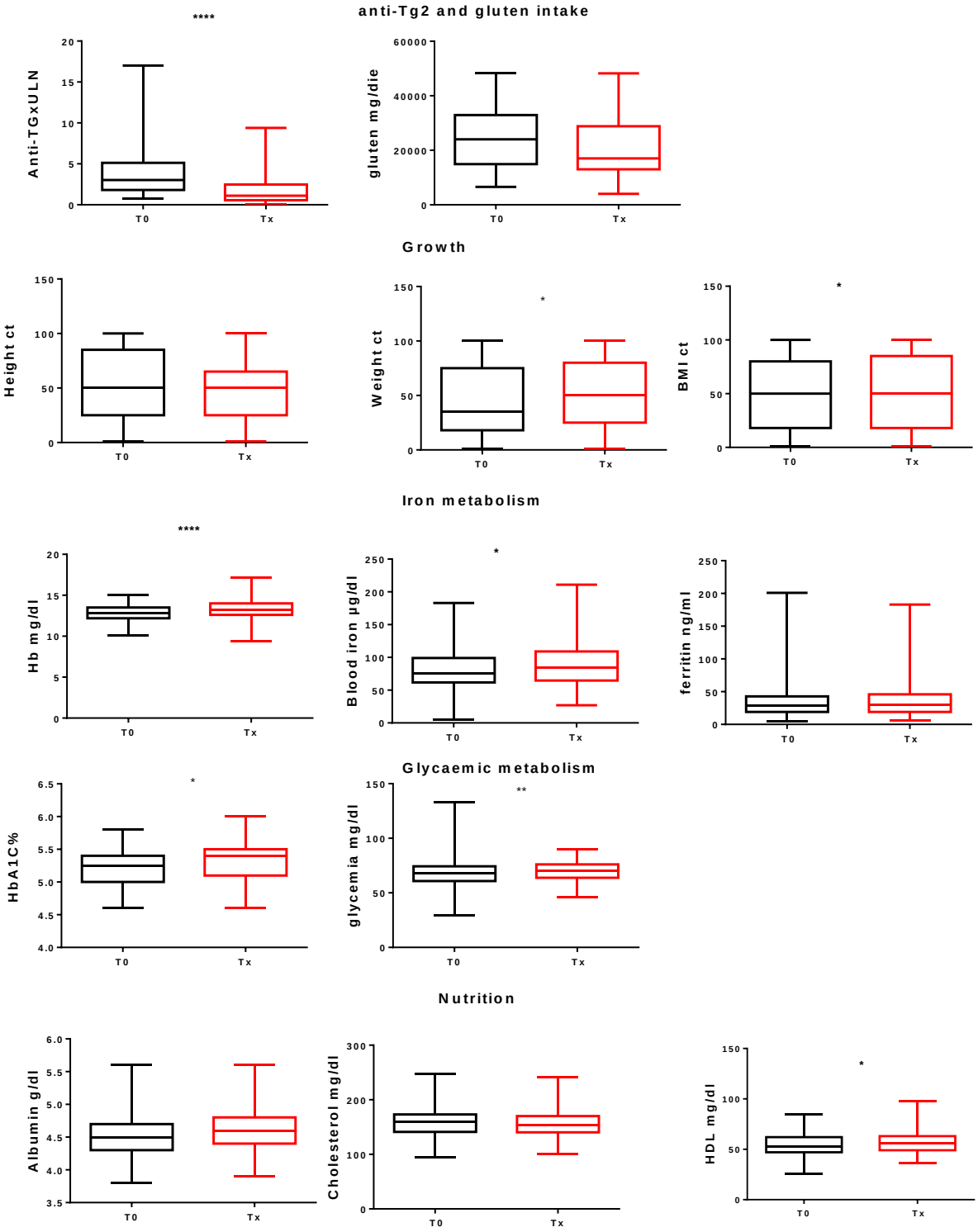


Figure 1: Flow chart of study cohort. GFD: gluten free diet. VA: villous atrophy


anti-Tg2 and gluten intake
****
Anti-TGxULN
20
15
10
5
0
T0
Tx
gluten mg/die
60000
40000
20000
0
T0
Tx
Growth
Height ct
150
100
50
0
T0
Tx
Weight ct
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0
T0
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BMI ct
*
150
100
50
0
T0
Tx
Iron metabolism
****
Hb mg/dl
20
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10
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Tx
Blood iron µg/dl
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250
200
150
100
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ferritin ng/ml
250
200
150
100
50
0
T0
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Glycaemic metabolism
HbA1C%
*
6.5
6.0
5.5
5.0
4.5
4.0
T0
Tx
glycemia mg/dl
**
150
100
50
0
T0
Tx
Nutrition
Albumin g/dl
6.0
5.5
5.0
4.5
4.0
3.5
T0
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Cholesterol mg/dl
300
200
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HDL mg/dl
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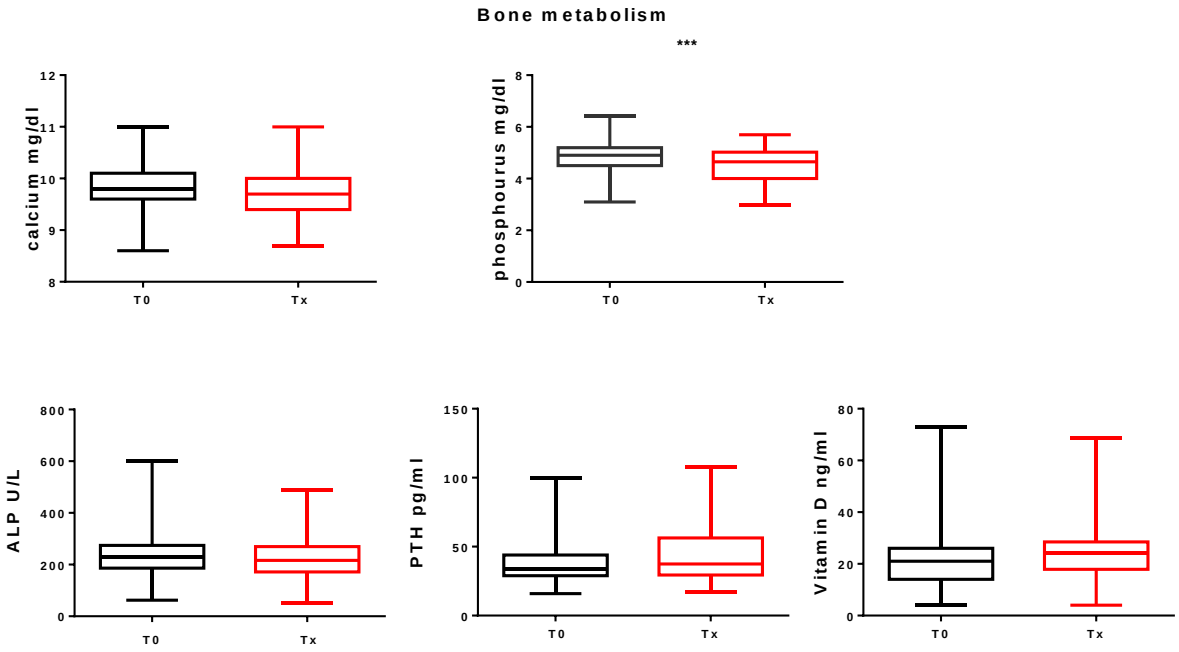

Figure 2: Parameters between time of diagnosis (T0) and in occasion of last follow-up (Tx). * level of significance

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Chapter 5: Prediction of villous atrophy in PCD children at the time of diagnosis

5.1 Precision medicine and machine learning towards the prediction of the outcome of potential celiac disease

This work has been published as:

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INTRODUCTION

Potential Celiac patients are characterised by genetic predisposition to celiac disease (CD), presence of CD specific antibodies (anti-human tissue transglutaminase antibodies and anti-endomysium) in the serum, but no morphological changes in the small bowel mucosa (1–7). Only a small percentage of them showed significant clinical symptoms (and are started on a gluten free diet at time of diagnosis), while the majority progressed over several years (up to a decade) without any clinical problem or a progression of the small intestinal mucosal damage even if they continued a gluten containing diet, on long term follow up one third of them progressed to a clear pattern of CD mucosal damage. The real issue was to attempt to predict, at enrolment, who was more likely to progress to villous atrophy disease in order to prevent clinical and histological damage related to the disease. In a previous paper, we developed a traditional multivariate approach to predict, on the base of the information at enrolment (time 0), subjects more likely to develop the full-blown disease. Overall, a discriminant analysis model allowed to correctly classify, at entry, 80% of the children who would not develop a flat mucosa over follow-up, whereas approximately 69% of those who did develop flat mucosa are correctly classified by the starting parameters (1).

As discussed by Wasserstein et al. in (8), making conclusions based uniquely on linear models can give unhelpful information when clinical data are used. Among others, some of the well-known limitations of the linear models are: assumption about the distribution of the variables not controlled; non independency of the variables selected in the model; the models obtained, being hypothesis driven, may not respect the uncertainty about the biological significance of the variable selected; relative weakness of sample size leading to very large confidence intervals on follow up.

In this second phase, we adopted a machine learning approach to validate an innovative method to predict the outcome.

ML techniques were proposed to support clinical decision in studies where multiple features can affect outcomes. Recently, several studies produced seminal papers that invite the community to use such methods (9–19). Obermeyer, Rajkomar et al.(9-11) reviewed Artificial Intelligence methods currently used in medicine, while the impossibility to use large amount of data without an automatic code was discussed by Schwalbe and Wahl (12). Also, the description of the "The All of Us Research Program" in (13) and the recent review on deep learning by Piccialli et al.(14) focused on these issues. The editorial office of The Lancet Respiratory Medicine (15) gave some guidelines for ML, as done by other authors (16–18). Beam et al.(19) focused on guidelines for reproducibility of results. What was noticed in such studies was that ML is a powerful set of tools that help the extraction of significant features for the prediction of outcomes. Nevertheless, because of its wide range applicability, considerable caution in the interpretation of models was required to produce an innovative approach to clinical data. Common pitfalls and roadmap for the application of ML methods in the medical domain were deeply reviewed (20–23).

Many studies adopted ML techniques effectively in various clinical frameworks for the prediction of outcomes. The main domain, where ML and other Data Analysis techniques are widely used, is cancer research and rheumatology, as pointed out respectively in the review by Hinkson et al.(24) and Radstake et al. in (25): in this case, also images are used to enrich the available data set. Images are also used in the detection of CD with the use of ML in (26,27).

What is the most important feature and maybe also the main drawback is that the application of the ML techniques is model-free, data-driven, and intrinsically non-linear. ML takes advantage of all the available data, uses the different features known in the learning process: for example, the fields with

categorical values can be converted into different numeric fields so that they are treated separately, without the need of ordering.

Our data set presents a temporal pattern due to the follow-up, with an increasing sparsity of the data as the follow-up is increased. For this reason, a ML strategy which considers the features collected at the enrolment allow to obtain a not increasing confidence interval for the final prediction, despite the decreasing sample size as the number of follow-ups increases. This provides a robust methodology, compared to the usual statistical parameters estimates.

In this study, we used ML for feature selection and for classification in a new condition such as CD and its multifactorial pathogenetic elements. Feature selection gives indications on the best predictive items in the dataset, while the classification result is given via threshold: it will give 1 (high risk) if the model output for a given value exceeds 50%, 0 otherwise.

Aims of this work was, starting from a follow-up dataset available for PCD, to apply Machine Learning (ML) to select most influent features and introduce predictive models to distinguish patients who developed duodenal atrophy from those who remained potential on a gluten containing diet.

MATERIALS AND METHODS

Prospective cohort features

A prospective cohort of potential celiac disease children (340) was followed up from diagnosis till maximum of 12 years (1). Diagnosis was confirmed when children showed at least 2 positive anti-transglutaminase IgA and anti-endomysium serological tests and all duodenal biopsies performed (1 from the bulb and 4 from distal part of the duodenum) were not atrophic, according to Marsh-Oberhuber classification. All patients enrolled were also HLA DQ2 and/or DQ8 positive. Symptomatic children started a gluten-free diet at time of diagnosis. The others (280/340) continued

a gluten containing diet and had clinical and serological evaluation every 6 months and histological examination every 2 years (1). 42/280 (15%) developed a flat duodenal mucosa during follow-up, while 89/280 (32%) became completely seronegative and 149 remained potential during follow-up. Risk factors associated with development of villous atrophy were investigated by log-rank test to compare the effect of factors on survival and a multivariate analysis was used to deal with the correlations among the variables considered. The study was carried out according to the Helsinki II Declaration and was approved by the Ethical Committee of the School of Medicine of the University of Naples Federico II, Protocol n. 191/06. The present research involving human participants under the age of 18 years (including donors of tissue samples). Each parent (and/or legal guardian) gave a fully informed consent to the participation of their child to the study and to the use of their biological samples for research purpose. The form is available on request at 'r.auricchio@unina.it'. The datasets analysed during the current study are available from the corresponding author on reasonable request.

Data cleaning and preprocessing

Starting from the available dataset, a data cleaning and pre-processing step was required. The analysed dataset contains both categorical and numerical features. Some of them present missing data values, however these features have still been considered either in feature reduction and classification tasks. In this context, results are mainly affected by the poor filling of the data and the imbalance of the predicted targets. Both issues can be easily explained: for the first one, the follow ups are available at different time lengths according to different individuals (censored data); for the second issue, the diagnosis of the overt CD happens in about 30% of the cases, while ML works better if the outcomes are balanced. In Figs. 1 and 2 we reported disposable data for each time point of the follow-up and the distribution of the patients by the available follow-up. In this work, the results of the clinical tests in the successive follow ups were not considered for two main reasons: first of all, the objective of this work is to make a prediction of the outcome at the time of the diagnosis of PCD; furthermore, there were not enough patients whose sequence of clinical results in the consecutive follow ups is consistently present.

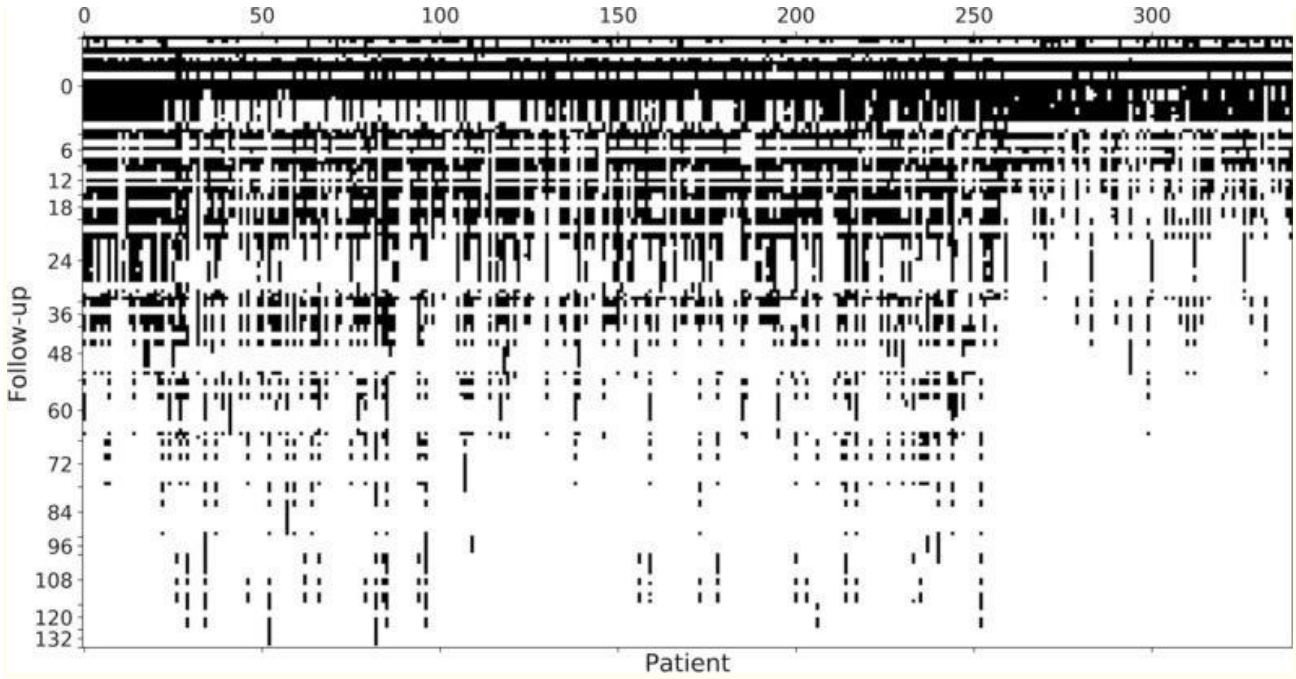

Figure 1

A spy plot about the presence of values on the whole dataset: black indicates available data, white missing. The numbers on the ticks on the vertical axis indicates the follow-up months, while the ticks without numbers indicate the gap between two separate follow-ups. The pattern is typical of longitudinal studies: such distribution highlights the problem when results are produced via the classical descriptive statistical approach, where the model is confirmed in terms of confidence intervals and probability distributions with a quite limited data set. ML with tree techniques can overcome this feature using all the available data.

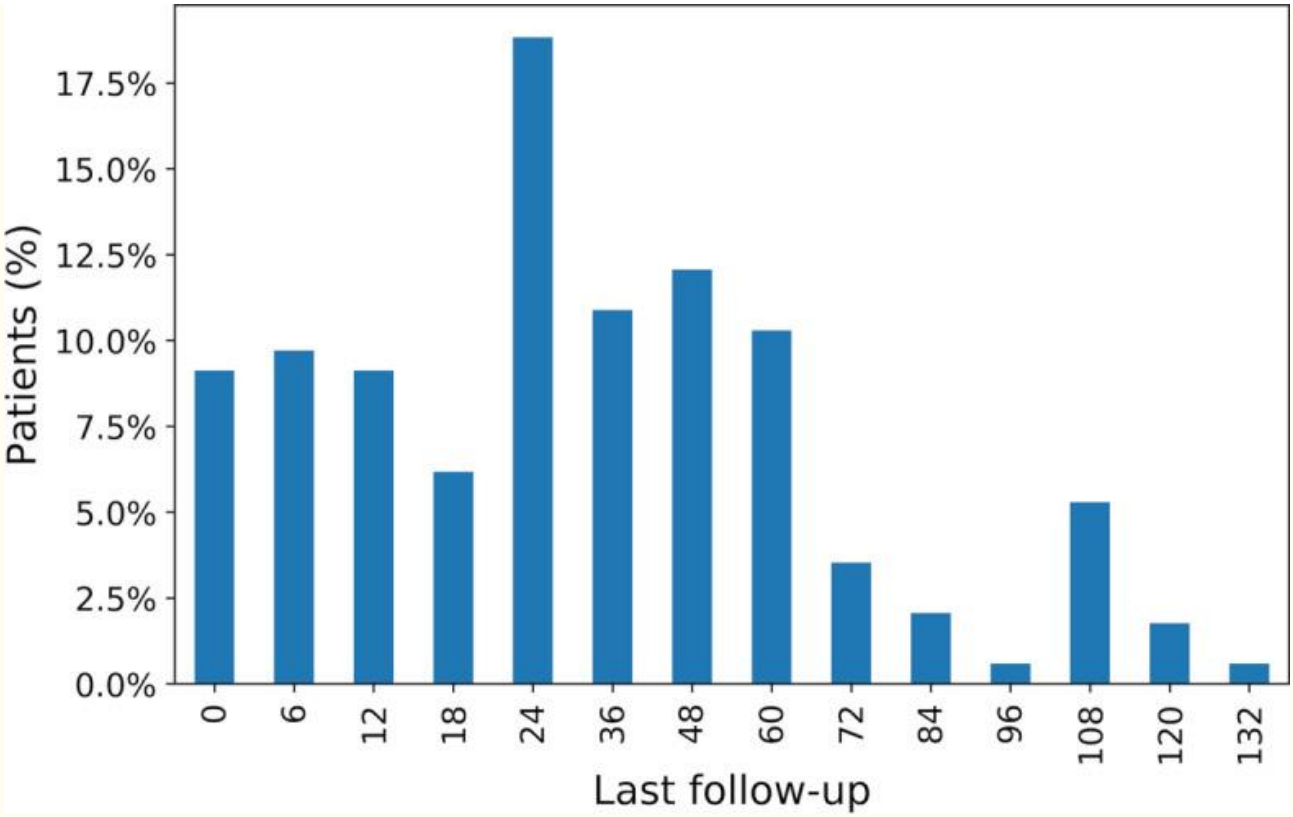

Figure 2

The percentage of patients with last follow-up. Different motivations can cause the interruption of follow-up, such as the onset of the disease, familial and logistic problems, mobility, unavailable blood sample.

The fields with categorical values were converted into multiple numeric fields through one-hot encoding (28); indeed, it would make no sense to apply ordinal encoding to the ordinal categories, because they may contain missing data, and therefore there could be a loss of significance if the missing data would be replaced with a numeric value. Finally, the considered features were those at the first follow up (at time 0).

Then, ML was applied to the dataset with two aims: feature selection and supervised classification for the villous atrophy development prediction. In both cases, ML methodologies are characterised in general by a model, whose parameters are adjusted by inferring on a subset of the dataset called *training set*, then the goodness of such model is evaluated by computing a score on a disjoint set of instances called *test set*. Each model also depends on non-trainable parameters called *hyperparameters* that influence the prediction; it is crucial to find good settings for them, and this operation is called hyperparameter tuning. Regarding this point, classical approaches consist in evaluating a model with different hyperparameters in a subset of the dataset, disjoint from both the training set and the test set, called *validation set*. Because of too few available instances, the procedure of splitting the dataset into train, validation and test sets is not recommended, because there is a risk of losing the statistical representativity of the training set. In order to alleviate this problem, a k-fold cross validation approach has been used, with k = 10. More in detail, the dataset was partitioned in 10 disjoint subsets. At each iteration of the cross validation, 9 subsets become the training set, while the remaining subset is chosen as the validation set. Since the number of occurrences of the diagnosed CD was 30% of the total number of samples, a more suitable version of the tenfold cross validation, called *stratified cross validation*, was deployed. This guarantees the same percentage of the distribution of the CD targeted instances in each of the 10 subsets. Then, for each choice of hyperparameters on a given model, the average of the validation scores obtained on all the iterations of the cross validation is computed, then, finally, the best hyperparameter configuration is chosen by taking the configuration corresponding to the best value between such scores. The justification of using this approach is that the classical split into train, validation and test sets presupposes the representativity of the entire dataset being preserved in each subset. Unfortunately, this assumption cannot be made for our data set, given the small number of PCD patients. Therefore, the same dataset has been used in both feature selection and classification, and the hyperparameter tuning has been validated by using the discussed k-fold stratified cross validation. This is a general strategy which limits the overfitting phenomena (29,30).

THE PROPOSED ML WORKFLOW

In Fig. 3 we present a ML workflow which briefly summarises the computational procedure for the PCD children categorization. The medical data are processed with a Feature Selection scheme (left

gray block), then in the obtained reduced feature space, a Classification phase (right gray block) is used to the outcome predictions. In this section, we recall the ML background about the models, the feature selection and the classification (28).

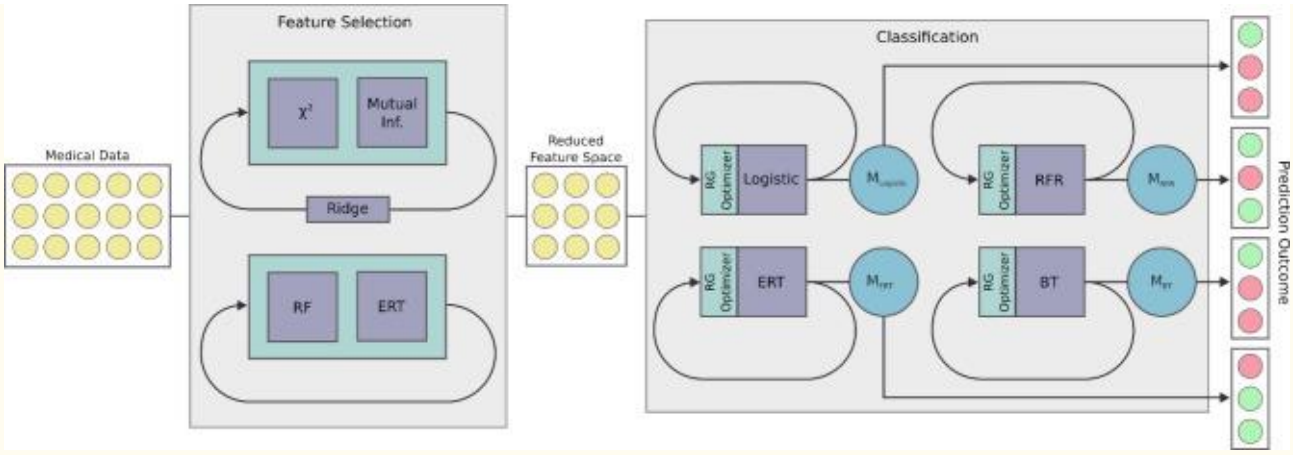

Figure 3

The proposed ML workflow for outcome prediction. The medical data are reduced in terms of features by a Feature Selection scheme (left grey box), then the predicted outcome is obtained by using ML models in a Classification procedure (right grey box). For abbreviations, see “ML models” section.

ML models

For the Feature Selection scheme, the following models have been considered: (i) univariate analysis via chi squared and mutual information statistical tests, with validation through the Ridge classifier, (ii) Random Forests (RF) and Extremely Randomized Trees (ERT). For the Classification phase, we consider: (i) RF, (ii) ERT, (iii) Boosted Trees (BT), (iv) Logistic regression (LR). Details of the ML models are here reported:

- The Ridge classifier, used in the statistical tests, is a linear model whose coefficients are obtained by solving the mean squared error optimization problem with a quadratic penalization term on the coefficients multiplied by a hyperparameter balancing the regularization.
- RF is a methodology relying on averaging random versions of decision tree models to reduce the inherently high variance from each tree model. The construction of each tree is done by satisfying properties related to discrimination criteria on each tree node. For the classification, the predicted result is obtained by a majority vote on the statement of each decision tree and for feature selection, the importance of the model feature is determined by the percentage of the features that are present in the decision tree nodes.
- ERT is based on the ensemble of more randomly built decision trees than RF, allowing less variance, paying a greater bias. The weight of the important features is assessed as described for RF.
- BT uses the idea to fit a sequence of simple decision trees with an assigned rule. Given an underlying function that maps the *feature space* in the *target space*, the boosting procedure approximates it through an *additive weighted expansions* technique; this procedure produces a good fit of the predicted values. In order to reduce the phenomenon of overfitting, a combined bagging-boosting procedure for the least-squares fitting of additive expansions is adopted.
- LR is a linear model which is used to predict the outcome in a probabilistic way. More in detail, the probability distribution of the predicted outcome is modelled by a logistic function. In this work, we adopted a modified version of LR where several penalization strategies are implemented, allowing to improve the training of the model through the optimization.

Feature selection scheme

We detail the feature selection block in Fig. 3. Given a data set, the objective is to extrapolate a subset of the features which are most representative. This methodology has a double significance: in the context of clinical diagnosis, it allows to detect risk factors; in the context of ML, it is a way to alleviate the problem of the curse of dimensionality, where the dimensionality of the features is

numerically comparable to the dimensionality of the samples. Since the number of samples is relatively low, a procedure of cross validation has been used to validate the choice of the features. More in detail, given the 10 splits of the cross validation, at each step the feature importance of the trained model is computed; then, for each model, the average of the feature importance in the whole cross validation procedure is considered. Finally, we reordered the features according to the sum of the feature importances from the four considered models. In conclusion, the reduced feature space is obtained so that the cumulative importance value (CIV), with respect to the overall sum, reached the value of 75%.

This approach can be justified as follows. Since a single Feature selection model is not able to extract the whole set of complex relationships between our data, an ensemble of the four methods is considered to enhance the generalisation of the best feature detection process. Furthermore, the CIV criteria are used because it has the advantage of not choosing aprioristically the cardinality of the best features set, but rather to adaptively determine it depending on the magnitude of the feature importance obtained.

Classification phase

The reduced feature space is processed in a classification phase. Indeed, the attempt to classify, at enrolment (time 0), who was more likely to progress to villous atrophy can be treated as a binary classification problem. For each ML model Λ in Fig. 3 (with Λ = Logistic, RFR, ERT, BT), an optimization through the hyperparameter tuning is done, as previously described, in order to generate the optimized model MΛ. MΛ that has been used for the prediction of the final outcome. The results of the classification were validated through a tenfold cross validation. The models that have been considered for this type of problem are those based on tree methodologies: Random Forest, Extremely Randomized Trees, and Boosted Trees. The Boosted Trees method, unlike the other two, is based on the progressive training of trees in a sequential way, i.e. a tree was trained starting from the previously trained tree through gradient boosting. This approach was considered to confirm that selected features

have powerful predicting efforts. In this work, the classification problem can hardly be seen as a regression problem, since the label had only two values. Linear logistic regression was used in this report. The goal was to find linear coefficients such that the logistic distribution obtained from the linear combination of the features with these coefficients can approximate the output to a correct prediction.

RESULTS

As far as the feature selection is concerned, univariate analysis allowed to select only the features that satisfy hypothetical statistical tests, hence chi-square tests and mutual information were chosen. The most relevant features were obtained by a grid search strategy by ranking the number K of the selected features from 10 to 30. The adopted grid search criterion was the maximum of the cross-validated AUC score by training a Ridge classifier with the regularization strength equal to 0.01. The optimal value for the number of features was found to be 15 for the Chi-square test and 19 for the mutual information test. Results obtained for the top 34 features are reported in Fig. 4, while the description of such features is summarised in Table 1.

Feature	Type	Description
F1	Categorical	Age group at diagnosis (grouped in below 3/between 3 and 10/ over 10): between 3 and 10 years
F2	Categorical	Anti-tTG2 IgA deposit in duodenal mucosa at time of diagnosis: low positivity
F3	Categorical	HLA haplotype: DQ2/DR7
F4	Categorical	IL2/IL21 haplotype: GG
F5	Categorical	Anti-tTG2 IgA deposit in duodenal mucosa at time of diagnosis (grouped in present/absent/weak): weak
F6	Categorical	Age at first biopsy/diagnosis (grouped by integer age)
F7	Categorical	IL12 haplotype: TT
F8	Categorical	Height of villi in the first biopsy (grouped in normal/pathological/variable): variable
F9	Categorical	SH2B3 haplotype: TT
F10	Categorical	CCR haplotype: TC
F11	Categorical	Intra-epithelial lymphocytes in first biopsy < 34 cells/mm^2
F12	Categorical	RGS1 haplotype: AC
F13	Categorical	Anti-endomysium antibodies at the first biopsy (grouped in absent/present/weak/very weak/patchy): weak
F14	Numerical	Gamma delta intra-epithelial infiltration in first biopsy
F15	Categorical	OLIG3 haplotype: AG
F16	Categorical	Villi/crypt ratio in first biopsy (grouped in normal/pathologic): normal
F17	Categorical	Thyroiditis in family
F18	Categorical	LPP haplotype: AC
F19	Categorical	Inflammatory infiltration in the lamina propria (grouped in present/absent): present
F20	Categorical	Depth of crypts in first biopsy (grouped in normal/pathologic): normal
F21	Numerical	CD3 in crypts
F22	Categorical	IL18RAP haplotype: TT
F23	Categorical	TAGAP haplotype: TC
F24	Categorical	REL haplotype: AA
F25	Categorical	Marsh in first biopsy (grouped in M0/M1/M3): M1
F26	Numerical	Anti-tTG2 value compared to the upper limit of the normal
F27	Categorical	Celiac disease in family
F28	Categorical	Villi/crypts ratio (grouped in normal/pathological): normal non è categorical?
F29	Categorical	Sex: male
F30	Categorical	SCHIP1 haplotype: AA
F31	Numerical	CD25 infiltration in the lamina propria in the first biopsy
F32	Categorical	Vitiligo in family
F33	Categorical	Hypercholester in family
F34	Categorical	Diabetes in family

Table 1. Feature description. Features are numbered in order of relevance, as obtained by feature selection and reported starting from the most important feature. Te frst 19 are the one selected for the classifcation process, a red line has been added to divide the selected features among the others. It can be noticed that the selected features include mainly features of the child (age, age at biopsy), his genetic profle and data related to the infltration of the Small Intestinal Mucosa (including mucosal production of anti-tTG antibodies).

Figure 4 shows the values via the cumulative weight of each variable giving the relevance of features, where the one hot encoding is applied to categorical ones.

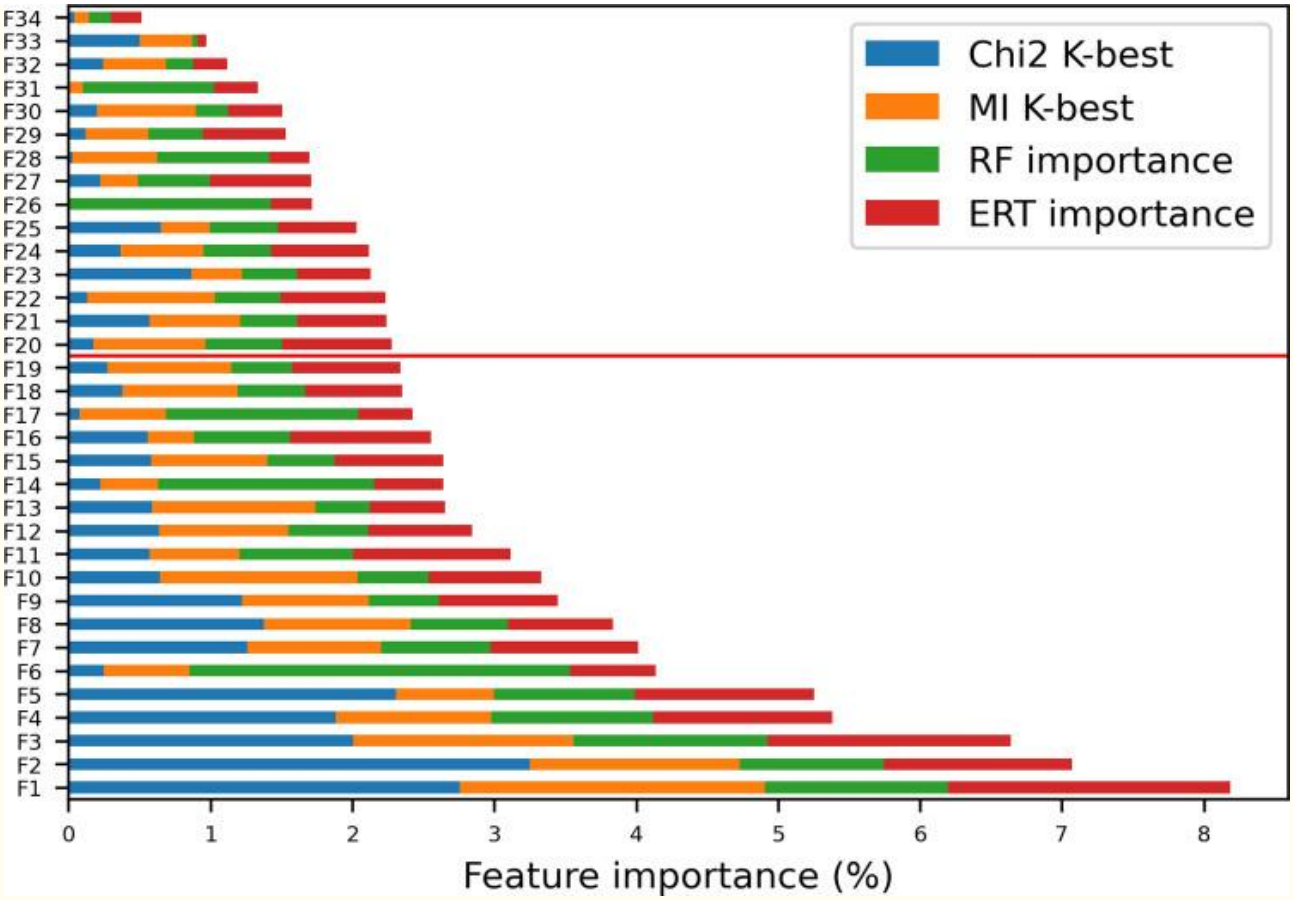

Figure 4

Cumulative feature importance. This graph was obtained by normalizing each feature relevance value by selecting a model for overall relevance and then by sorting the normalized relevance on the considered models. By using this methodology, the best selected features were chosen so that the Cumulative Importance Value (CIV), concerning the overall sum, reaches the value of 75%: the red line divides the selected features from the others. For the description of the features, see Table

Features are numbered in order of relevance, as obtained by feature selection and reported starting from the most important feature. The first 19 are the one selected for the classification process, a red line has been added to divide the selected features among the others. It can be noticed that the selected features include mainly features of the child (age, age at biopsy), his genetic profile and data related to the infiltration of the Small Intestinal Mucosa (including mucosal production of anti-tTG antibodies).

According to the threshold set to 75% of CIV, 19 features were chosen, denoted by F1–F19 (see Table 1). In Fig. 5 the contribution of the selected features to the four models is shown.

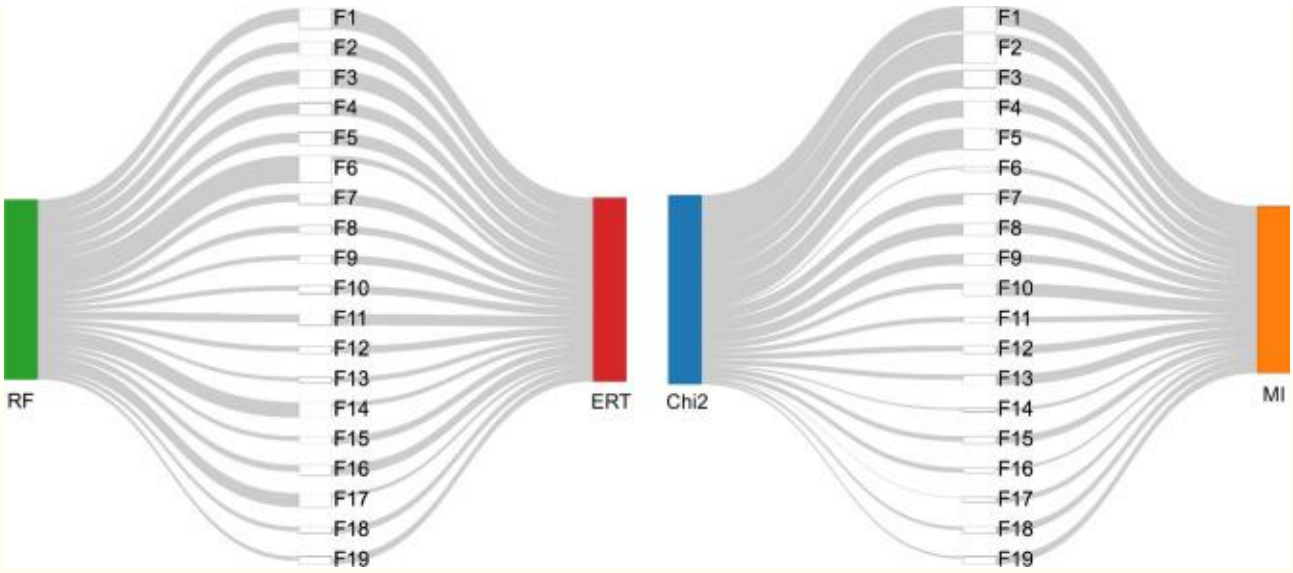

Figure 5

Feature contribution to the four models. In this graph, the thickness of the grey lines indicates how much the feature impacts the results obtained by the model. For legend on the models, see Fig. 3, for

the description of the features, see Table 1It can be noted that the selected features include mainly features of the child (age, age at biopsy), his genetic profile and data related to the infiltration of the Small Intestinal Mucosa (including mucosal production of anti-tTG2 antibodies) at time 0.

The validation of the model was given in terms of accuracy, sensitivity, specificity. Then, the Area Under the Curve (AUC) and the Receiver Operating Characteristics (ROC) were computed to estimate the performance of the proposed methodology. The results of classification are reported in the form *mean* ± *standard deviation* of the 10 cross validations in Table 2. All the methods report an accuracy above 75%, but there are deep differences when other parameters are considered. Both Random Forest (RF) and Extremely Randomized Trees (ERT) have the highest scores in terms of accuracy and specificity, but because of the low specificity score, they do not perform sufficiently for predicting true positives. Instead, both Boosted Trees (BT) and Logistic Regression (LR) provide the best scores for specificity without a great loss in terms of True Negative cases. In particular, we observe that BT has a higher value of accuracy, specificity and AUC than LR, at the small cost in terms of sensitivity, but since its standard deviation is smaller than LR, BT can be considered as the best model. Building sequential decision trees through bagging-boosting techniques has been proven helpful for this task. Furthermore, if we consider the ROC AUC score, the two aforementioned methods reported the best results, showing that such models have a good predictive power, even though the data set is unbalanced. As expected, all the optimized models report the highest ROC AUC value than the non-optimized values (except for the ERT). The results related to this application showed that there are some Supervised Learning Models, like in this case BT an LR, which can detect patterns which were peculiar only to the relatively few cases that develop CD. The trained model can be used for future classification of PCD starting from the clinical data, giving an indication to the paediatrician in the domain of precise medicine.

Model	Accuracy (TP+TN)/N	Sensitivity TP/(TP+FN)	Specificity TN/(TN+FP)	ROC AUC
RF	0.84±0.04	0.06±0.12	**0.98±0.03**	0.52±0.06
RF (optimized)	0.83±0.05	0.22±0.21	0.94±0.05	0.58±0.11
ERT	**0.86±0.03**	0.16±0.18	**0.98±0.03**	0.57±0.09
ERT (optimized)	0.85±0.03	0.06±0.12	**0.98±0.02**	0.52±0.05
BT	0.81±0.07	0.38±0.17	0.89±0.07	0.63±0.10
BT (optimized)	0.80±0.08	0.58±0.18	0.84±0.08	**0.71±0.09**
LR	0.77±0.07	0.58±0.20	0.80±0.08	0.67±0.10
LR (optimized)	0.75±0.08	**0.60±0.24**	0.78±0.10	0.69±0.11

Table 2. Classification results. TP=true positive, TN = true negative, FP=false positive and FN=false negative. The results of classification are reported in the form mean±standard deviation of the parameters obtained by 10 cross validations. All the methods report an accuracy above 75%. Specifcity also is always above 75%, with two of the considered methods over the 98%, while the best performance in terms of sensitivity is 60%. Ten, the Area Under the Curve and the Receiver Operating Characteristics are reported to estimate the performance in terms of accurate classification of the proposed methodology. For all results, the best-obtained scores are highlighted in bold. For the ROC AUC score, two methods (BT & LR) report better results, showing that such models have a good predictive power even though the data set is unbalanced. As expected, all the optimized models report the highest ROC AUC value than the non-optimized values (except for the ERT), due to the hyperparameter tuning

Comparison with previous work on PCD. In the previous paper1 a stepwise discriminant analysis was used to select variables able to differentiate children who became celiac from those who remained potential over 8 years follow-up. A Discriminant score (D-Score) was calculated by multiplying the normalised value of each variable included in the stepwise discriminant equation to its respective regression coefficient. From the score, the individual probability to be assigned to one or the other group was derived: we classified (predicted) the individuals into CD or not-CD group, using the selected variables, blinded to the final diagnosis. In this work we are able to categorise PCD that can more likely develop CD using ML. Starting from the available dataset, the models are trained by the items and can be used for the outcome prediction. Tis overcomes the previously available linear model and proposes a novel classification of PCD based on ML.

DISCUSSION

ML methods showed that some clinical and laboratory features have an important predictive power to forecast the development of villous atrophy. We wish to highlight this statement in order to guide the reader that could be sceptical about automatic indicators: what we found, is that the features selected by ML are roughly the same that give the important information in the linear model available in1, but the prediction that ML offers is significantly more accurate when compared to previous methods. The issue of this domain of research is to support the clinical decision making for the management of potential celiac children at entry, based on informations/variables available at the first clinical and laboratory work up. Indeed, the majority of potential CD do not develop a full-blown disease within 8 years of follow up. Up to one third decrease the production of their main feature: the anti-human transglutaminase antibodies (Anti tTG2). On the other end, for the 30–35% who eventually develop villous atrophy over follow-up, an accurate prediction at time 0 (diagnosis) might prevent the progressive pathological process leading to a full small bowel mucosal destruction. We previously used a traditional multivariate approach to estimate, by a discriminant model, at diagnosis, which individual is more likely to develop villous atrophy over time, reaching an accuracy of prediction close to 70%. But the multivariate approach requires assumptions about the quality of variables used to develop the model which might be not fully appropriate to many clinical data. The independency of each variable by the other variables, which is a requirement for the best multivariate model is rarely respected: there is, at the end, at least a 30% misclassification. Alternatively, a hypothesis free method does not require a specific distribution of each variable neither it requires mutual independency of the variables. It may be finally simpler to fit the clinical judgement of the physician. For example, our trained BT (optimized) model can categorize PCD starting from the 19 selected features with an accuracy of 0.80, sensitivity of 0.58 and specificity 0.84. This gives a clear indication to physician on the relevance of collecting data on genetic profile and infiltration of the Small Intestinal Mucosa for PCD and raises the question about the opportunity to put a child on gluten-free diet starting from these features before the development of the full blown CD. ML indications can move towards precision medicine also the detection of CD, as done in other diseases with similar workflows, as shown for the evaluation of cardiometabolic risk and risk of developing diabetes (2–7,31–34).

Celiac Disease automated diagnosis is not new to computer-assisted systems, which have been explored since 2008 (35); spatial domain, transform domain, scale-invariant and and spatio-temporal features have been applied to several aspects of CD diagnosis, especially to the subjective interpretation of the intestine small mucosal immaginery (36). But artificial intelligence, machine learning and deep learning do require large amount of data, in order to produce reliable results, and this is often one of the major caveat of clinical studies. This work also presents some limitations. The relatively low number of data samples, with the outcome being unbalanced, and the lack of test samples from an external cohort are critical issues. It is indeed known that ML applied to small, sparse and heterogeneous data is challenging in terms of model contextualization, validation procedure and the classification accuracy. Moreover, about the limitations of the proposed ML workflow, we are working on a semi-automatic strategy of hyperparameter tuning in both the feature selection scheme and the classification phase, since not all the possible combinations of hyperparameters have been tested. Strategies, like the usage of the cross validation for the choice of the best hyperparameters in both feature selection and classification, should allow improving the model performance.

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5.2 Proteomic biomarkers in serum predict villous atrophy development in potential celiac disease at time of diagnosis

This work has been submitted on Gastroenterology as:

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INTRODUCTION

Potential celiac disease (PCD) is a clinical condition characterized by the presence of a positive celiac disease associated serology and an architecturally normal intestinal mucosa, with (Marsh1) or without (Marsh 0) an increased number of intraepithelial lymphocytes (IELs) (1). From a pathogenetic point of view, this is explained by the fact that in PCD, gluten induces a CD4+ T cell specific response that in turn allows a specific B cell response, but intestinal damage sustained by cytotoxic cells is somehow prevented (2).

PCD has been considered a form of early-stage disease, but recent evidence highlights that PCD represents a heterogeneous condition in which evolution to villous atrophy (VA) is not mandatory for all cases Indeed, when children are considered, less than half of them will develop a full-blown disease, even when a long follow-up on a gluten containing diet is considered. Most of them will keep on producing celiac disease (CD) specific antibodies without never developing intestinal damage (3). Furthermore, around one third of them, will permanently stop producing antibodies (3). Many risk factors have been investigated for their power to predict, from diagnosis, patients' future evolution, including both clinical, genetic and immunological parameters (3–8). Unfortunately, up-to-now they cannot represent for clinicians more than a guide and management of PCD patients remains a challenging issue to be handled (9). Thus, there is a constant need for new biomarkers able to define subgroup of PCD patients that will develop a full-blown disease and for that should immediately start a gluten free diet (GFD). In this context, serum proteome analysis offers a wide look to the metabolic and molecular pathways that characterizes a specific phase of development of health and disease in

very small children. For instance, in children at risk for type 1 diabetes (T1D) enrolled in the context of the TEDDY study, both targeted and untargeted strategies of serum protein profiling revealed a satisfying accuracy in the prediction of the development of the disease: a machine learning analysis predicted both the development of persistent autoantibodies and T1D onset 6 months before autoimmunity initiation, with an area under the receiver operating characteristic curve of 0.871 and 0.918, respectively (10). Also, Bergemalm at al (11) measured 92 proteins related to inflammation using a proximity extension assay in the pre-clinical stage of ulcerative colitis. Six proteins (MMP10, CXCL9, CCL11, SLAMF1, CXCL11 and MCP-1) were up-regulated ($P < .05$) in preclinical ulcerative colitis compared with controls. They concluded that a set of inflammatory proteins are up-regulated several years before a diagnosis of ulcerative colitis. These proteins were highly predictive of the development of the disease.

Starting from the hypothesis that an immune dysregulation underlies the progressive development of CD in genetically predisposed children, we designed a study with the primary aim to evaluate how a specific set of 92 serum inflammatory proteins could help differentiating among PCD patients, those who will eventually develop VA in future from those who will not and contemporary investigate how specific changes in serum inflammatory proteins associate with small intestinal damage to give some insights on CD pathogenesis

MATERIALS AND METHODS

Study design

We performed a monocentric case–control study at the Paediatric Department of University Federico II in Naples comparing serum samples from PCD patients who developed full blown celiac disease during the follow up (cases = CEL) with those from individuals who remained potential over time (controls = POT). More in detail the study included 31 asymptomatic PCD patients, longitudinally followed for up to 8 years (mean time 5.85 years) on a gluten containing diet. The diagnosis was

received according to the European Society of Gastrointestinal Hepatology and Nutrition (ESPGHAN) guidelines12 based on a positive CD associated serology (anti-tissue transglutaminase and anti-endomysium antibodies) tested twice on two different blood sample and a normal intestinal duodenal architecture (villi/crypts ratio higher then 2) on all the 5 biopsy samples analysed: 14 cases were Marsh 0 at diagnosis and 17 cases were Marsh 1 ($p<0.05$). Seventeen children remained potential (POT) over time while 14 children developed flat mucosa (CEL) having respectively a mean age of 8,85 years (CI 7,1-10,6) vs 8,38 years (C.I 5,7-11), with no statistically significant difference. We compared serum inflammatory protein abundance at time of PCD diagnosis between children who remained potential (n=17) versus those who developed VA (n=14) (mean follow-up time: 6 years).

Ethical approval.

The study was approved by the regional ethical boards. All patients gave their written and informed consent before participating in this study.

Proteomic profiling and quality control

Serum was collected from blood samples, fractionated and stored at − 80 °C until analysis. Serum samples were analyzed for relative concentrations of 92 inflammation-linked proteins using the Olink multiplex arrays (INFII panel, Proseek multiplex arrays; Olink Bioscience, Uppsala, Sweden) according to the manufacturer's instructions 13. In brief, a standard 96-well microplate format is used by the assay, including 90 samples and six external quality control standards. Ninety-two oligonucleotide-labelled pairs of antibodies were mixed with each sample. When high-specificity antibodies bind the target protein, the attached oligonucleotides form a unique DNA reporter sequence which is subsequently quantified and amplified with standard PCR. Samples were randomly distributed and analyzed on seven plates. PCR values above the fluorescence detection limit were log2-transformed and corrected for technical variations based on negative and inter-plate controls. With the negative control samples (buffer with antigen) the lower limits of detection (LOD) were determined. Olink proteomics performed a quality control per sample: samples that deviated less than 0.3 normalized protein expression (NPX) from the plate median passed the quality control.

Pathway analyses

We used STRING Network pathways, to identify canonical pathways and upstream regulators of dysregulated preclinical protein markers. Gene Ontology enrichment analysis (GOEA) and Functional Annotation Clustering analysis were performed on these 10 proteins by using DAVID Bioinformatic Resources restricting the output to Biological Process (BP) and Molecular Function (MF) Clusters. • The threshold for statistical significance of GOEA was FDR<5% and Enrichment Score >1.5. The 'Kyoto Encyclopedia of Genes and Genomes' (KEGG Pathway) 14 and REACTOME pathway analyses were also performed. The threshold for statistical significance for the Pathway analysis was FDR < 0.1. A detailed description of the analysis can be found in the Supplementary Material (Supplementary Figure 4).

Statistics

Statistical analyses were performed using the MetaboAnalyst 5.0 platform (https://www.metaboanalyst.ca), the R statistical platform (https://www.R-project.org)

For comparisons of continuous distributions among two groups, a two samples Student's t-test was performed. The obtained p-values were then adjusted with Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to assess the False Discovery Rate.

Partial least squares discriminant analysis (PLS-DA) was performed to examine the differences between the two sample groups using combination of various proteins, which correlate significantly among themselves. PCA is an unsupervised method aiming to find the directions that best explain the variance in a data set (X) without referring to class labels (Y). The VIP (Variable importance for projection) scores are statistical estimates of importance of each variable in the PLS-DA model.

The Random Forest Classification model was built considering as main features of number of classification trees and maximal predictors of 5000 and 7 respectively. Before using the model, all features were log10 transformed and subjected Pareto scaling (more specifically, each feature was mean-centered and then divided by the square root of the standard deviation). The model evaluation was generated by Monte Carlo Cross Validation (MCCV) approach. Briefly, two thirds of the samples were used to evaluate the feature importance, and the remaining third of the samples was used to validate the generated model. The procedure was repeated multiple times to calculate the performance and the confidence interval of the model.

Linear Discriminant analysis was used to identify variable able to discriminate, at time of enrolment (T0) between the children who remained potential (POT) by those who developed flat mucosa (CEL).

Wilks lambda shows the ability of each protein to discriminate starting from 1 = complete overlap between the groups to 0 = complete separation. By the discriminant score, obtained by the linear combination of the selected variable, cases are assigned blindly to one of the two groups. The discriminant score obtained by the linear multivariate model was used to compute the probability of each subject to be predicted in the group of CEL or that of POT. To control for the bias related to the classification of the subject by the equation obtained by the same group to be classified a jack-knife approach was adopted, where each subject is excluded from the computation of the equation and then classified by the model obtained without his own presence.

RESULTS:

Different distribution of serum inflammatory proteins at the time of diagnosis between children who will remain potentials (POT) and those who will develop villous atrophy (CEL).

In Figure. 1 the Volcano Plot shows the selection of inflammatory proteins at the time of diagnosis in children who will remain potentials (POT) versus those who will develop villous atrophy (CEL). The log fold increase (CEL > POT) is reported by their level of significance according to the Student t test. Il20, Il2, SIRT2, LIF, IL22 RA1, CST5, IL17RA, IL15RA, CDCP1, IL14 showed a significant difference between means (by t-test) and significant fold changes CEL > POT.

The box plots of the distribution of these proteins in the two groups are shown in Supplementary Figure 1. It may be noted that children who later develop flat mucosa (CEL) show, in comparison to those who remain potential (POT), an increased concentration of a set of 10 inflammation-related proteins several years before the development of their intestinal damage.

Figure. 2 shows the graphic separation at time 0 (enrolment) of the children who develop flat mucosa from those who remain potential at long term follow up obtained by the proteins selected through the partial least square discriminant analysis

Random Forest classification is shown in Figure. 3, where in green are children who develop flat mucosa (CEL) and in blue those who remain potential over a t long term follow up (POT

Finally, a linear discriminant analysis has been performed, obtaining, by the multivariate combination of 7 biomarkers (CDCP1, IL2, LIF, IL-10RA, SIRT2, CST5, IL4) a discriminant equation that allows to correctly classify 96.8% of children. (Table 1 first 2 columns, supplementary Table 1 a and b). The distribution of the Discriminant Scores, obtained by the multivariate combination of the 7 proteins, shows a remarkable discrimination between CEL and POT (Supplementary Figure 1). Only one child expected to be CEL was wrongly assigned to POT (marked with the n. 19 in the Supplementary Figure 1). Scrutinizing the data of this misclassified subject, we noted that all his biomarkers at time 0 (diagnosis 16/06/2005) were more in the range of the group of children that remained with a normal mucosa (POT) than with the range of those who developed mucosal atrophy (CEL): (CDCP1 = 2.4, IL2 = 1.21, LIF = 0.52, IL10RA = 1.23, SIRT2 = 4.16, CST5 = 5.22, IL4= 0.48). This child after 8 years' follow-up (age) showed a small intestinal mucosa of Marsh grade 1, not flat. Reviewing the charts of this child, an error in the records was found.

Figure 4 shows the probability to be predicted in one of the two groups (CEL or POT) by their individual discriminant score obtained by the combination of the 7 selected biomarkers. Most of the children are assigned to the predicted group with a very safe probability. The level of correct discrimination is fully maintained (> 90%) also when a jack-knife procedure is applied, where each subject is excluded iteratively from the computing of the discriminant equation and then classified according to the remaining cases. When the misplaced case was corrected, we obtained a 100% correct classification (prediction) of children who later developed flat mucosa (CEL) from those who remained potential (POT). After the independent jack-knife procedure 93,4% of cases were correctly predicted by this set of biomarkers.

Table 1 shows the summary of the comparisons by the various methods. The first column shows the selected biomarkers. The second column shows the ability to discriminate between the CEL or POT (Wilk's Lambda). The third column give the univariate F, which estimates the contribution of each single variable before the multivariate analysis, with relative level of significance (4th column). The fifth column shows the increase (fold changes) of CEL over POT. The 6th column give the significance of the difference between means (CEL-POT). The 7th column give the amount of explained variance in the first factor of the Partial Least Square Discriminant Analysis, and the last column show the Mean Decrease in accuracy of the Random Forest model.

By exploring the residual correlation among the 7 protein selected by the multivariate analysis, we found no residual correlation in the group of children who remain potential (POT), but a strong correlation in children who develop flat mucosa (CEL) between LIF and SIRT2 and CST5. (Figure 2

supplement). This residual correlation, which should have been abated in the multivariate equation, appears to be a significant signal of a pathogenic pathway leading to flat mucosa.

Pathway analysis

In this study we used different Bioinformatics tools to obtain details and functional connections into our group of proteins of interest. First, we used STRING database to identify known and predicted protein-protein interactions in our protein subset. This analysis allowed us to discover connections based on three STRING sources (text mining, experiments and databases). These interactions shown in Figure 5 include both physical and functional associations. Some canonical pathways and upstream regulators of dysregulated preclinical protein markers were identified. Second, we took advantage also of the GOEA, KEGG and REACTOME pathway analysis (Supplementary Figure 4). In details, in the GOEA we were able to isolate the negative regulation of defense response and the positive regulation of the JAK-STAT cascade processes in which our protein subset of interest was mainly enriched. Moreover 4 proteins have cytokine activity (IL4, IL20, LIF, IL2) and thanks to KEGG and REACTOME we obtained additional evidence of the role of many proteins in the JAK-STAT signalling pathway (IL22RA1, IL4, IL20, IL10RA, LIF, IL2) and immune system (IL22RA1, IL4, CD274, IL20, IL10RA, LIF, IL2). The Enrichr tool (XZ) was also used to perform the analysis on our sub-categories of proteins of interest (this part in shown in the supplementary material part of the study). Gene Ontology suggested a significant enrichment in the Positive Regulation of Tyrosine Phosphorylation, Leucocyte Differentiation and isotype switching to IgG isotypes; the regulation of protein phosphorylation is also involved. The biological process of gluten-induced inflammation is then already on stage, including the initial phases of leucocytes differentiation to invade the small intestinal mucosa.

KEGG pathway suggests the relevance of the involvement of the JAK-STAT pathway in inflammation, while the Molecular Function prioritize the cytokine activity. Finally, signalling by Interleukins is suggested by the Reactome Pathway (Supplementary Figure 4).

Figure 5 shows the molecular pathway obtained by the STRING database for the 10 key biomarkers selected by the previous analysis. Three out of 10 biomarkers (CDCP1, SIRT2 and CST5) are not related to the others due to their main involvement in different and specific function in this domain. SIRT2 works in the regulation of protein phosphorylation process (15); CDCP1 is involved in tumor progression and metastasis and was recently identified as novel marker for leukemia diagnosis and

for immature hematopoietic stem cell subsets (16); CST5 encodes an inhibitor of cysteine proteases of the cathepsin family, important mediator of tumor suppression by p53 in colorectal cancer (17).

Supplementary Figure 4 shows The Enrichment Score for each Biological Process (BP) and Molecular Function (MF) term (A). The gene count for each KEGG (B) and REACTOME (C) pathway is plotted, the first pathways is the most significant (lower FDR value, refer to Supplementary Table).

DISCUSSION

Potential Celiac Patients offers the unique opportunity to observe the progression of the gluten-induced tissue damage in Celiac Disease (18). The immune system of these patients produces a specific response to gluten, as demonstrated by the production of anti-tissue transglutaminase antibodies, but does not elicit the intestinal damage. Hence the scenario of recognition of the gluten peptide, activation of T-cell induced stimulation of B cells to produce antibodies is already clearly present, but the further progression, to the activation of intra-epithelial T-cells and Natural Killer cascade, is not yet present (18). We previously observed that the expression of some inflammatory cytokines, such as IL21, is markedly suppressed in PCD patients compared to full blown celiac (18). Over a long follow-up time (up to 12 years), only around 40% of these patients, on gluten containing diet, do progress to full blown disease and flat mucosa3. Notably 25-30% of them even stop producing anti-tTG antibodies, while the others keep on producing antibodies but preserve a normal mucosa. Considering these data, the decision to put a PCD patient on GFD since time of diagnosis is quite critical. The ideal aim would be to precisely predict patients that will develop the full-blown disease in order to start from the beginning a GFD and avoid a long-term exposure to gluten. Our previous model, mainly based on small intestinal mucosa features 3 moved a step forward, but it does require a mucosal biopsy and the accuracy of prediction did not get over 80% of cases, somewhat uncertain to take such a life-long clinical decision.

Serum proteome analysis offers a wide indirect look to the metabolic and molecular pathways that characterizes a specific phase of different disorders (19): both targeted and untargeted proteomic strategies have been used to study the evolution of immune dysregulatory conditions such as T1D and ulcerative colitis (10,11), but no studies are available in the context of celiac disease and potential celiac disease.

We thus decided to analyse a set of 92 serum proteins known to be involved in the pathway of inflammasome with the aim of discriminating since the time of diagnosis the subgroup of PCD

patients that will evolve to full blown disease. Indeed, this analysis appeared to give a robust reinforcement to the prediction of those who are most likely to develop CD and have to start immediately a GFD.

The multivariate linear combination of the serum concentration of Il2, SIRT2, LIF, CST5, IL10RA, CDCP1, IL14 provides a predictive ability to classify patients in those who will develop flat mucosa (CEL) or those who remain potential (POT) with a > 95% accuracy, either in the original cohort as well as in the jack-knife cross-validated cohort.

Alternative methods of analysis (ex. Volcano Plot, Partial Least Square Discriminant Analysis, Random Forest, Linear Discriminant Analysis) do converge in the same selection reinforcing the robustness of the prediction. It should be considered that these proteins are not independent from each other, hence each multivariate model may easily accept a replacement of a single protein with another which is in some way correlated to the metabolic process underlying the inflammatory status of the individual: hence we expected variations in the list of predictive biomarkers, but the repeated analysis with different methods confirmed the selection obtained by the Linear Discriminant Analysis.

Besides the great clinical opportunity that the study provides allowing to precisely differentiate since diagnoses, PCD children that will develop intestinal damage in future and to avoid in patients a continues gluten exposure, it also highlights how specific changes in abundance of inflammatory proteins in circulation associate with small intestinal damage and suggests interesting insights into the pathogenies of the development of mucosal lesions.

Pathways analysis performed by the full set of the 10 selected biomarkers showed the early activation of the gluten-induced immune response, with production of cytokines as well as of proteins involved in the initial recruitment of leucocytes that eventually infiltrate the small intestinal mucosa. Gene Ontology suggested a significant enrichment in the Positive Regulation of Tyrosine Phosphorylation, Leucocyte Differentiation and isotype switching to IgG isotypes; the regulation of protein phosphorylation is also involved. The biological process of gluten-induced inflammation is then already on stage, including the initial phases of Leucocytes differentiation to invade the small intestinal mucosa. KEGG pathway suggests the relevance of the involvement of the JAK-STAT pathway in inflammation, while the Molecular Function prioritize the cytokine activity. Finally, Signalling by interleukins is suggested by the Reactome Pathway (Supplementary Figure 4)

7/10 biomarkers are connected significantly within a network of immune response, cytokine activation and inflammation, while for 3/10 (CST5, CDCP1 and SIRT2) String could not find a

relation with the other proteins. Interestingly, CST5 and SIRT2 are strongly related to LIF only in children who develop flat mucosa (CEL). This is an area to be explored, since these proteins might be specifically implicated in the gluten induced process preparing the mucosal damage.

Among the proteins found to be differentially expressed between POT and CEL,) Cystatin D (CST5) is a lysosomal proteases and a candidate tumor suppressor gene 20. It was also reported to be an ultra-early inflammatory biomarker of traumatic brain injury (ZXS). Proteases belonging to the same family have been reported to be involved in intestinal permeability (21).

These data, together with the fact that CST 5 is expressed also at the level of the intestine (22), suggest that barrier alterations appears very early in Potential patients that will develop VA.

It has been suggested that CUB domains play essential roles in developmental processes such as embryogenesis and organogenesis (23). Interestingly members of the CUB domain proteins are described as "multifunctional epithelial receptors" and at the level of the intestine play a nodal role for the absorption on vitamins, iron and lipids. It is also one of the inflammation-related proteins associated with T cell immune recovery during chronic HIV-1 infection (RSZ). CDCP1 is a robust serological biomarker for risk stratification of NASH in obese individual (DXS).

Sirtuin 2 (SIRT2) contributes to the regulation of intestinal cell proliferation and differentiation (DSXZ). It is decreased in intestinal tissues from IBD patients where it can regulate WNT-β-catenin signaling. Knockout of SIRT2 alleviates ileal injury via enhanced autophagy under cold exposure (XZQ) (24).

Leukemia Inhibitory Factor (LIF) is a member of the IL-6 cytokine family and is expressed in almost every tissue type within the body (25). Although LIF was named for its ability to induce differentiation of myeloid leukemia cells, studies of LIF in additional diseases and solid tumor types have shown that it has the potential to contribute to many other pathologies. LIF expression has been linked to proliferation, metastasis, and chemoresistance. The mechanisms behind these effects of LIF are not well understood and can differ between different tissue types.

LIF exerts a crucial regulatory role in immunity and functions as a protective factor against many immunopathological diseases, such as infection, inflammatory bowel disease (IBD), and graft-verse-host disease (GVHD) (RTE) (26). LIF is essential for intestinal stem cells ISC function and protects against radiation-induced gastrointestinal syndrome.

In intestinal lamina propria lymphocytes (LPLs), STAT4 activation by LIF blocks STAT3 dependent Il17a/Il17f promoter activation, whereas in IECs, LIF bypasses the extraordinarily low level of

STAT4 to induce YAP gene expression via STAT3 activation (27). Thus, LIF effectively inhibits Th17 accumulation and promotes repair of damaged intestinal epithelium in inflamed colon. Due to this activity it has been regarded as a potential target for therapy in IBD.

In conclusion the healthy potential celiac children who will develop mucosal damage over time (CEL), compared to those who will still have a normal small intestinal mucosa after a long term follow up (POT), do show since diagnosis the full machinery for the progression of mucosal damage: they, as the others, do recognize the antigen and produce anti-tTG antibodies, but show also in their sera (which may reflect only 5% of their immunological processes) an increased expression of inflammatory molecules, including the activation of lymphocyte infiltration in the mucosa, Jak-Stat signalling and proliferation of intestinal steam cells. Here, we provide a comprehensive characterization of the inflammatory profile by analysing plasma samples from individuals who developed VA and identify a protein signature mainly consisting of CDCP1, LIF, IL10-RA, IL2, CST5, SIRT2, IL4.

The different expression of these proteins is able to differentiate PCD children that will develop VA with an accuracy higher than 95% and appears thus to be sufficient to suggest the avoidance of the almost certain evolution to intestinal damage by starting a GFD at time of diagnosis.

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Protein	Discriminant Analysis						
	Wilk's lambda^	Variance Ratio F	p	**Fold Changes**	**t-test p**	**PLS-DA (f1)***	**Random Forest ****
CDCP1	,565	22,351	,000	1,23	0,071	1,32	0,0124
IL2	,383	22,581	,000	1,32	0,006	1,12	0,0067
LIF	,346	17,018	,000	1,35	0,025	0,81	0,0047
IL-10RA	,323	13,617	,000	1,3		0,88	0,0053
SIRT2	,302	11,541	,000	1,53	0,013	2,5	0,0016
CST5	,272	10,689	,000	1,25	0,036	1,91	0,0039
IL4	,256	9,537	,000	1,76	0,08	1,57	0,00021

Table 1. Wilk's Lambda estimate the cumulative capacity of adding each variable to the first most effective in increasing the discrimination between children who develop fat mucosa (CEL) and those who remain potential (POT) ranging from1 = total overlap to 0 total distance.

* Explained variance by PLSDA factor 1

** Mean Decrease in accuracy

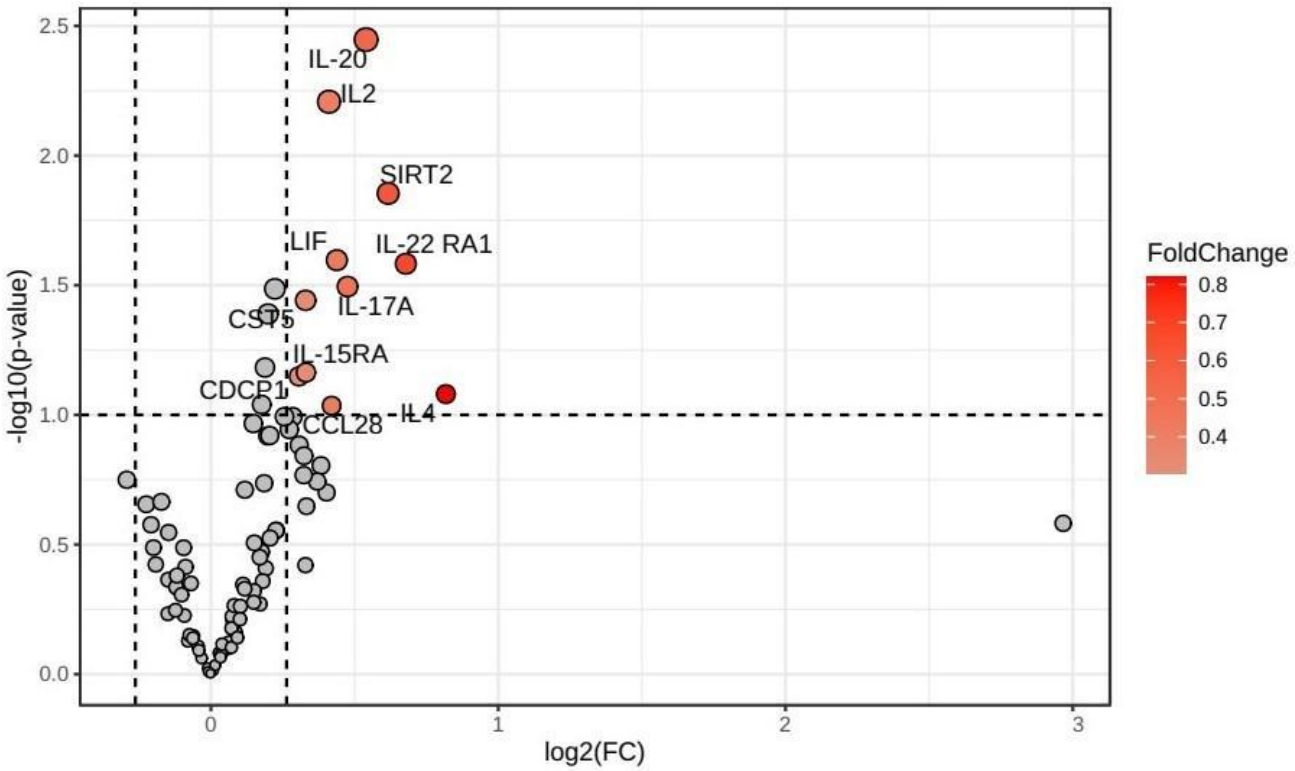


Figure 1. VOLCANO PLOT: Fold Changes (% CEL increments over POT) by Student T-test p value. Important features selected by volcano plot with fold change threshold (x) 1.2 and t-tests threshold (y) 0.1. The red circles represent features above the threshold. Note both fold changes and p values are log transformed. The further its position away from the (0,0), the more significant the feature is.

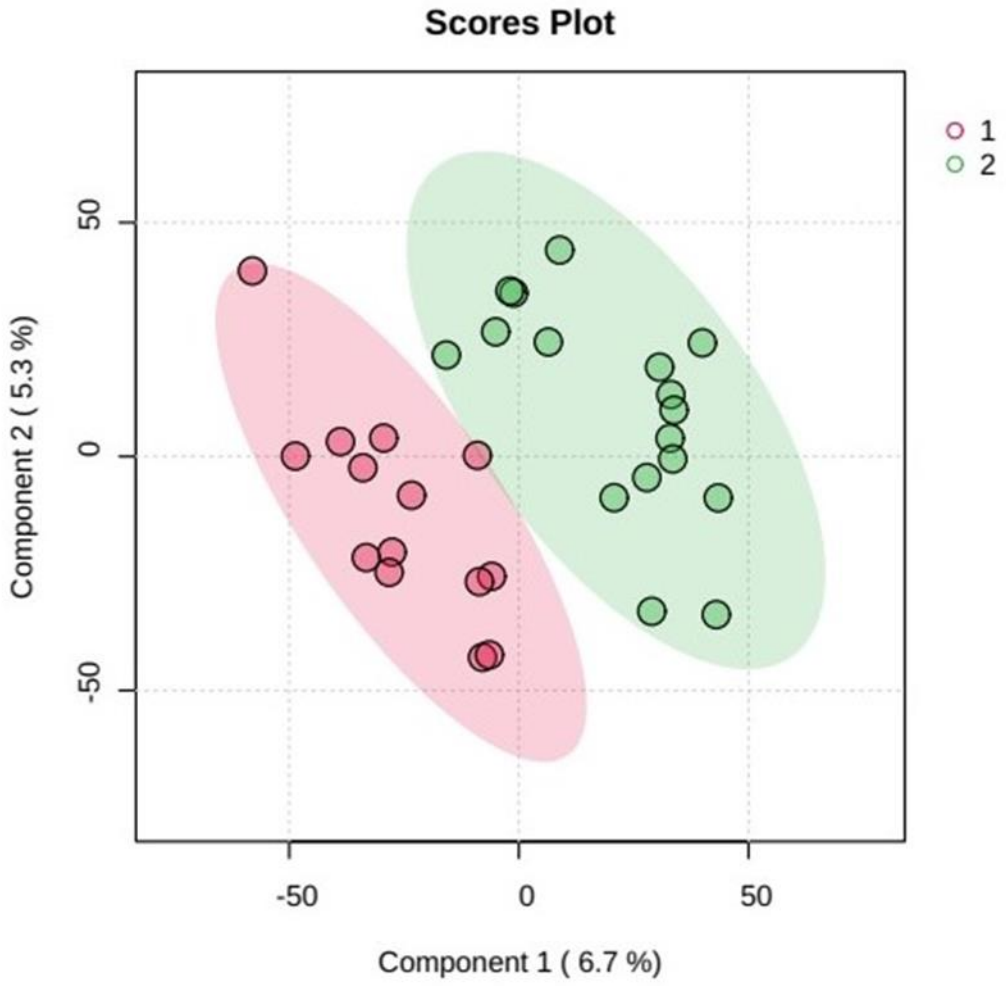


Figure 2. Partial Least Square Discriminant Analysis. Scores plot between the selected POT and CEL; 1 Pink: PO, 2 Green: CEL

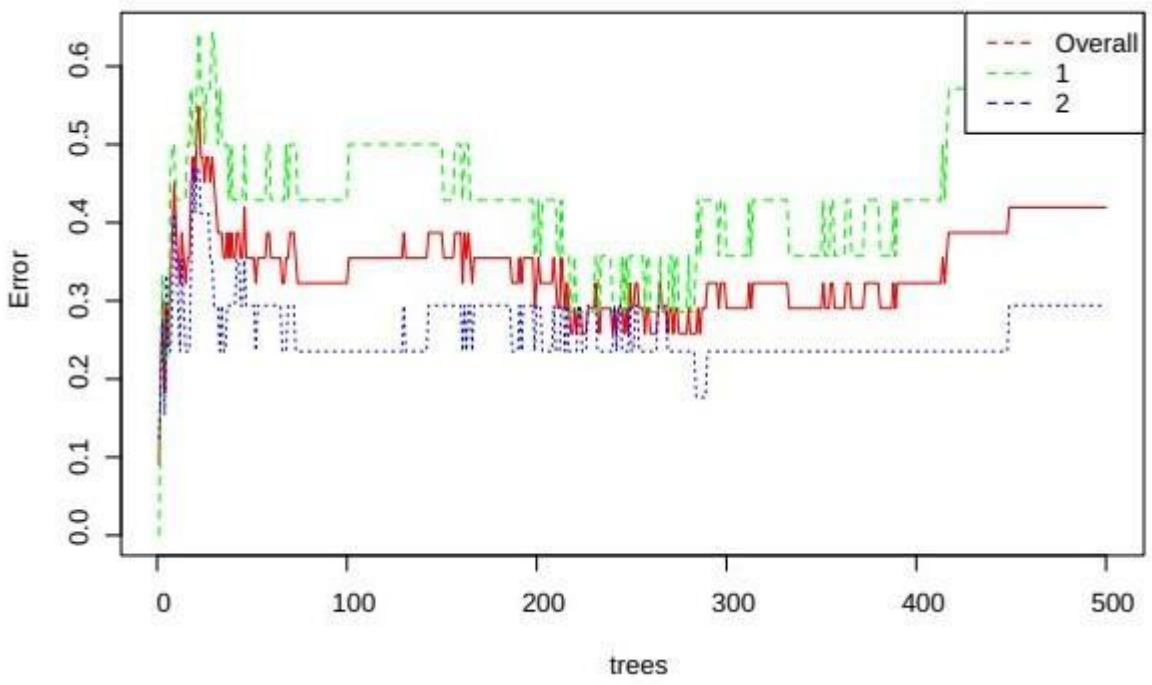


Figure 3. Cumulative error rates by Random Forest Classification. The overall error rate is shown as the black line: the red and green lines represent the error rates for each class. 1 Green: CEL, 2 Blue: POT

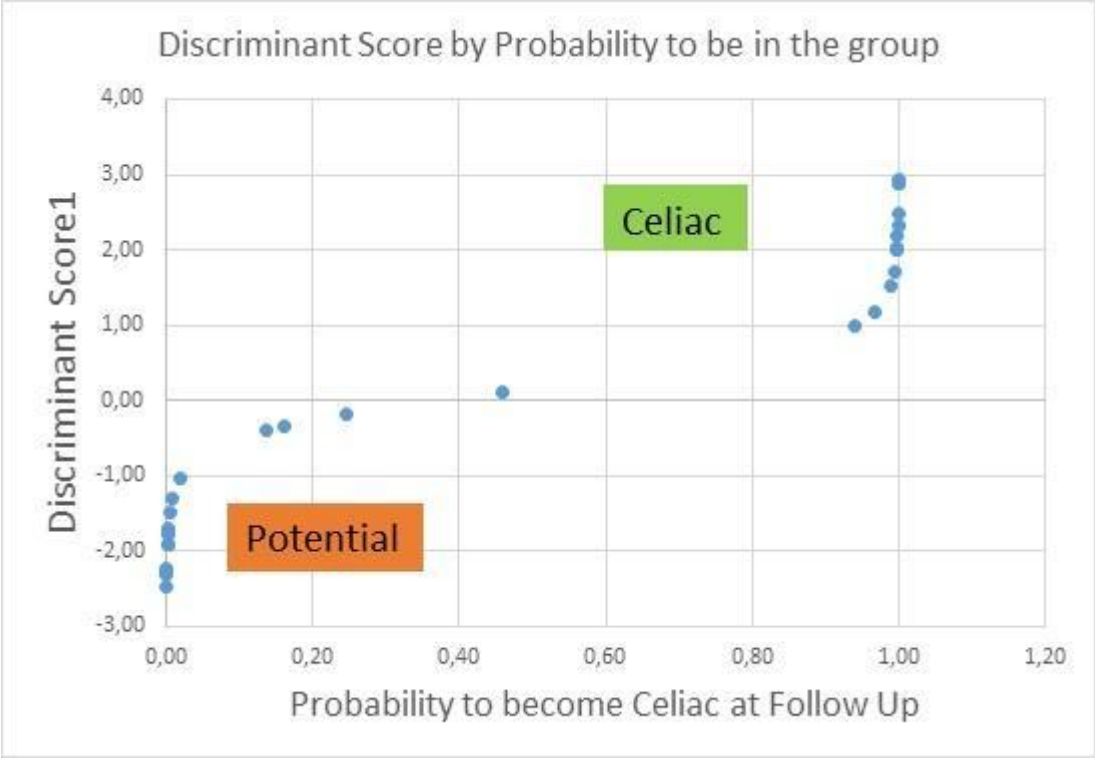


Figure 4. Probability (y axis) to be either in the POT or the CEL group by the Discriminant Score of each individual (x axis).

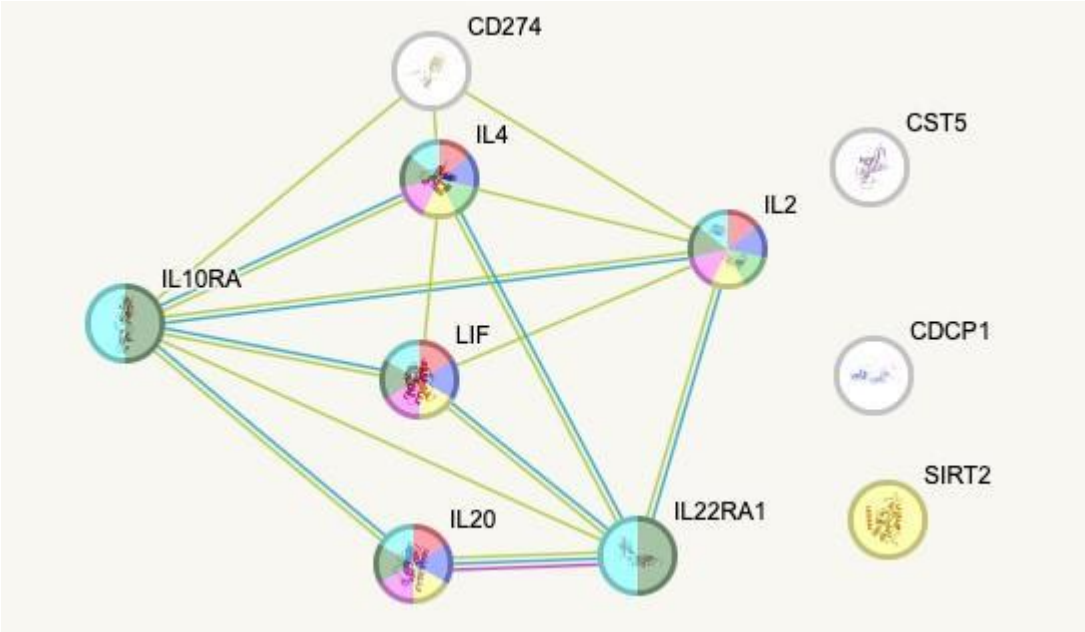


Figure 5. STRING network of the selected Biomarkers

SUPPL270124

Supplementary Table 1 a. LINEAR DISCRIMINANT ANALYSIS

Step	Protein	Wilk's Lambda	Variance Ratio F	p
1	CDCP1	,565	22,351	,000
2	IL2	,383	22,581	,000
3	LIF	,346	17,018	,000
4	IL-10RA	,323	13,617	,000
5	SIRT2	,302	11,541	,000
6	CST5	,272	10,689	,000
7	IL4	,256	9,537	,000

At each step the protein that minimize Wilk's lambda is added

Supplementary Table 1 b. Classification results

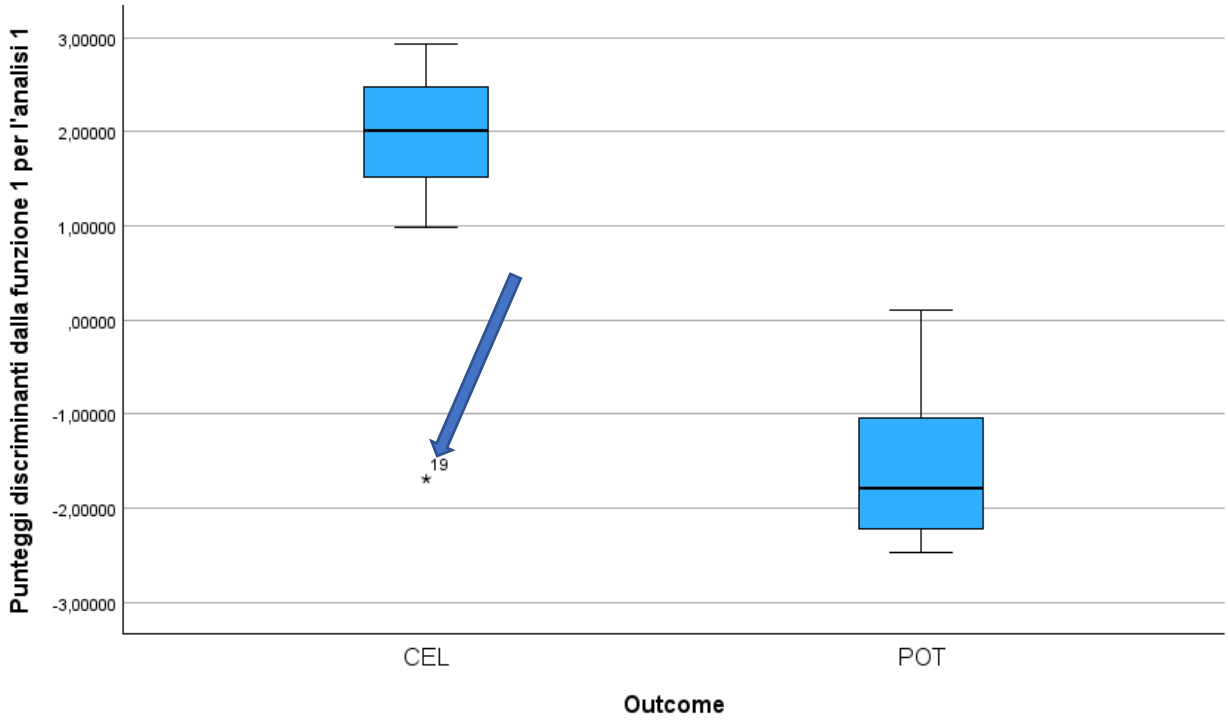
Classification results					
			Predicted Group		
		Outcome	CEL	POT	Total
Original group[a]	n	CEL	13	1	14
		POT	0	17	17
	%[a]	CEL	92,9	7,1	100,0
		POT	,0	100,0	100,0
Cross Validated[b]	n	CEL	13	1	14
		POT	2	15	17
	%[c]	CEL	92,9	7,1	100,0
		POT	11,8	88,2	100,0

a. 96,8% correctly classified

b. Cross-validation is performed only for the cases in the analysis. In cross-validation, each case is classified based on the features derived from all other cases except that one

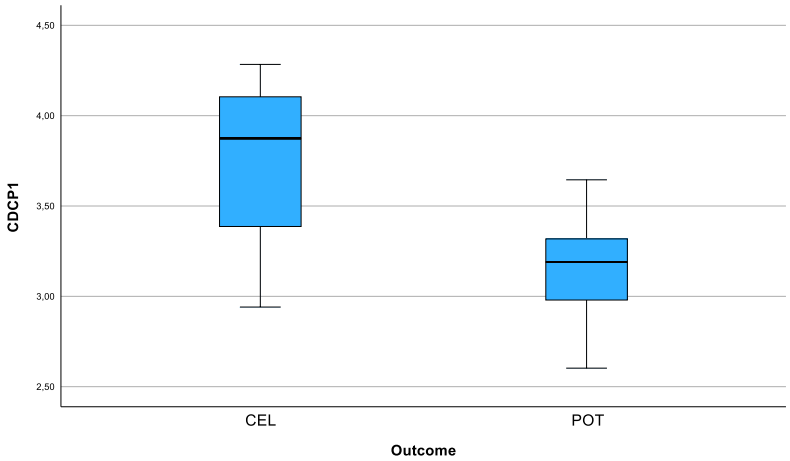
c. 90,3% correctly classified

Supplementary Figure 1. Distribution of the Discriminant Scores of cases predicted as CEL or POT

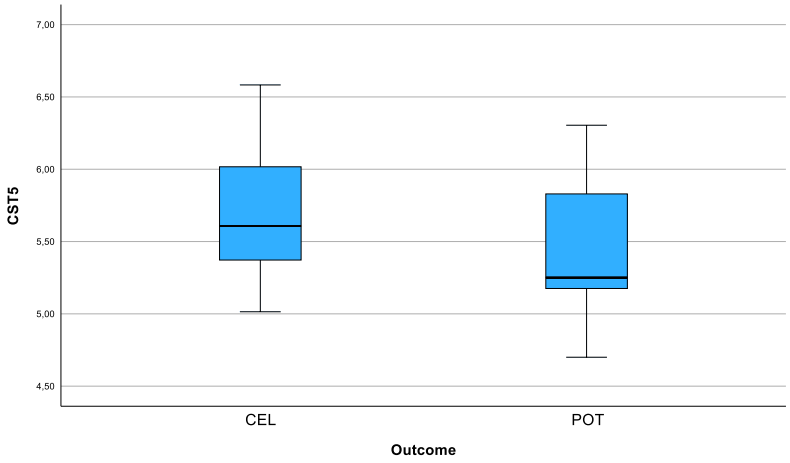


Supplementary Figure 2. BOX PLOT OF THE SELECTED PROTEINS

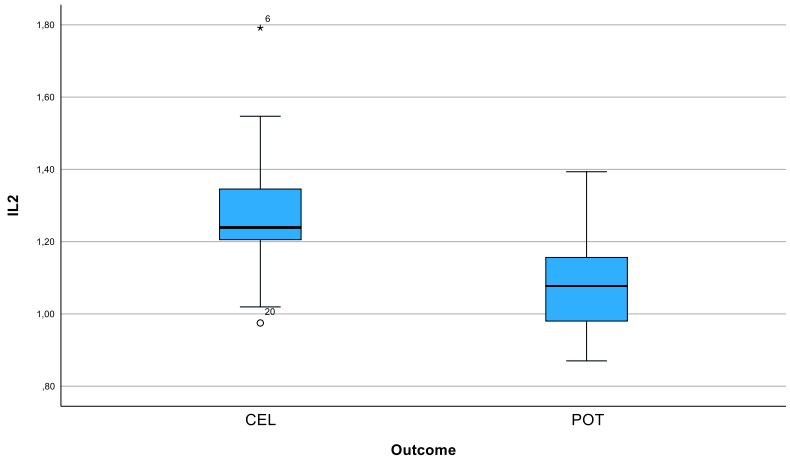
CDCP1



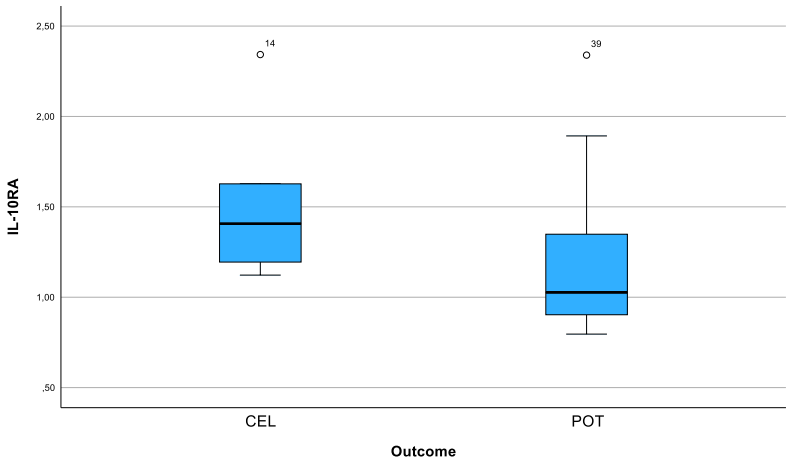
CST5


7,00
6,50
6,00
5,50
5,00
4,50
CST5
CEL
POT
Outcome

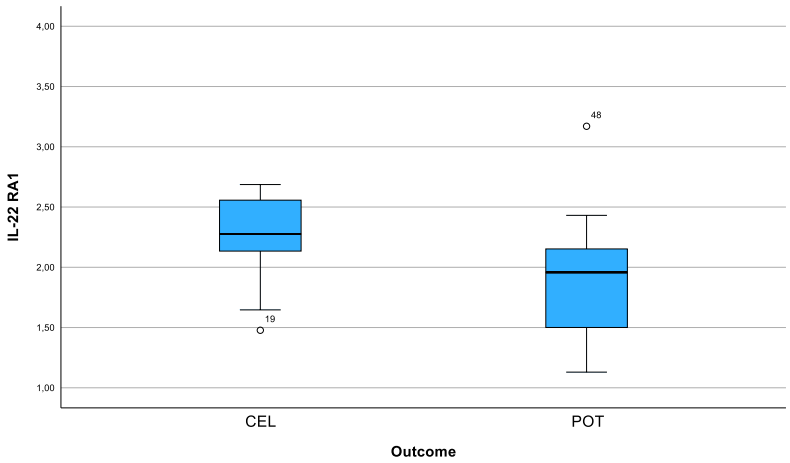
IL2


1,80
1,60
1,40
1,20
1,00
,80
6
20
IL2
CEL
POT
Outcome

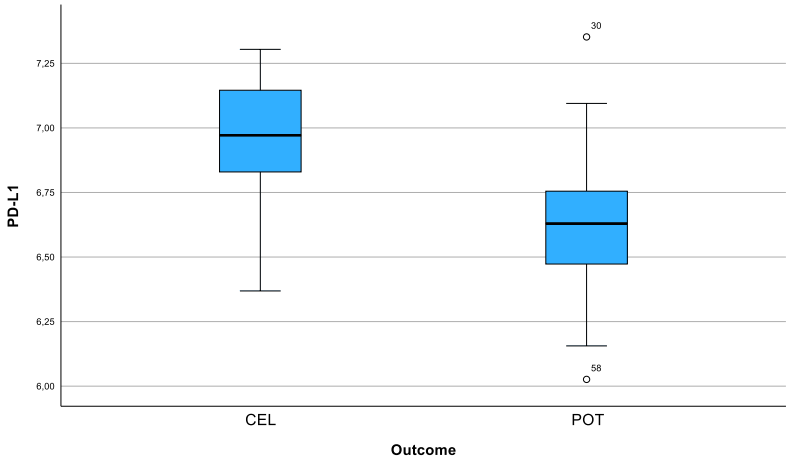
IL-10RA



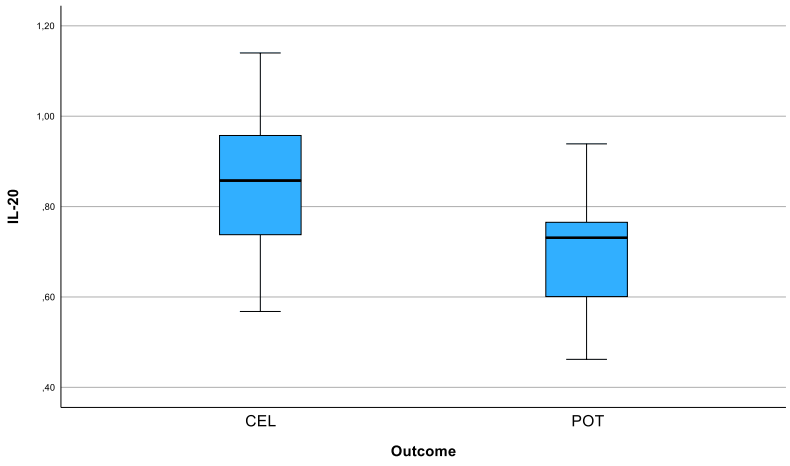
IL-22 RA1



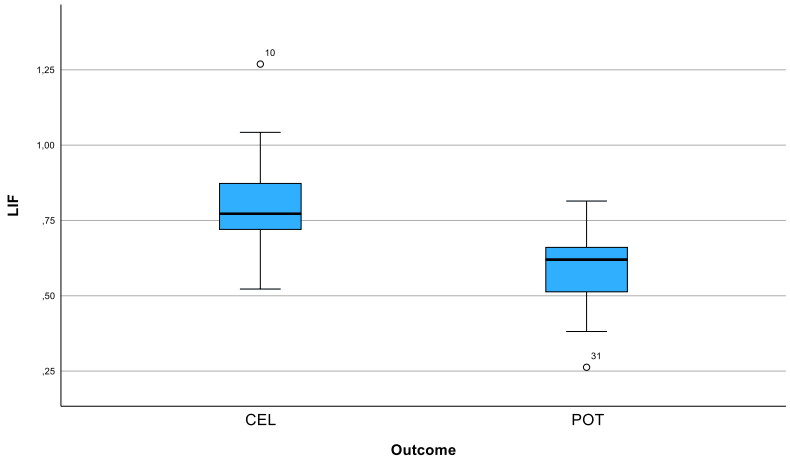
PD-L1


PD-L1
7,25
7,00
6,75
6,50
6,25
6,00
30
58
CEL
POT
Outcome

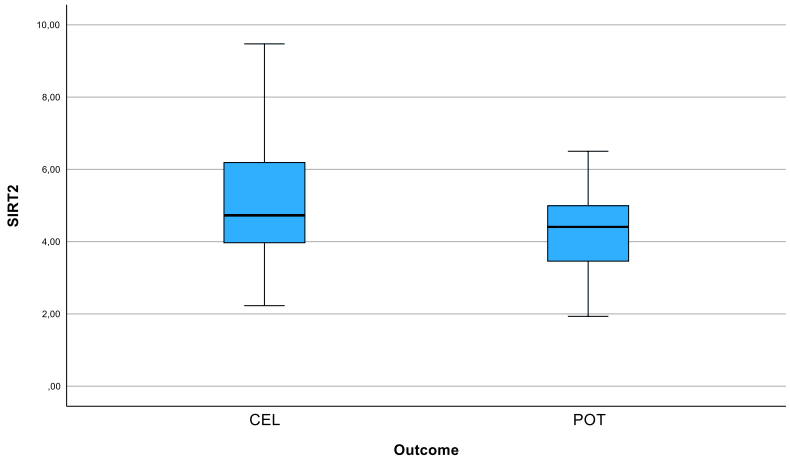
IL-20


IL-20
1,20
1,00
,80
,60
,40
CEL
POT
Outcome

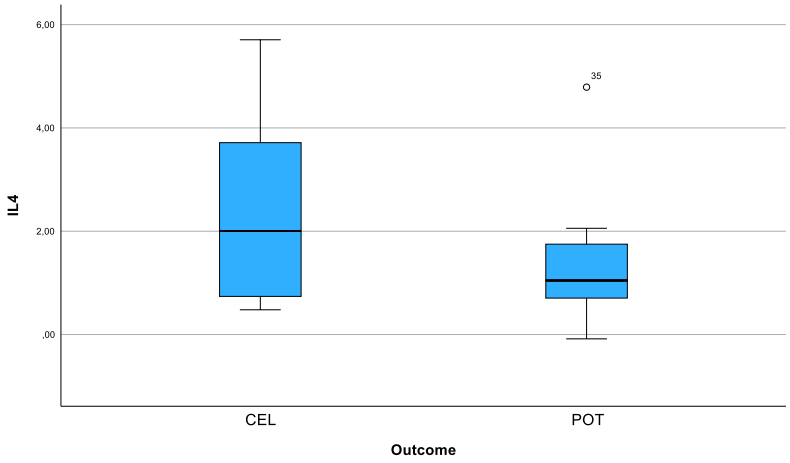
LIF


10
1,25
1,00
,75
,50
,25
LIF
31
CEL
POT
Outcome

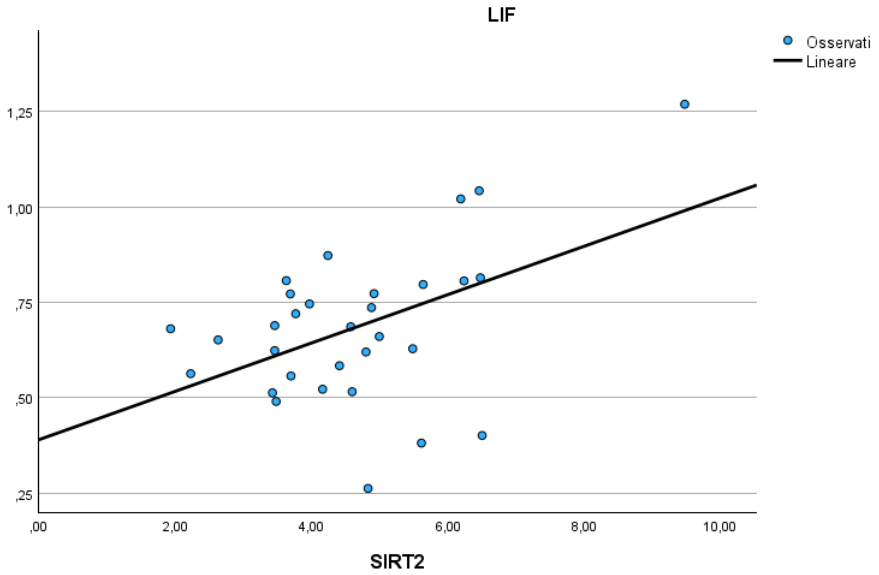
SIRT2


10,00
8,00
6,00
4,00
2,00
,00
SIRT2
CEL
POT
Outcome

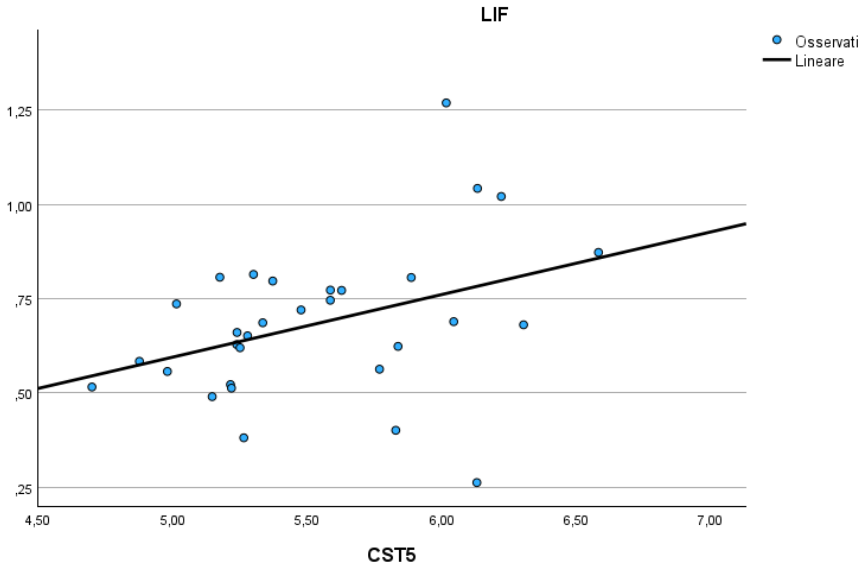
IL4



Supplementary Figure 3. CORRELATION BETWEEN LIF AND SIRT2 AND CST5 ONLY CELIAC AT ENROLMENT



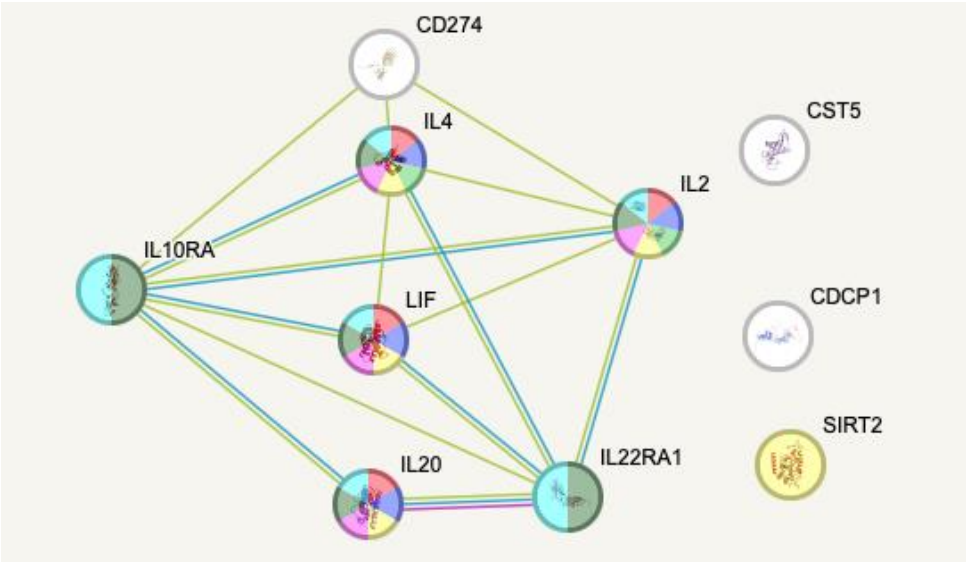
R = 0,613 p = 0,020 R^2 = 0,376



R =0,867 p = < 0,001 R^2 = 0,752

Supplementary Figure 4 PATHWAY ANALISIS BY STRING. Gene Ontology analysis restricting the output to BP and MF = Molecular Function (A), and KEGG (B) and REACTOME pathways analysis (C) on 10 proteins.

The Enrichment Score for each BP and MF term is plotted (A). The gene count for each KEGG (B) and REACTOME (C) pathway is plotted, the first pathways is the most significant (lower FDR value, refer to Supplementary Table).



Functional enrichments in your network

explain columns

	Biological Process (Gene Ontology)			
GO-term	*description*	*count in network*	*strength*	*false discovery rate*
GO:0042531	Positive regulation of tyrosine phosphorylation of STAT prot...	4 of 65	2.08	0.00045
GO:2000515	Negative regulation of CD4-positive, alpha-beta T cell activat...	3 of 36	2.22	0.0045
GO:1902107	Positive regulation of leukocyte differentiation	4 of 181	1.64	0.0059
GO:0007166	Cell surface receptor signaling pathway	7 of 2040	0.83	0.0183
GO:0048304	Positive regulation of isotype switching to IgG isotypes	2 of 10	2.6	0.0199
GO:2000320	Negative regulation of T-helper 17 cell differentiation	2 of 11	2.55	0.0217
GO:0007260	Tyrosine phosphorylation of STAT protein	2 of 13	2.48	0.0227
GO:0042102	Positive regulation of T cell proliferation	3 of 108	1.74	0.0227
GO:0002824	Positive regulation of adaptive immune response based on ...	3 of 110	1.73	0.0227
GO:0002761	Regulation of myeloid leukocyte differentiation	3 of 123	1.68	0.0227
GO:0002521	Leukocyte differentiation	4 of 396	1.3	0.0227
GO:0002573	Myeloid leukocyte differentiation	3 of 134	1.64	0.0238
GO:0002684	Positive regulation of immune system process	5 of 874	1.05	0.0238
GO:0045597	Positive regulation of cell differentiation	5 of 876	1.05	0.0238
GO:2000561	Regulation of CD4-positive, alpha-beta T cell proliferation	2 of 24	2.22	0.0322
GO:0045785	Positive regulation of cell adhesion	4 of 485	1.21	0.0322
GO:0018108	Peptidyl-tyrosine phosphorylation	3 of 176	1.53	0.0348
GO:0001932	Regulation of protein phosphorylation	5 of 1108	0.95	0.0399
GO:0040020	Regulation of meiotic nuclear division	2 of 32	2.09	0.0412
GO:0097192	Extrinsic apoptotic signaling pathway in absence of ligand	2 of 36	2.04	0.0487

Enrichment Table Columns

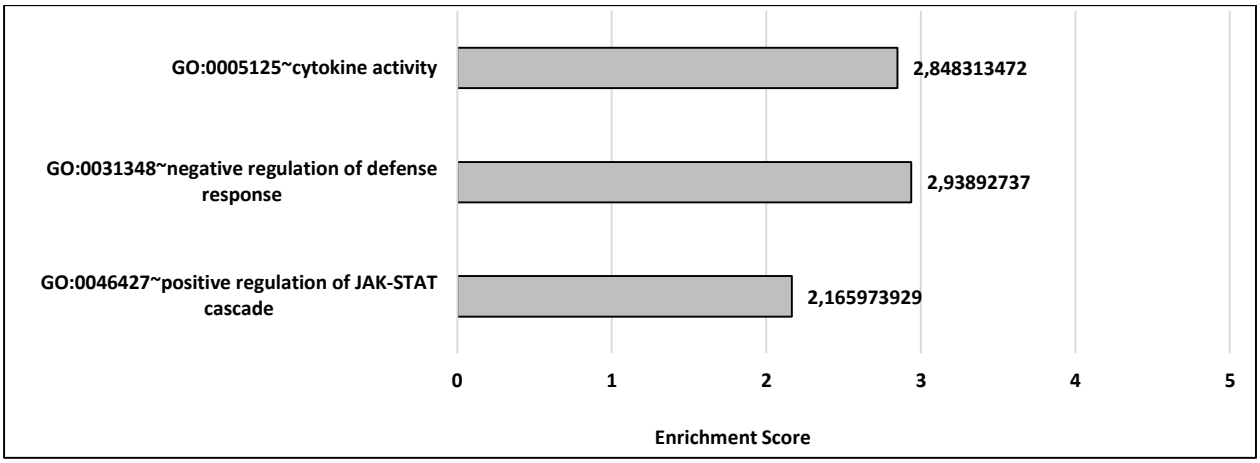
Count In Network:
The first number indicates how many proteins in your network are annotated with a particular term. The second number indicates how many proteins in total (in your network and in the background) have this term assigned. You can click on the numbers to see the network view of the gene sets behind them.

Strength:
Log10(observed / expected). This measure describes how large the enrichment effect is. It's the ratio between i) the number of proteins in your network that are annotated with a term and ii) the number of proteins that we expect to be annotated with this term in a random network of the same size.

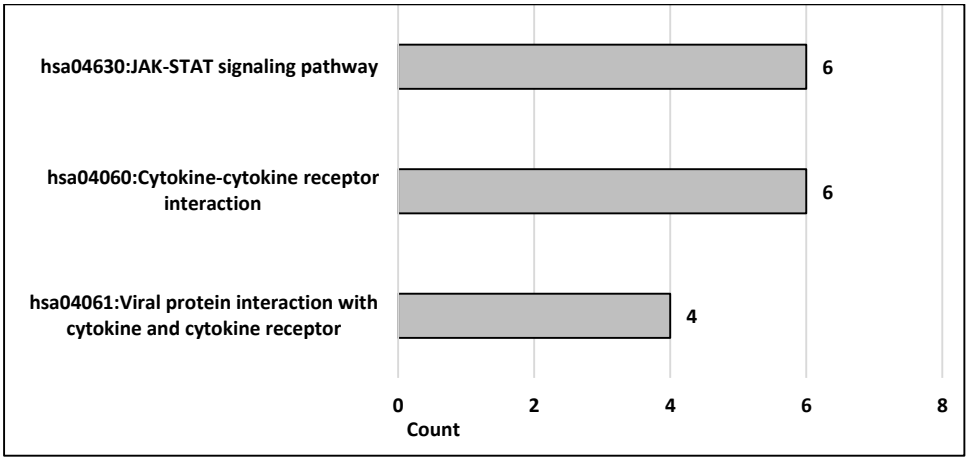
False Discovery Rate:
This measure describes how significant the enrichment is. Shown are p-values corrected for multiple testing within each category using the Benjamini–Hochberg procedure.

	KEGG Pathways			
pathway	*description*	*count in network*	*strength*	*false discovery rate*
hsa04630	JAK-STAT signaling pathway	6 of 158	1.87	2.08e-08
hsa04060	Cytokine-cytokine receptor interaction	6 of 282	1.62	3.10e-07
hsa04061	Viral protein interaction with cytokine and cytokine receptor	4 of 96	1.91	1.43e-05
hsa05330	Allograft rejection	2 of 34	2.06	0.0121
hsa04672	Intestinal immune network for IgA production	2 of 43	1.96	0.0152
hsa05320	Autoimmune thyroid disease	2 of 48	1.91	0.0157
hsa05321	Inflammatory bowel disease	2 of 59	1.82	0.0200
hsa04658	Th1 and Th2 cell differentiation	2 of 85	1.67	0.0356
hsa04659	Th17 cell differentiation	2 of 99	1.6	0.0425
hsa04660	T cell receptor signaling pathway	2 of 100	1.6	0.0425

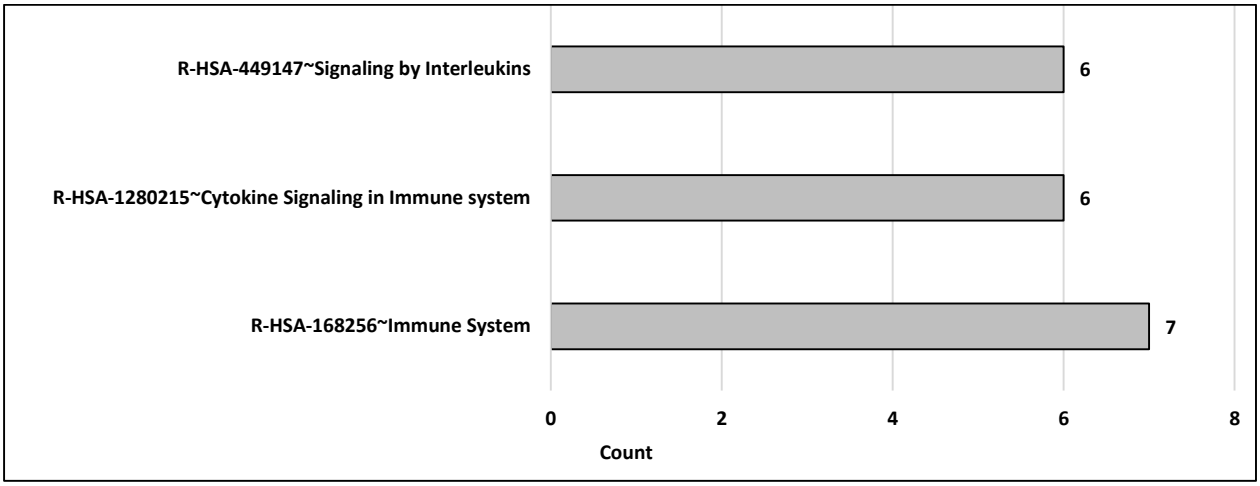
A)


GO:0005125~cytokine activity
2,848313472
GO:0031348~negative regulation of defense response
2,93892737
GO:0046427~positive regulation of JAK-STAT cascade
2,165973929
0
1
2
3
4
5
Enrichment Score

B)


hsa04630:JAK-STAT signaling pathway
6
hsa04060:Cytokine-cytokine receptor interaction
6
hsa04061:Viral protein interaction with cytokine and cytokine receptor
4
0
2
4
6
8
Count

C)


R-HSA-449147~Signaling by Interleukins
6
R-HSA-1280215~Cytokine Signaling in Immune system
6
R-HSA-168256~Immune System
7
0
2
4
6
8
Count

Chapter 6. Other considerations on the relationship between celiac disease and intestinal histology

6.1 Mucosal healing after a long-term gluten free diet in CD

This work has been published as:

Mandile R, Maglio M, Mosca C, Marano A, Discepolo V, Troncone R, Auricchio R. Mucosal Healing in Celiac Disease: Villous Architecture and Immunohistochemical Features in Children on a Long-Term Gluten Free Diet. Nutrients. 2022 Sep 7;14(18):3696. doi: 10.3390/nu14183696. PMID: 36145072; PMCID: PMC9504881.

1. INTRODUCTION

Celiac disease (CD) is an immune-mediated systemic disorder elicited by gluten and related prolamins in genetically susceptible individuals (1). Its incidence is progressively increasing in adult and pediatric populations, due to both an improvement in diagnostic accuracy and a real increase in the incidence (2). Nowadays, the only available treatment is represented by a strict adherence to a lifelong gluten free diet (GFD), which is the only way to prevent short- and long-term complications of untreated CD (3). In fact, severe mucosal damage with villous atrophy correlates with the development of future complications and complete mucosal recovery is essential to guarantee a good prognosis (4).

There is considerable heterogeneity across studies assessing the completeness of mucosal recovery achieved by a GFD in CD (5,6,7,8,9). Some celiac patients are reported to fail to achieve complete mucosal recovery even on a strict dietary regimen, despite this issue seeming to be much more relevant in adult than in pediatric populations (10). To this regard it must be noted that the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) recently recommended against routinely repeating biopsies in children on a GFD (11). Moreover, the "definition" of mucosal recovery remains hard to establish. Historically, mucosal damage was defined as an alteration of normal intestinal architecture, with shortened or even absent villi, hyperplastic crypts, and an increased number of intraepithelial lymphocytes (IELs) upon histological evaluation (12). Now, more sophisticated techniques, such as flow cytometry and immunohistochemistry have allowed a more extensive evaluation of mucosal damage in CD through the characterization of single types of cells infiltrating both the epithelium and lamina propria (13). Indeed, a damaged intestinal

mucosa in CD is characterized in the epithelium by an increase in ILEs expressing the CD3 surface marker, with around 10% of them also expressing the γδ receptor, and in the lamina propria by an increase in inflammatory mononuclear cells that express the CD25 surface marker (14). These cells tend to decrease on a GFD, but many studies suggest that some stigmata of the disorder remain despite clinical and serological remission (15,16,17,18). In addition, it remains to be established whether these subtle alterations may influence the patient's prognosis.

In the present study, we aim to investigate histological and immunohistochemical features in CD patients on a long-term GFD and to correlate them to the duration of the GFD.

2. METHODS

2.1. Patients and Study Design

This retrospective study was carried out at the University Federico II of Naples, a tertiary care centre and the reference centre for the diagnosis and management of CD in Campania, Italy. The study group (GFD group) included children with a diagnosis of CD on a long-term GFD (inclusion criteria: more than 2 years, mean duration time 8 years, range 1.9–18 years). The adherence to a correct dietary regimen was assessed both by CD specific serology and by an expert nutritionist performing a detailed nutritional interview. We selected two control groups: CD patients with villous atrophy at diagnosis (ACD group), and healthy subjects with normal small intestinal mucosa architecture (CTR group). This latter group included subjects with negative CD serology and a diagnosis of one of the following clinical conditions: eosinophilic esophagitis, type 1 diabetes, functional gastrointestinal disorders, and first-degree relatives of CD patients. Patients with inflammatory bowel diseases or other inflammatory conditions of the lower gastro-intestinal tract were excluded. Patients received a duodenal biopsy during an esophagogastroduodenoscopy (EGDS) performed for diagnostic purposes, after giving their informed consent. The demographic and clinical features of the patients are summarized in Table 1.

Table 1. Clinical features of enrolled patients.

2.2. Histological Evaluation

In each patient, EGDS with 5 biopsies, 1 from the bulb and 4 from the distal duodenum, was carried out. According to our protocol, 4 of 5 fragments, including 1 from the bulb, were fixed in 10% formalin, embedded in paraffin, sectioned at a 5 μm thickness and then stained with

hematoxylin/eosin. Histological and morphometrical analyses with measurement of villi and crypts by light microscopy were performed by two experienced pathologists. A villous height:crypt depth ratio equal or higher than 2 was considered normal, as stated in latest ESPGHAN guidelines (1). Among biopsies with a normal villous height:crypt depth ratio, Marsh 0 was defined by the presence of less than 25 intraepithelial lymphocytes (IELs) per 100 enterocytes, and Marsh 1 by the presence of more than 25 IELs per 100 enterocytes. The Marsh score was given based on the score of the fragment with the worst picture. The pathologists were blinded to the serology results.

2.3. Immunohistochemical Evaluation

One fragment (not from the bulb) was added to an optimal cutting temperature compound (Killik; BioOptica, Milan, Italy), stored at −80 °C, and used for immunohistochemical staining for CD3+, TCRγδ+, and CD25+ cells, as previously reported (19). The number of stained cells per millimeter of epithelium determined the density of the cells expressing CD3 and TCRγδ in the intraepithelial compartment. The cut-off values for CD3+ and TCRγδ+ cells were 34 mm and 3.4 mm per epithelium, respectively. On the other hand, the number of cells expressing CD25 in the lamina propria was evaluated within a total area of 1 mm2. The cut-off value for CD25+ cells was 4 per mm2 of the lamina propria. To determine the cut-off values, 100 children with untreated celiac disease and 50 non–celiac disease control children were studied. The percentiles were obtained using the Statistical Package for the Social Sciences SPSS software (IBM, Chicago, IL, USA). The cut-off values represented the 90th percentile of control patients (19).

2.4. Statistical Analysis

All variables were chosen before the analyses because of their possible relevance for the study aims. Quantitative data were expressed using means with 95% confidence intervals. When comparing means, an independent-sample t test with 2-tailed significance was used in normally distributed variables, and the Mann-Whitney U test with nonparametric variables. The Chi-square $\chi 2$ test or Fisher's exact test were used to test differences between categorical parametric and non-parametric variables, respectively. To test correlation, the Spearman rank-order was used.

3. RESULTS

3.1. Patients

The study group included 38 CD subjects in clinical remission and already on a strict GFD (mean duration time 8 years, range 1.9–18 years), as assessed by nutritional interview performed by an

expert dietician. The single most frequent reason (in 33/38 patients) to obtain a biopsy from patients on the GFD was the need to have a pre-challenge biopsy in the context of clinical studies. After the gluten challenge, all patients involved in the study eventually relapsed. These patients had a negative CD-associated serology and did not complain of any symptoms. Only 4/38 patients repeated a biopsy because of the persistence of a positive CD serology (EMA and/or anti-TG2) despite the nutritional interview assessing good adherence to the correct dietary regimen and these patients also being completely asymptomatic. In 1/38 patient, an upper endoscopy with biopsies was performed because of suspected gastroesophageal reflux disease. For the control groups, we selected both patients with a negative CD associated serology (N = 44) and CD patients at diagnosis (N = 31). There were no significant differences between the three groups in the sex distribution or other demographic features (Table 1).

	GFD Group	ACD Group	CTR Group
Number	28	31	43
Sex (female/male)	16/12	20/11	20/23
Age at diagnosis Mean yrs (range)	6.8 (0.5–17.4)	7.5 (2.1–15.5)	6.6 (0.8–17.2)
GFD duration Mean yrs (range)	8.6 (1.9–18)	-	-
Anti-TG2 Mean ULN value	0.67xULN	8.17xULN	0.1xULN

GFD: gluten-free diet; ACD: atrophic celiac disease; CTR: controls; anti-TG2: anti-transglutaminase antibodies; yrs: years; ULN: upper limit of normality.

3.2. Histological and Immunohistochemical Evaluations

In total, 23/38 duodenal samples from the GFD group were defined as a Marsh0 at the histological evaluation, while 15/38 were Marsh1. No villous atrophy was found in our cohort of patients, and the V/C ratio was always higher than 2 (Table 2).

Table 2. Histological and Immunohistochemical features.

	GFD Group	ACD Group	CTR Group
V/C	3.23	1.3	2.85
CD3+ cells/mm (mean ± SD)	37.0 ± 1.41	66.8 ± 36.99	26.4 ± 13.77
TCRγδ+ cells/mm (mean ± SD)	9.8 ± 0.98	20.3 ± 14.77	2.6 ± 5.59
CD25+ cells/mm^2 (mean ± SD)	8.9 ± 10.95	45.8± 36.03	7.3 ± 10.9

GFD: gluten-free diet; ACD: atrophic celiac disease; CTR: controls; V/C: villous/crypts ratio; SD: standard deviation.

The V/C ratio significantly improved after the GFD and completely normalized in all patients, becoming even higher than in the CTR group (median value 3.2 vs. 3, $p < 0.01$) (Figure 1A) though measurement of villus height and crypt depth did not show appreciable differences between the first (mean ± SEM: 333.5 ± 11 and 99.7 ± 3 µm, respectively) and the second (336.2 ± 7.5 and 107.7 ± 3.5 µm) group (Figure 1B). In parallel, densities of CD3+ and TCRγδ+ cells in the epithelium were significantly reduced in the GFD (37 ± 2.8 and 9.8 ± 1.1 cells/mm of epithelium, respectively) compared to the ACD group (66.8 ± 7 and 20.32 ± 2.9 cells; $p < 0.001$ and $p < 0.01$, respectively), even if it remained higher than in the CTR group (26.3 ± 1.8 and 2.6 ± 0.4 cells, respectively; $p < 0.01$ and $p < 0.0001$ respectively) (Figure 2A,B).

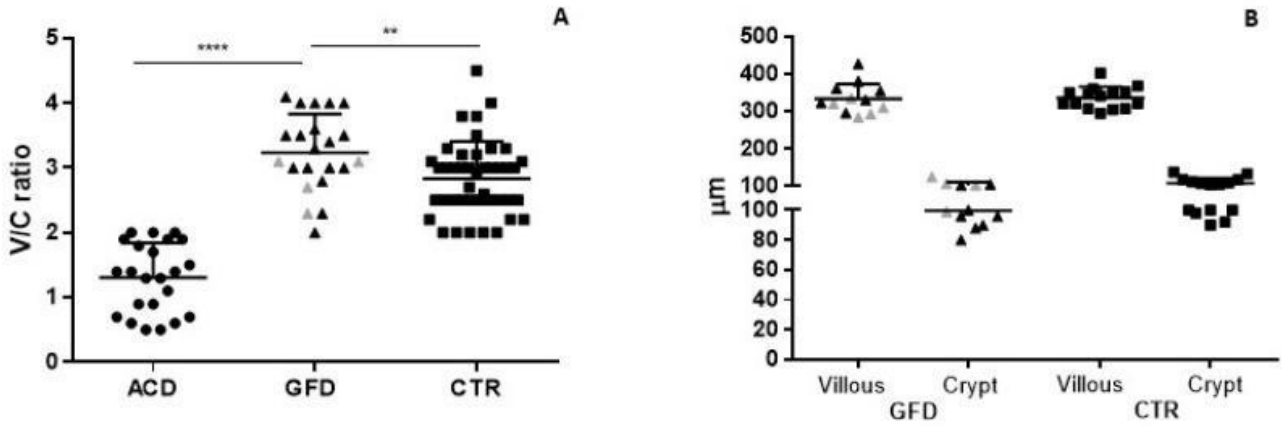


Figure 1. (A) Villous/crypts ratio in patients with villous atrophy, on a gluten free diet and in control patients. (B) Villi and crypts length in patients on a gluten free diet and in control patients. Grey dots represent patients on a gluten free diet with a persistently positive CD-serology. V/C: villous/crypts ratio; ACD: atrophic celiac disease; GFD: gluten free diet; CTR: controls. *: level of statistical significance; ** means $p < 0.005$, **** means $p < 0.00005$.

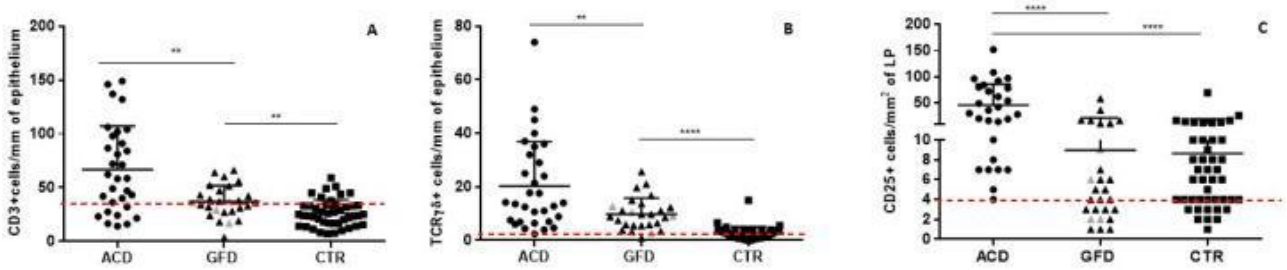


Figure 2. Immunohistochemical parameters in different patients group. (A) CD3+ cells/mm of intestinal epithelium. (B) TCRγδ+ cells/mm of intestinal epithelium. (C) CD25+ cells/mm2 of

intestinal lamina propria. Grey dots represent patients on a gluten free diet with a persistently positive CD-serology. Dashed horizontal line represents cut-off limits of normality for each immunohistochemical parameter. ACD: atrophic celiac disease; GFD: gluten free diet; CTR: controls. *: level of statistical significance; ** means $p < 0.005$, **** $p < 0.00005$.

In the study group there was not only a higher density of intraepithelial lymphocytes (IELs) but also a higher percentage of subjects with an increase in CD3+ (15/27, 56%; Figure 3A) and TCRγδ+ (23/27, 85%, Figure 3B).

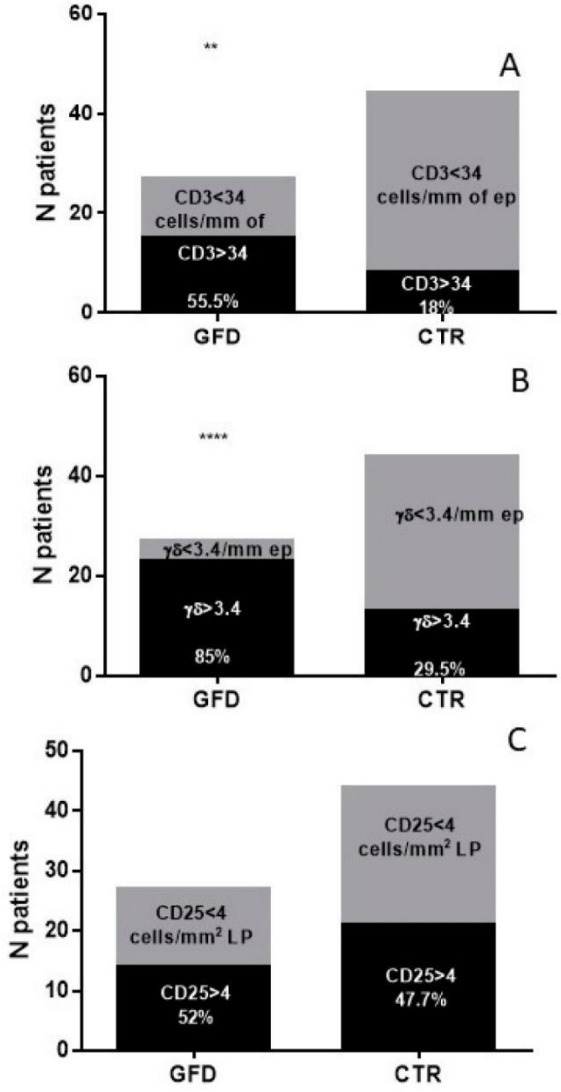


Figure 3. Percentage of patients with altered immunohistochemical parameters. (A) patients with altered CD3+ cells/mm of intestinal epithelium. (B) patients with altered TCRγδ+ cells/mm of intestinal epithelium. (C) patients with altered CD25+ cells/mm2 of intestinal lamina propria. N: number; GFD: gluten free diet; CTR: controls. *: level of statistical significance; ** means $p < 0.005$, **** $p < 0.00005$.

Regarding the IELs compared to the CTR group (8/44, 18%, p < 0.0.01; 13/44, 29%, p < 0.0001, respectively), of note is that the density of the TCRγδ+ IELs remained altered even after years of a gluten free diet (Figure 2B). In contrast, the number of CD25+ cells in the lamina propria were significantly reduced after following a GFD (8.9 ± 2.4 cells/mm2 of lamina propria) and became comparable to the CTR group (8.6 ± 1.6) (p = 0.9) (Figure 2C). The percentage of GFD patients showing increased CD25+ cells in the lamina propria was also comparable to the CTR subjects, being 52% (14/27) in GFD vs. 47.7% (21/44) in the CTR, (Figure 3C). In the GFD group, 7/27 (26%) patients showed all three immunohistochemical parameters (CD3+, TCRγδ+ IELs, CD25+) altered at the same time. Interestingly, no differences were observed in the immunohistochemical parameters between seropositive GFD and seronegative GFD patients (Figure 2). Furthermore, in seropositive children, the V/C ratio was always higher than 2, half of them (50%) showed an increased number of CD3+ and CD25+ cells, 75% had an increased number of TCRγδ+ IELs. No correlation was shown between the densities of the cells (CD3+, TCRγδ+ and CD25+) and GFD duration (Figure 4).

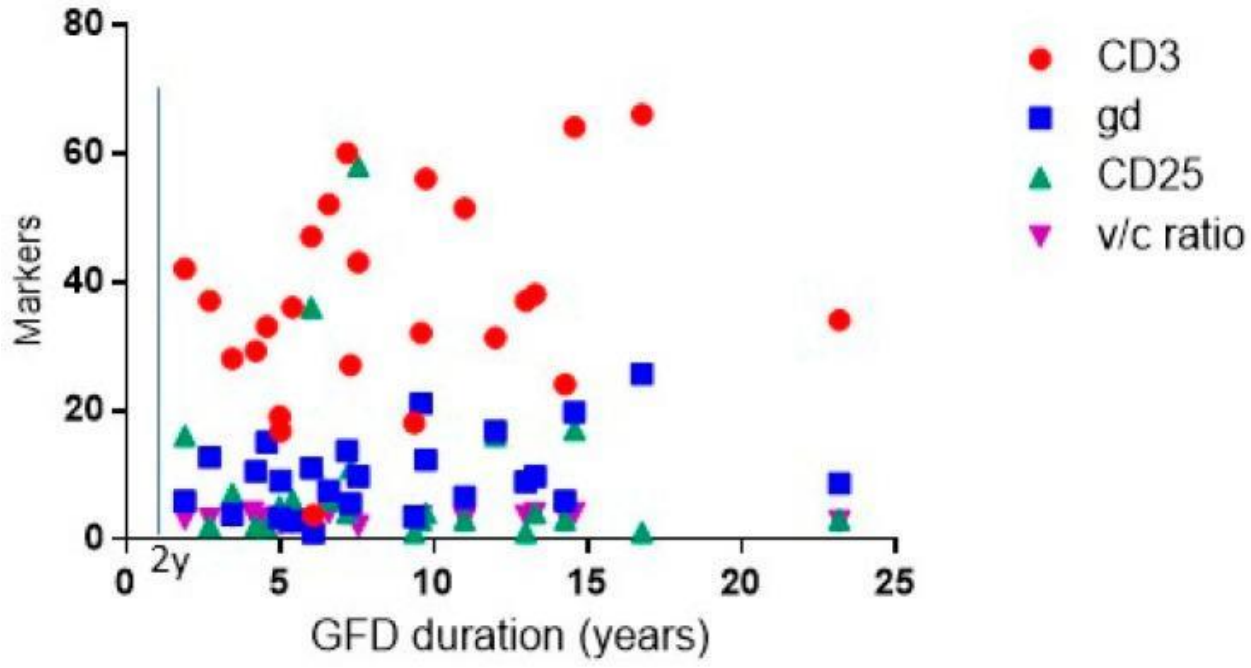


Figure 4. Linear correlation among different immunohistochemical markers and gluten free diet duration. Red circle represents CD3+ cells; blue squares TCRγδ+ cells, green up-pointed triangles CD25+ cells, violet down-pointed triangles villous/crypts ratio. Vertical blue line represents the minimum period of gluten free diet (2 years). GFD: gluten free diet.

4. DISCUSSION

In CD, gluten activates an adaptive immune response that ultimately causes damage to the small intestinal mucosa and its exclusion from the diet is currently accepted as the only effective treatment. Different studies carried out in adults and in children, suggest however, that diet is not always able to restore the normal intestinal architecture (4,5,6,7,8,9,10). Moreover, even when it happens, more subtle signs of mucosal inflammation can persist. Our data, retrospectively obtained from a cohort of celiac children on a long-term GFD, demonstrated that a GFD is effective in restoring a normal intestinal architecture in children, even in those who underwent a second duodenal biopsy for a persistently positive serology despite good adherence to the dietary regimen. Indeed, morphometrical analysis revealed that, in patients on a GFD, the V/C ratio became even higher than in controls. This apparently surprising result can be explained by the fact that enterocyte proliferation in crypts is notably increased in CD patients in remission (20), whose cells are committed to "repairing" damaged tissue, compared to controls. Our results are in contrast with those recently published by Leonard et al. in a retrospective study on 103 CD patients from two children's hospitals in Boston, who showed that twenty percent of children with CD do not heal on a GFD (21). In all our patients, the V/C ratio reverted to a normal value after a GFD was commenced. Vice versa, in line with the ESPGHAN (11), our data support the notion that there is no need to repeat biopsies as routine practice in CD children in remission, with a positive impact on patients' comfort, quality of life and on medical costs.

In contrast with the morphometrical analysis, our immunohistochemical analysis revealed that, despite following a long-term GFD, some signs of inflammation persist in the epithelium of CD patients, while disappearing from the lamina propria (22). Furthermore, we demonstrated that IELs are significantly reduced in patients on a GFD compared to CD patients at diagnosis but remain significantly increased compared to controls. This is particularly evident for the TCRγδ+ IELs, that remain altered in 85% of patients on a long-term GFD, while lamina propria CD25+ cells completely normalize on a GFD and become comparable to controls.

It is obvious to expect that GFD causes a reduction in the IELs number; however, the literature is not clear whether they completely return to normal values. IELs represent a large population of antigen-experienced "innate-like" T cells that are typically recalled in the intestinal epithelium by inflammatory cytokines released by gluten specific CD4+ T cells activated by gluten in the lamina propria (15,22). Most IELs express an αβ-receptor and their classical function is to kill stressed epithelial cells in an antigen independent manner. A small proportion of IELs, around 10% of the total, bear the γδ-receptor: the precise functional role of these cells is not fully understood, but recent studies suggest they could have a cytolytic phenotype, expressing high levels of granzyme B under basal conditions and undergoing a reshaping of their function when tissue damage occurs, acquiring

a proinflammatory INF γ-producing phenotype in overt CD (23). Furthermore, the authors revealed, via transcriptional studies, a permanently altered program in TCRγδ+ cells of CD patients irrespectively of a strict adherence to a GFD, suggesting that TCRγδ+ cells represent a hallmark of CD that persist even upon gluten withdrawal from the diet. Besides, it has been previously demonstrated by our and other groups that interleukin (IL)15, that has a prominent role in the recruitment of TCRγδ+ IELs (24), is persistently elevated in patients on a GFD (25,26,27) and could thus be partially responsible for the persistence of TCRγδ+ cells in the intestinal epithelium.

The concept that some inflammatory features persist in CD patients in remission is not completely new in the literature. In a recently performed clinical trial on 19 well-treated adult celiac patients, proteomic analysis of total tissue or the isolated epithelial cell compartment from intestinal biopsies collected before and after a 14-day gluten challenge, demonstrated that patients with a stronger mucosal response to the challenge already displayed signs of ongoing tissue inflammation before the gluten challenge (28). This minimal tissue inflammation in basal conditions is paralleled by increased gluten specific CD4+ T-cell frequencies in the gut and the presence of a low-level blood inflammatory profile. Thus, apparently, in well-treated subjects, the disease cannot be completely quiescent, with the presence of low-grade inflammation and anti-gluten immunity in the gut mucosa and histological evaluation not necessarily correlated with a full recovery (28). In addition, from a genomic point of view, Dotsenko et al. recently published that 167 genes were differentially expressed in the intestinal mucosa of CD patients on a GFD and after a gluten challenge. In particular, genes encoding proteins that transport small molecules were expressed less, suggesting that GFD patients were not completely "healed" despite histological remission (29).

Our data also suggest that mucosal healing is independent of both the duration of the diet and the persistence of a positive CD associated serology in patients that correctly follow the dietary regimen. We noticed, in fact, that the number of CD25+ cells and IELs did not correlate to the years of following the GFD. Since the biopsy in remission was performed after at least 2 years on a GFD (mean time 8 years, range 2–18 years), we could speculate that, after that period of time, the intestinal mucosa acquires its definitive shape. Moreover, in the small subset of children that repeated the intestinal biopsy because of the persistence of a positive CD associated serology despite a good adherence to the GFD (as assessed by the nutritional interview), the immunohistochemical features were comparable to the whole cohort. The presence of circulating anti-transglutaminase antibodies could be due to persistent intestinal antibody production by specific plasma cells even years after gluten withdrawal and is not necessarily indicative of insufficient dietary compliance (30,31).

In conclusion, our work supports the idea that a GFD is an effective strategy both to restore normal intestinal architecture and to reduce inflammation in the lamina propria of CD children; however, the epithelium maintains some stigmata of the disorder. The increased number of TCRγδ+ cells despite a long-term GFD enforces the concept that these cells represent a hallmark of the disease, and this compartment remains altered regardless of gluten consumption. Moreover, the mucosal reshaping associated with tissue healing takes place mostly in the first 2 years of a GFD and does not necessarily correlate with the presence of a positive CD serology. Further studies are needed to address the issue of whether these subtle immunohistochemical alterations, despite the restoration of a normal intestinal architecture, can have a clinical impact in terms of the development of long-term complications in CD patients.

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6.2 Causes of non-celiac villous atrophies

This work has been published as:

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INTRODUCTION

Normal duodenal mucosa has numerous finger-like projections or villi and an equal number of crypts (1). Villi length to crypts depth ratio is typically more than 2 to 3 in normal conditions. In case of mucosal damage, several morphological changes can be observed, leading at the end to crypts hyperplasia and villous atrophy (VA) (2). Michael N. Marsh first described the progression of the intestinal damage in celiac patients after gluten exposure. Marsh classification ranges from a normal mucosa (preinfiltrative lesion, type 0), to a completely destroyed mucosa (hypoplastic lesion, type 4) (3). As stated by Marsh himself, flattening does not involve attrition of every villous to the level of the crypts openings. More than a simple "atrophic process," it is a complex tissue remodeling including expansion of intervillous ridges, mesenchyme, and crypts (4).

More than 90% of VA is attributed to celiac disease (CD), especially if associated to a positive serology, regardless of patients' age (5). An obvious exception is neonatal age, when gluten has still not been introduced in the diet and VA is more frequently related to a congenital disorder. The dilemma occurs when VA appears in the context of a negative CD serology. This clinical entity is termed as seronegative villous atrophy (SNVA) and is generally affected in adults by a worse prognosis compared with classical seropositive CD (mortality 6% people/year vs 0.2% people/year) (6).

Recently, this condition has been studied in the adult population. The single most frequent cause of SNVA is represented by seronegative celiac disease (SNCD): from 7 to 45% according to different studies (31% Aziz et al (7), 45% Volta et al (8), 7% Schiepatti et al (9)). A small percentage of celiac patients in fact display VA but are negative to specific serology. Diagnosis of this condition strictly relies on the histological response to a gluten-free diet (GFD), after other rare forms of enteropathy unrelated to gluten ingestion have been ruled out. Surprisingly, SNVA is typically characterized by a more severe degree of VA and clinical presentation and generally has a later onset in age compared with seropositive CD. Other causes of SNVA include infections, congenital or acquired immunodeficiencies, bacterial overgrowth syndrome, inflammatory bowel disease (IBDs), drugs (NSAID, olmesartan, immunosuppressors), autoimmune enteropathies and food allergies, with

variable frequencies depending on the authors (10). There are, however, few studies regarding SNVA in children.

The aim of our work was to describe the clinical presentation, histological, and immunohistochemical features of duodenal biopsies in a cohort of pediatric patients with SNVA.

METHODS

Patients

Over a 7-year period (from 2010 to 2017), we retrospectively collected data from all the children with intestinal villous atrophy but without CD-associated antibodies diagnosed in our centre, University Federico II, Naples. The identification of SNVA was based upon duodenal biopsies showing villous atrophy and negative CD serology. Reasons that led to the execution of an EGDS and a duodenal biopsy in these patients were failure to thrive, chronic diarrhoea, melena, unresponsive iron deficient anemia, upper abdominal pain and heartburn. In all the cases, duodenal biopsies were performed regardless of the presence of duodenal macroscopic lesions. For each patient age at diagnosis, clinical symptoms and final diagnosis were recorded. This study was approved by the ethical committee of the University Federico II, Naples (number 58/20).

Celiac Serology

All patients were tested for total serum IgA and twice for anti-tissue transglutaminase (anti-TG) IgA and anti-endomysium (EMA). In case of IgA deficiency, IgG antibodies were evaluated in order to exclude CD. To measure serum anti-TG antibodies, an enzyme-linked immunosorbent assay kit was used, based on a human recombinant antigen (Eu-tTg IgA, Eurospital, Trieste, Italy)

Duodenal Biopsy and Immunohistochemical Analysis

In each patient, esophagogastroduodenoscopy with 5 biopsies (1 from the bulb and 4 from the distal duodenum) was carried out. According to our hospital protocol, 4 of 5 fragments, including 1 from the bulb, were fixed in 10% formalin, embedded in paraffin, and then stained with hematoxylin-eosin. The histological and morphometrical analysis by light microscopy was performed by 2 experienced pathologists. No double check was performed. Only correctly oriented samples were evaluated. Not correctly oriented specimens were recut in order to make them readable. A villous height: crypts

depth ratio ≥2 was considered normal (11). Among biopsies with a normal villous height: crypts depth ratio, Marsh 0 was defined by the presence of less than 25 intraepithelial lymphocytes (IELs) per 100 enterocytes and Marsh 1 by the presence of 25 or more intraepithelial lymphocytes (IELs) per 100 enterocytes. Marsh score was assigned based on the fragment with the most severe lesion.

The evaluation of these 4 fragments was made blinded to any serology results. One fragment (not from the bulb) was put in an optimal cutting temperature compound (Killik, Bio-Optica, Milan, Italy), stored in liquid nitrogen, and used for immunohistochemical staining for CD3+, TCRγδ+, and CD25+ cells,. The number of stained cells per millimeter of epithelium determined the density of cells expressing CD3 and TCRγδ in the intraepithelial compartment. Cutoff values for CD3+ and TCRγδ+ cells were 34 and 3.4 mm per epithelium, respectively. On the other hand, the number of cells expressing CD25 in the lamina propria was evaluated within a total area of 1 mm2. The usual cutoff value for CD25+ cells is 4 mm2 lamina propria. To determine the cutoff values to be used, 100 children with untreated CD and 50 non-CD control children were studied. Percentiles were obtained using the SPSS software (IBM, Chicago, IL). Cutoff values represented the 90th percentile of non-CD patients.

Intestinal Deposits of Anti-TG2 IgA Antibodies

Duodenal biopsies were also investigated for the presence of extracellular deposits of anti-TG2 IgA antibodies as previously described (12). The evaluation of the deposits, performed considering the pattern and the intensity of the staining, was graded semiquantitatively as follows: negative, very weak, weak with patchy distribution, strong with patchy distribution, and strong with a homogenous distribution.

RESULTS

Villous Atrophy Etiology and Histology Analysis

Over a 7-year period, between 2010 and 2017, VA was found in 1282 patients. One thousand two hundred eighteen out 1282 had a positive CD serology whereas 64/1282 (5%) were defined as SNVA (55% boys, mean age at diagnosis: 5.9 years). Clinical diagnoses were: inflammatory bowel diseases (IBD) (21/64), gastro-esophageal reflux disease (GERD) (12/64), food allergies (8/64), infections (7/64, of which 3 HIV infections), immunodeficiencies (3/64), short bowel syndrome (3/64), congenital diarrhea (2/64), other/inconclusive diagnosis (8/64) (Fig. 1). Mean V/C ratio was 1.63.

Forty-four, 15, and 5 showed Marsh 3a, 3b, and 3c lesion, respectively. The latter category included 2 patients with Crohn disease, 2 patients with immunodeficiencies, 1 with lymphohistiocytosis (Table 1).

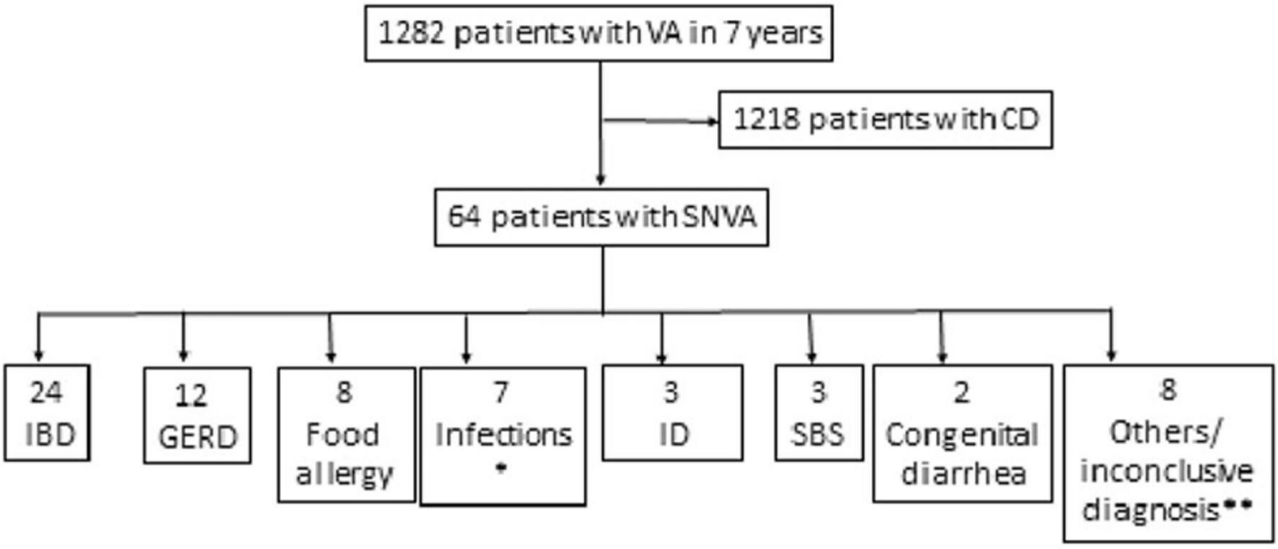

FIGURE 1. Patients enrolled in the study. CD = celiac disease; IBD = inflammatory bowel disease; GERD = gastro-esophageal reflux disease; ID = immune deficiency; SBS = short bowel syndrome. *Seven infections that included 3 HIV patients. ** Included cases of Down syndrome, lymphohistiocytosis, duodenal membrane, and duodenal fistula, ectodermic dysplasia, 2 inconclusive diagnosis.

TABLE 1. Clinical, serological, and immunohistochemical features of patients affected of seronegative villous atrophy

Patient No.	Sex	Diagnosis group	Age at diagnosis	CD serology	Marsh stage	V/C	CD3	γδ	CD25	Anti-tg staining	Notes
1	M	Congenital diarrhea	1	neg	M3b	0.9	3.2	0.8	36	Absent	Tufting enteropathy
2	M	Congenital diarrhea	0.6	neg	M3b	1.1	n.e	n.e	n.e	n.e	Unknown origin*
3	M	Food allergy	2.6	neg	M3a	1.8	46	6.6	8	Absent	Eosinophilic esofagitis
4	F	Food allergy	0.5	neg	M3a	1.9	n.e	n.e	n.e	n.e	Cow's milk protein allergy
5	F	food allergy	1.1	neg	M3a	1.7	35	4.5	34	Absent	Cow's milk protein allergy
6	F	Food allergy	7.9	neg	M3a	1.3	5	0.4	14	Absent	Eosinophilic gastroenteritis
7	F	Food allergy	10.9	neg	M3a	1.8	28	2	38	n.e	Cow's milk protein allergy
8	M	Food allergy	0.7	neg	M3a	1.8	24	3.5	5	Absent	Cow's milk protein allergy
9	F	Food allergy	1.1	neg	M3a	1.8	31	2	7	Absent	Cow's milk protein allergy
10	M	Food allergy	5.4	neg	M3a	2.0	42.4	1.2	61	n.e	Cow's milk protein allergy
11	M	GERD	2.7	neg	M3a	1.6	40	4.1	17	Absent	
12	F	GERD	14.4	neg	M3a	1.7	33.2	4.8	18	Weak	
13	M	GERD	6.5	neg	M3a	1.8	27	1.4	23	Absent	
14	F	GERD	10.0	neg	M3a	1.6	23.4	1.1	15	Weak	
15	M	GERD	12.3	neg	M3a	1.6	17	2.8	11	Weak	
16	F	GERD	4.8	neg	M3a	1.8	86	2.5	26	Absent	
17	M	GERD	2.4	neg	M3b	1.2	n.e	n.e	n.e	n.e	
18	F	GERD	12.0	neg	M3b	1.6	26	1.2	21	Absent	
19	M	GERD	1.5	neg	M3a	1.7	8.2	2	112	Weak	
20	M	GERD	7	neg	M3a	2.0	53.3	2.5	79	Absent	
21	F	GERD	12.6	neg	M3a	1.8	21.7	1.6	13	Absent	
22	M	GERD	8.8	neg	M3a	1.7	34.75	2.8	14	Absent	
23	F	IBD	10.3	neg	M3c	0.8	28	0.44	16	Absent	Crohn
24	F	IBD	13.4	neg	M3b	1.4	n.e	n.e	n.e	n.e	Crohn
25	F	IBD	6.7	neg	M3a	1.7	n.e	n.e	n.e	n.e	UC
26	M	IBD	13.6	neg	M3a	1.8	n.e	n.e	n.e	Weak	UC
27	F	IBD	13.5	neg	M3a	1.8	28	0.8	19	Absent	Crohn
28	M	IBD	10.7	neg	M3a	2.0	26	1.3	10	Absent	UC
29	F	IBD	17.7	neg	M3a	2.0	25.2	6.6	5	Weak	Crohn
30	M	IBD	11.2	neg	M3a	1.7	75	0.36	8	Absent	Crohn
31	M	IBD	15.9	neg	M3b	1.2	34	3.2	10	Weak	UC
32	M	IBD	15.9	neg	M3c	n.e.	24	0.8	30	Absent	Crohn
33	F	IBD	11.7	neg	M3a	1.9	20	0.36	26	Absent	UC
34	M	IBD	13.3	neg	M3a	1.6	n.e	n.e	n.e	n.e	UC
35	M	IBD	16	neg	M3b	1.5	n.e	n.e	n.e	n.e	UC
36	F	IBD	8.2	neg	M3a	1.8	36	1.9	11	Absent	Crohn
37	F	IBD	16.4	neg	M3a	1.6	n.e	n.e	n.e	n.e	Crohn
38	F	IBD	25.5	neg	M3a	2.0	10.7	2	4	Absent	UC
39	M	IBD	13.5	neg	M3a	1.6	19.8	1	28	Weak	UC
40	F	IBD	13.6	neg	M3b	1.2	16.4	2.1	35	Absent	Crohn
41	M	IBD	12.5	neg	M3a	2.1	7.5	1.9	62	Patchy	Crohn
42	F	IBD	12.6	neg	M3a	2.1	11.6	1.9	68	Absent	UC
43	F	IBD	12.9	neg	M3a	1.6	27	2.1	14	Absent	UC
44	M	Immunodeficit	2.3	neg	M3c	0.8	n.e	n.e	n.e	n.e†	IgA deficiency
45	M	Immunodeficit	0.6	neg	M3a	1.0	n.e	n.e	n.e	n.e	SCID
46	M	Immunodeficit	1.1	neg	M3c	n.e.	n.e	n.e	n.e	n.e	Chronic granulomatous disease

Patient No.	Sex	Diagnosis group	Age at diagnosis	CD serology	Marsh stage	V/C	CD3	γδ	CD25	Anti-tg staining	Notes
47	M	Infections	15.8	neg	M3a	1.6	14	0.6	7	Absent	Hp infection
48	M	Infections	11.1	neg	M3a	2.0	7.6	1.8	11	Absent	Yersinia infection
49	F	infections	10.8	neg	M3a	2.3	n.e	n.e	n.e	n.e	Hp infection
50	M	Infections	0.3	neg	M3a	1.8	n.e	n.e	n.e	n.e	HIV
51	M	Infections	2.8	neg	M3b	1.2	15.3	5	47	Absent	SIBO
52	F	Infections	17	neg	M3b	1.5	37.6	3.8	18	Absent	HIV
53	M	Infections	9.1	neg	M3a	2.3	n.e	n.e	n.e	n.e	HIV
54	F	Other	3.2	neg	M3a	1.8	67	1.4	n.e	Absent	Down syndrome
55	M	Other	0.5	neg	M3b	1.0	n.e	n.e	n.e	n.e	ectodermal dysplasia
56	F	Other	0.2	neg	M3b	1.1	14.6	3.6	74	Absent	duodenal membrane
57	M	Other	0.2	neg	M3c	0.6	17	2.1	42	Absent	lymphohistiocytosis
58	M	Other	0.2	neg	M3b	1.0	n.e	n.e	n.e	n.e	Down syndrome
59	F	Other	0.3	neg	M3a	2.0	13	1.8	24	Absent	Undiagnosed*
60	M	Other	0.5	neg	M3b	1.1	25.5	6.5	94	Absent	Melena of unknown origin*
61	F	Other	1.7	neg	M3a	n.e.	29.3	3.6	2	Absent	Duodenal fistula
62	M	SBS	0.4	neg	M3a	1.8	30	10.8	38	Absent	Postsurgery
63	F	SBS	5.3	neg	M3b	2.6	12.7	0.8	11	Weak	Postsurgery
64	M	SBS	1.8	neg	M3a	1.5	n.e	n.e	n.e	n.e	Postsurgery

TABLE 1. Clinical, serological, and immunohistochemical features of patients affected of seronegative villous atrophy

image

Villous Atrophy Immunohistochemistry and Immunofluorescence Analysis

In 46 out 64 patients, immunohistochemistry on duodenal frozen sections has been performed. Lamina propria mononuclear cells expressing IL2 receptor (CD25 positive) were counted as marker of mucosal inflammation. In 41/46 (89%) patients, mononuclear CD25-positive cells were above the cut-off >4 cells/mm2 (Fig. 2A). Intraepithelial lymphocytes expressing γδ T-cell receptor (TCR) were counted as high counts are indicative of gluten-dependent enteropathy. In 11/46 (24%), there was an excess (>34 cell/mm) of CD3+ intraepithelial lymphocytes (IELs) (Fig. 2B) and in 11/46 (24%) there was an excess (>3.4 cell/mm) of TCRγδ+ IELs (Fig. 2C). All subjects with elevated counts of TCRγδ+ IELs had in fact still low level of these cells (<3 times the cutoff of normality). Eighteen of 46 (39%) patients had CD3+ IELs and/or TCRγδ+ IELs above limits of normality. In 10/46 (22%) patients, a positive immunofluorescence indicated the presence of anti-TG2 mucosal antibodies: in 9 patients with a very weak staining and in 1 patient, with Crohn disease, with a weak staining and patchy distribution. In 2 cases, patients had concurrently a very weak positive staining for anti-TG2 mucosal antibodies and high TCRγδ+ IELs (Fig. 2C, arrows): 1 patient with GERD and 1 patient with Crohn disease.

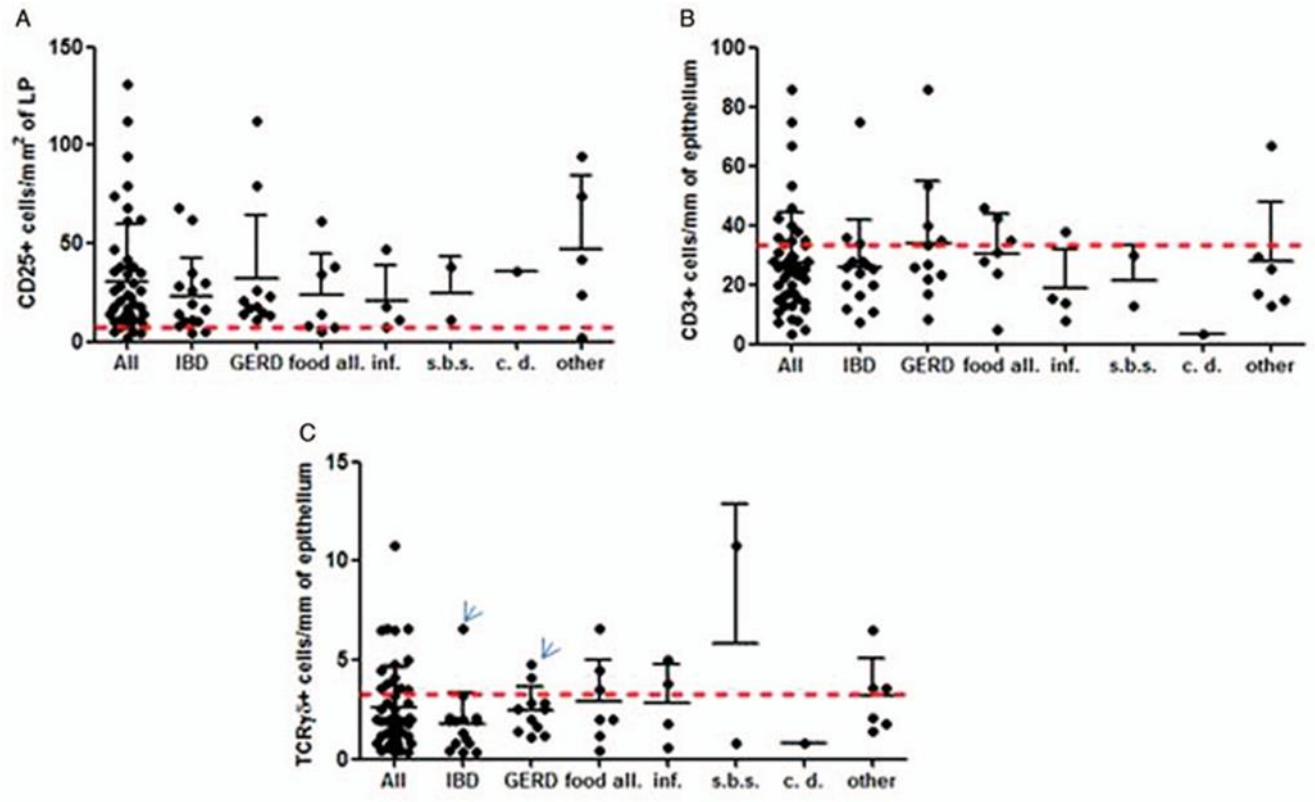

FIGURE 2. CD25+ mononuclear cells, CD3+ and TCRγδ+ intraepithelial lymphocytes density in duodenal mucosa of patients affected of nonceliac villous atrophy. The red lines represent cut-off values. IBD = inflammatory bowel disease; GERD = gastroesophageal reflux disease; SBS = short bowel syndrome; food all = food allergy; c.d. = congenital diarrhea; inf. = infections. The arrows indicate the 2 patients who had both positive staining for mucosal deposits of anti-TG2 antibodies and high TCRγδ+ cells.

DISCUSSION

Celiac disease remains the single most frequent cause of VA in the adult as well as in the pediatric population. Nonetheless, SNVA is not such a rare condition as it represents up to 5% of the diagnosis of VA in children. In most cases (70%), VA is mild (Marsh3a). One could argue that this could be a "false" VA, related to a not proper orientation of the duodenal biopsy. In fact, to correctly analyze biopsies, it is essential that the plane of the section is perpendicular to the luminal surface, as judged by the fact that the crypts of Lieberkuhn are cut longitudinally and not in cross section (13). To avoid this, together with qualitative histology (Marsh-Oberhuber classification), we also analyzed the quantitative ratio between villi and crypts, confirming the real presence of VA (mean V/C ratio 1.6). As in CD, most patients with a SNVA had an inflamed lamina propria, with an increase of CD25+ mononuclear cells (mainly lymphocytes and macrophages, expressing IL2 receptor and indicating an

inflamed intestinal mucosa). This is not surprising as the mucosa remodeling leading to VA is a feature of many conditions sustained by T-cell activation, such as IBD and graft-versus-host reaction (14, 15).

On the contrary, unlike CD, only about one quarter of patients had an increased number of IELs and of TCRγδ+ IELs. These cells typically increase in the epithelium of CD patients, and are thus considered as an immunohistochemical marker of CD. Moreover, all subjects with elevated counts of TCRγδ+ IELs had in fact relatively low level of these cells (<3 times the cutoff of normality).

A small proportion of patients (22%) present anti-TG2 mucosal antibodies, most of them with a weak intensity. Actually, we know that intestinal anti-TG2 antibody production does not show absolute specificity for CD. It has often been seen in association with inflamed mucosa. In a recent study, it has been shown that anti-TG2 mucosal antibodies can be present in up to 24% of seronegative patients with diagnosis other than CD (16).

In our cohort of patients, the single most frequent cause of SNVA is IBD. There could be, however, a selection bias as our center is the regional reference center. Surprisingly VA occurred similarly in Crohn and ulcerative colitis disease (11 vs 12 patients). In fact, even if only in Crohn disease, the upper gastrointestinal tract is generally macroscopically involved, duodenitis may develop in both Crohn and ulcerative colitis disease, even in the absence of upper gastrointestinal symptoms.

Other causes of SNVA are in the order food allergies (8/64), infections (7/64, of which 3 HIV infections), immune deficiency (3/64), short bowel syndrome (3/64), congenital diarrhea (2/64), others/inconclusive diagnosis (8/64). All these causes, because of their pathogenesis, are more frequent in pediatric then in adult patients, with the exception of the infections. On a more general note, it should be emphasized that most of those nonceliac biopsies show, a milder degree of remodeling in comparison to the more severe picture observed in CD.

The second most frequent clinical diagnosis associated with SNVA in our cohort is surprisingly represented by GERD. GERD should be more correctly considered as an association rather than the

cause of VA. We putatively hypothesize that hyperacidity could be the link between GERD and VA, as the first part of the duodenum can be regarded as an extension of the gastric antrum and consequently it is exposed to acidic gastric secretions (17). As in GERD, the esophageal damage can be explained by an excess of gastric acid secretions, similarly we could speculate that this same acid causes a duodenal damage. This remains a hypothesis that should be investigated. In any case in our cohort of patients, clinical features, the mild degree of the lesions and the immunohistochemistry data (when available) did not make a trial with gluten-free diet necessary.

Our cohort of patients did not include other causes known to determine VA, such as autoimmune enteropathy, tropical sprue, drug-induced enteropathy, and seronegative celiac disease (SNCD). Some of these causes are extremely rare when taken singularly (for instance, autoimmune enteropathy prevalence is 1:100000 (18)) and some other are rare in pediatric age. For example, drug-induced enteropathy is typically related to the use of antihypertensive drug like angiotensin II receptor blocker (olmesartan), that are commonly used in adults but of course not in children (19).

Unlike the adult population, where seronegative celiac disease is the most frequent cause of SNVA (4-7), this condition seems to be virtually absent in pediatric age, as also stated in a recent article published by the Finnish group (20).

Of course, this is a retrospective study and, as such, has some limitations. For instance, all samples were not reviewed by the same pathologist and immunohistochemistry staining was not performed for all patients, which limits the ability to interpret these results. Like in all retrospective studies, causality links cannot be demonstrated and it remains to establish whether GERD is a cause of SNVA or a casual association. Moreover, the percentage of SNVA could partially be overestimated by the fact that, since the year 2012, diagnosis of CD can be made without intestinal biopsy in selected cases (presence of high level of anti-TG, symptoms and HLA DQ2, and/or DQ8 genetics) (21), which actually represented almost half of the cases in our practice. If we analyze separately data collected between 2010 and 2012, we observe that the percentage of SNVA decreases to 4% (19 cases of SNVA over 463 cases of VA).

In conclusion, SNVA remains an area of active clinical research and, given the heterogeneity of the clinical conditions associated to VA, it calls for studies aimed to validate biomarkers both for CD and non-CD conditions, which may improve diagnostic accuracy.

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Chapter 7. Conclusions

Potential celiac disease is a relative common disorder where immune tolerance to gluten is already lost, but intestinal damage has not developed yet (*chapter 1*). Unfortunately there is still scarce knowledge on its natural history and on risk factors associated to progression of the disorder. In the present project of thesis we studied on a very large cohort of Italian children with a very long follow up (up to 17 years) what happens to PCD patients prospectively monitored on a gluten containing diet. Confirming our previous studies, we demonstrated that evolution to VA is not the rule as most of the patients remain asymptomatic without developing intestinal damage (*chapter 3*). Moreover, it dose not seem that chronic gluten exposure increases in these children the risk of developing clinical or subclinical manifestations of malnutrition. Equally, it does not seem to increase the risk of developing a concomitant autoimmune disorder (*chapter 4*). The most crucial point in its management remains the possibility to predict since diagnosis future evolution to VA or, viceversa, the interruption of autoantibodies production. This could allow an individualised therapeutic strategy, for instance directly suggesting GFD to those committed to develop VA. In this context, we tested a panel of 92 serum inflammatory proteins known to be involved in the inflammasome pathway with the primary aim of distinguishing at entry patients destined to evolve to an over form of disease: their combined evaluation allowed a correct classification of patients in the two groups (evolution to villous atrophy vs remaining potential) in up to 94% of the cases. This study also highlighted how specific changes in abundance of inflammatory proteins in serum associate with small intestinal mucosa damage. The deep comprehension of pathogenic mechanisms that sustain this condition is mandatory to implement prevention strategies and remains the main challenge for the future.