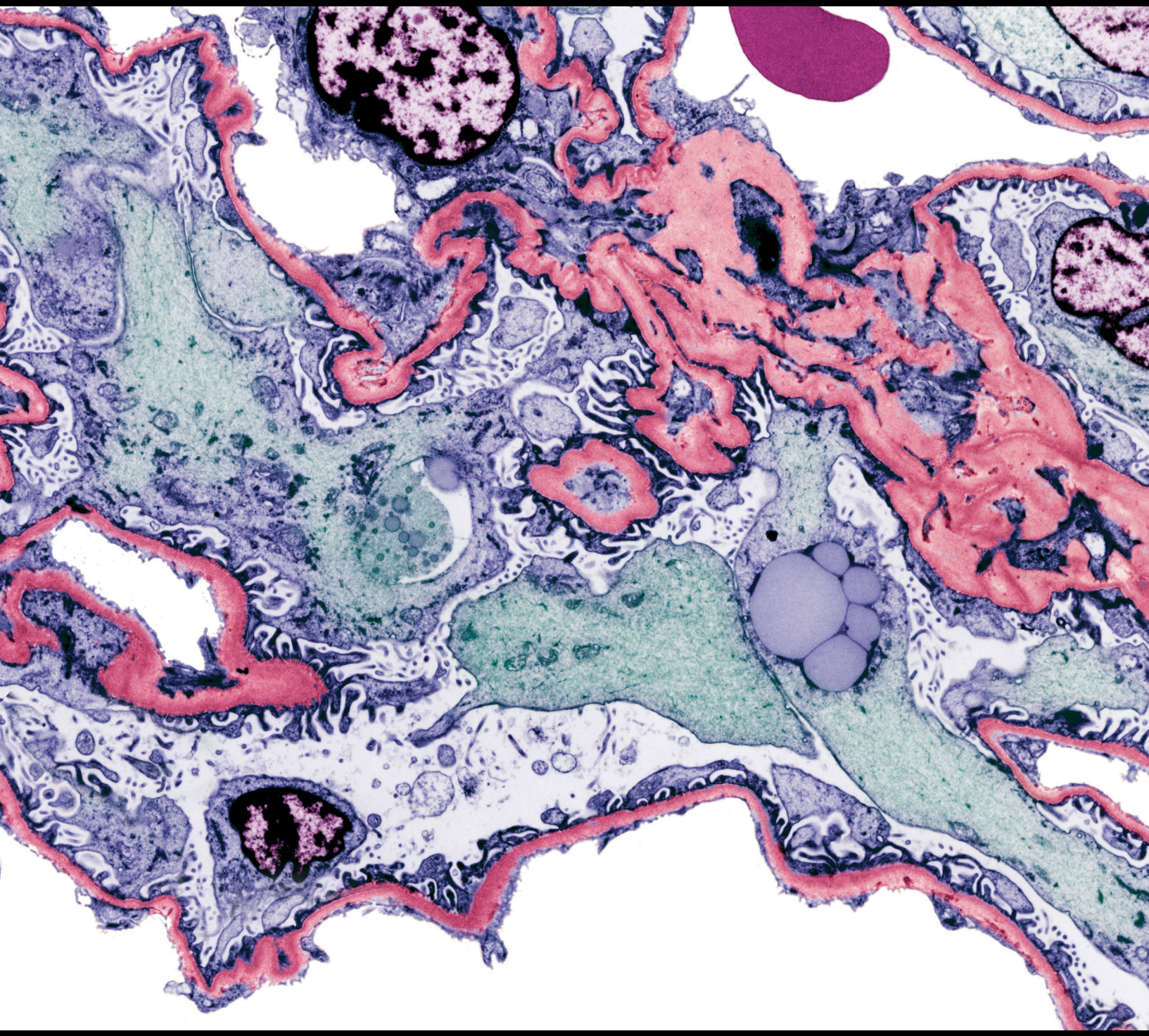
Autoimmune Diseases

Coeliac Disease

Guest Editors: Raffaella Nenna, Stefano Guandalini, Alina Popp, and Kalle Kurppa



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Editorial Board

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Editorial
Coeliac Disease

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Celiac disease is an autoimmune disorder, caused by a permanent intolerance to gluten contained in wheat and to similar prolamines present in barley and rye. It is a common disease as its prevalence, in Caucasian populations, is about 1%. There are several patterns of clinical presentation: typical (with gastrointestinal symptoms), atypical (extraintestinal manifestations), and silent forms, all characterized by typical histological lesions in the small bowel mucosa. Nowadays the gluten-free diet for life is the only therapy available for CD. Considerable progress has been made due to advances in molecular biology, which have allowed better understanding of the genetic mechanisms involved in CD and the identification of new pathogenetic pathways that have the potential to be targeted by new drugs. In this special issue, we have invited authors to contribute original papers that will stimulate the continuing efforts to understand the pathogenesis and heterogeneous clinical presentation of celiac disease.

Immunogenic peptides, created by deamidation of food-derived gliadin peptides by small intestinal tissue transglutaminase, are presented by antigen-presenting cells, mostly dendritic cells bearing HLA-DQ2 and DQ8 molecules, to proinflammatory CD4+ T cells, activating them. E. Liu et al. investigated peripheral T cell responses from young children with newly diagnosed CD prior to treatment with a gluten-free diet, finding that T cell reactivity is heterogeneous but favors reactivity with α-gliadin epitopes more than γ-gliadin.

Metabolomics is a rapidly emerging new concept in science that appears to have relevant application also in the field of celiac disease. In their meticulous review, A. Calabrò et al. highlight the metabolomics perspective on celiac disease. Furthermore, the growing recognition of the important role of gut microbiota in the development of celiac disease and other food-related disorders is addressed and thoroughly reviewed.

Healing of the small bowel mucosa is directly dependent on the adherence to the gluten-free diet, and its assessment by noninvasive tools has been a long-time objective of research in celiac disease, with the aim of establishing suitable minimally invasive methods both for diagnosing and for adequate follow-up of celiac disease and of the adequacy of the gluten-free diet. In their study, E. Trigoni et al. concluded that anti-endomysium antibodies had better ability, at least in adult individuals, than anti-tissue transglutaminase to predict celiac disease and to assess the gluten-free diet in the critical period of the first semester after diagnosis and the beginning of the diet.

Among the atypical clinical forms of CD, oral signs are often neglected but could offer important diagnostic clues in challenging diagnoses. Lesions in the oral mucosa or defects in dental enamel can in fact be present in celiac patients. The paper of M. Erriu et al., evaluating oral signs and different DQ2 haplotypes, highlights the fundamental role that dentists can play in early recognition of CD.

It is well known that untreated celiac disease patients develop specific serum autoantibodies against transglutaminase 2. Recently it has been discovered that corresponding circulating antibodies against transglutaminase 6 (TG6) may play a role in particular gluten-related neurological disorders. In their study, R. Stenberg et al. demonstrate that the presence of anti-TG6 antibodies is increased in subjects with early

neurological injury resulting in cerebral palsy, suggesting that an early brain insult may lead to subsequent TG6 autoimmunity.

Due to their common genetic background, celiac disease, type 1 diabetes mellitus, and autoimmune thyroiditis are often associated. M. van der Pals et al. confirmed three times higher prevalence of thyroid autoimmunity markers (autoantibodies against thyroid peroxidase) in celiac disease children compared with 12-year-old healthy controls but no difference in thyroid autoimmunity between clinically detected and screen-detected celiac disease children.

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Clinical Study

Celiac Disease in Adult Patients: Specific Autoantibodies in the Diagnosis, Monitoring, and Screening

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The increasing prevalence of celiac disease (CD), especially in adults, its atypical clinical presentation, and the strict, lifelong adherence to gluten-free diet (GFD) as the only option for healthy state create an imperative need for noninvasive methods that can effectively diagnose CD and monitor GFD. *Aim*. Evaluation of anti-endomysium (EmA) and anti-tissue transglutaminase IgA (tTG-A) antibodies in CD diagnosis, GFD monitoring, and first degree relatives screening in CD adult patients. *Methods*. 70 newly diagnosed Greek adult patients, 70 controls, and 47 first degree relatives were tested for the presence of EmA and tTG-A. The CD patients were monitored during a 3-year period. *Results*. EmA predictive ability for CD diagnosis was slightly better compared to tTG-A ($P = 0.043$). EmA could assess compliance with GFD already from the beginning of the diet, while both EmA and tTG-A had an equal ability to discriminate between strictly and partially compliant patients after the first semester and so on. Screening of first degree relatives resulted in the identification of 2 undiagnosed CD cases. *Conclusions*. Both EmA and tTG-A are suitable markers in the CD diagnosis, in the screening of CD among first degree relatives, having also an equal performance in the long term monitoring.

1. Introduction

Celiac disease (CD) (*coeliac*, from Greek κοιλιακός koiliakos, "abdominal") was first described in the second century AD by the Greek physician Aretaeus of Cappadocia as a malabsorptive syndrome with chronic diarrhea [1]. Nowadays it is well known that CD is an immune-mediated systemic disorder elicited by gluten and related prolamins in genetically susceptible individuals and characterized by the presence of a variable combination of gluten dependent clinical manifestations, CD-specific antibodies, HLA-DQ2 or HLA-DQ8 haplotypes, and enteropathy. The typical but not pathognomonic lesions of the small intestinal mucosa resolve with the removal of gluten from the diet [2].

The autoantigen which is also the molecule recognized by anti-endomysium antibodies (EmA) has been identified as the enzyme "tissue transglutaminase" (tTG) [3, 4]. tTG induces the deamidation of gluten peptides and the formation of novel epitopes that, in association with HLA II antigens, induce the antibody response to gliadin and tTG antigens, resulting in the damage of the small intestinal mucosa [5, 6]. Anti-tissue transglutaminase antibodies recognize the same antigen as EmA, from which they differ in terms of detection method. EmA are tested by the indirect immunofluorescence method (IF) and directed against "reticulin-like" fibres in connective tissue around smooth muscle fibres in the oesophagus, liver, stomach, and bladder of monkeys, in the sections of the jejunum and kidneys of rats and in sections of the human umbilical cord (HUC). However, commercially primate GIT tissue is used in almost all (but not exclusively) centers. For the determination of anti-tissue transglutaminase IgA and IgG antibodies, ELISA with human extractive or recombinant transglutaminase is recommended. Both EmA

and anti-tissue transglutaminase antibodies are very specific and sensitive [7–9].

Adult patients with CD rarely present with malabsorption related symptoms. Far more commonly they describe nonspecific or subtle gastrointestinal symptoms or they present with extraintestinal manifestations (atypical or silent form); thus they may initially be overlooked [10, 11].

The increasing prevalence of CD, especially in adults, its atypical clinical presentation, and also the lifelong adherence to a gluten-free diet (GFD) as the only option for healthy state create an imperative need for proper immunological tests that can easily, timely, and effectively diagnose CD and monitor GFD [12–16].

2. Aim

The aim of the present retrospective study was (a) to evaluate the efficacy of specific autoantibodies in the diagnosis and monitoring of celiac disease in Greek adult patients, where the prevalent diet is the Mediterranean one, mostly based on whole grains and (b) to assess the frequency of undetected celiac disease among the first degree relatives of CD patients.

3. Materials and Methods

3.1. Patients and Controls. This long term study which took place in the Department of Immunology-Histocompatibility of "Evangelismos" General Hospital of Athens with the cooperation of the Celiac Disease Clinic of the same hospital included the following groups of individuals.

(a) 70 Greek adult patients, 50 women and 20 men with mean age 39 ± 11.1 years (range: 19–66) who were newly diagnosed with CD. The monitoring of the patients took place on the moment of the diagnosis when they had a regular unrestricted diet and consequently at 6, 12, 24, and 36 months after the initiation of GFD. 51 of the 70 patients (72.9%) followed a strict GFD while the remaining 19 (27.1%) had a partial compliance with GFD.

(b) 47 first degree relatives (10 parents, 8 siblings, and 29 offspring) of the aforementioned patients, 23 men and 24 women with mean age 24 ± 15.5 years (range: 1–59). In all these family members on gluten-containing diet, the serological tests were performed only once.

(c) 70 individuals who constituted the control group. 30 of them were patients with inflammatory and noninflammatory diseases of the intestine (8 with Crohn's disease, 7 with ulcerative colitis, 6 irritable bowel syndrome, and 9 with microscopic colitis), 20 women and 10 men with mean age 41 ± 11.3 years (range 21–55) and the remaining 40 were healthy blood donors, 22 women and 18 men with mean age 38 ± 12.1 years (range: 18–58). The control group was serologically tested also once.

No individual in this study had IgA deficiency.

3.2. Methods. The antibodies studied were as follows. (a) Anti-endomysium (EmA) which were determined semiquantitative by the technique of indirect immunofluorescence (IIF) using a commercial kit INOVA (NOVA Lite Monkey Oesophagus IFA Kit/Slides, USA) on a 5-μm-thin cryostat section of distal monkey oesophagus as antigen substrate. Patient samples were tested in dilutions ranging from 1 : 5 to 1 : 2560. The antibody titre was defined as the highest sample dilution yielding fluorescence. Titre below 1 : 5 was considered negative. (b) Anti-tissue transglutaminase class IgA (tTG-A) which were assayed using a commercial anti-tTG type IgA ELISA test kit (QUANTA LiteTM, INOVA Diagnostics, USA). The cut-off value provided was 25 U. ELISA was performed in duplicate according to the manufacturer's instruction. Serum samples were kept frozen at −80°C until assays were performed.

The final diagnosis of CD as well as the inflammatory and noninflammatory diseases of the intestine in all these patients was based on currently accepted criteria [17, 18], after thorough clinical and laboratory investigation, including endoscopy and biopsies from the upper and lower gastrointestinal tract. Histological damage observed in the intestinal biopsy samples in CD patients was graded according to Marsh's classification [18]. Moreover, all patients in the study underwent clinical evaluation at each follow-up visit and experienced dietitians assessed their compliance to the GFD, through a standard questionnaire.

3.3. Statistics. EmA were expressed using the negative logarithms of measured values while for the comparison of proportions chi-square and Fisher's exact tests were used. Differences in changes of EmA and tTG-A during the follow-up period in total and between the two studied groups were evaluated using repeated measurements analysis of variance (ANOVA) and Bonferroni correction was used. tTG-A was log-transformed for the analysis of variance due to its skewed distribution. Receiver operating characteristic (ROC) analysis was used and area under the curve (AUC), optimal sensitivity, and specificity were determined. All P values reported are two tailed. Statistical significance was set at 0.05 and analyses were conducted using SPSS statistical software (version 18.0).

4. Results

4.1. Evaluation of EmA and tTG-A in CD Diagnosis. The frequency of autoantibodies tested in the control group and in CD patients at the time of diagnosis before they started GFD is presented in Table 1.

All CD patients but 3 presented with positive EmA while increased values of tTG-A were detected in 66 out of the 70 CD patients. Two of the three EmA negative patients were also tTG-A negative while the third one had increased values of tTG-A (155 U). In these three EmA negative patients the small intestinal biopsy showed Marsh I type lesions.

In the control group, however, none had positive EmA whereas 3 individuals showed borderline values of tTG-A (30 U, 32 U, and 28 U, resp.). Two of them were diagnosed

Table 1: Frequency of specific antibodies tested.

	Patients				P Pearson's χ^2 test
	CD patients		Controls		
	N	%	N	%	
tTG-A					
Negative	4	5.7	67	95.7	**<0.001**
Positive	66	94.3	3	4.3	
EmA					
Negative	3	4.3	70	100.0	**<0.001**
Positive	67	95.7	0	0.0	

Table 2: Evaluation of autoantibodies tested in CD diagnosis.

	Se (%) (95% CI)	Sp (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
tTG-A	94.3 (86.0–98.4)	95.7 (88.0–99.1)	95.7 (87.8–99.1)	94.4 (86.2–98.4)
EmA	95.7 (88.0–99.1)	100.0 (94.9–100.0)	100.0 (94.6–100.0)	95.9 (88.5–99.1)

with Crohn's disease and the third one with irritable bowel syndrome.

According to the antibodies frequencies found in the studied groups, sensitivity (Se), specificity (Sp), positive (PPV) and negative predictive value (NPV), and the intervals for CD were calculated (Table 2).

As presented in Table 2, EmA showed higher specificity (100.0%), sensitivity (95.7%), PPV (100.0%), and NPV (95.9%) than tTG-A in the diagnosis of celiac disease. Furthermore, their predictive ability for CD diagnosis for CD was slightly better in comparison to the one of tTG-A ($P = 0.043$).

4.2. Changes of EmA and tTG-A over Time after Initiation of GFD. The serological changes over time in all the patients, both strictly and partially compliant, are illustrated in Figure 1 which describes the proportions of positive samples at all follow-up time points. There was a significant change in the proportions of the positive samples over time for both strictly ($P < 0.001$) and partially ($P = 0.037$) compliant patients concerning EmA but for tTG-A the change was significant only for those who had a strict diet ($P < 0.001$).

Compared to partially compliant patients, strict compliance was associated with significantly lower proportions of positive EmA and tTG-A results in the first, second, and third year of the followup ($P < 0.001$). The strictly compliant patients' EmA presented a sharper decline of positive samples proportion ($P = 0.014$) than tTG-A, in the first six months. This decline also continued through the first and second year assessment. On the other hand, for the strictly compliant patients, tTG-A showed a less prominent decrease of positive samples in the first semester ($P = 0.268$) which, however, became significant, continuous, and progressive over time ($P < 0.001$).

More specifically, at the time of diagnosis 96.1% and 94.1% of the fifty-one patients who then started a strict GFD had positive EmA and tTG-A, respectively, while after 6 months

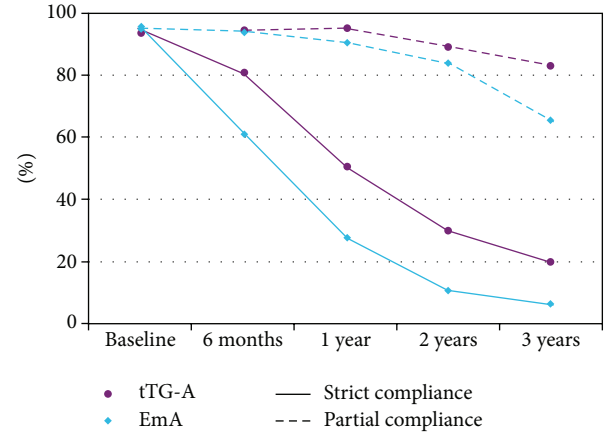

Figure 1: Changes in the percentage of positive samples for EmA and tTG-A over time in strictly compliant and partially compliant patients.

of strict GFD 60.8% and 80.4% of them were still positive compared with 27.5% and 51% after 1 year of diet. After 3 years only 3 of the patients (5.9%) had antibody titres over the cut-off level for EmA, while 10 (19.6%) remained with tTG-A positive.

4.3. Predictive Capacity of EmA and tTG-A for the Compliance with GFD. The ability of EmA and tTG-A to discriminate the degree of adherence to GFD at the follow-up time points, 6 months, 1, 2, and 3 years was assessed by ROC analysis and is shown in Figures 2(a), 2(b), 2(c), and 2(d), respectively.

We concluded that EmA can assess the degree of compliance with GFD in the first semester from the beginning of the diet, while both EmA and tTG-A have an equal ability to discriminate between strictly and partially compliant patients in the first, second, and third year.

4.4. Prevalence of Celiac Disease among the First Degree Relatives. Forty-seven family members of 28 patients with CD underwent serological testing for EmA and tTG-A. Two family members (2/47) (4.2%) had positive EmA while in 5 of them (5/47) (10.6%) increased values of tTG-A were detected.

More specifically, positive EmA was detected in 2 first degree relatives (4.2%), in dilutions 1 : 40 and 1 : 20, respectively, 1 12-year-old girl whose father had CD and 1 18-year-old boy with CD mother. The CD in these family members was confirmed by intestinal biopsy. The two EmA positive individuals also showed high levels of tTG-A (55.0 U and 65.0 U, resp.). However, there were 3 more individuals with tTG-A positive (40.0 U, 60.2 U, and 63.0 U) who were not diagnosed with celiac disease. In these individuals CD diagnosis was excluded by an intestinal biopsy and the fact that they were EmA negative.

5. Discussion

Celiac disease in adults presents with a variety of atypical symptoms so there is a need for sensitive and specific

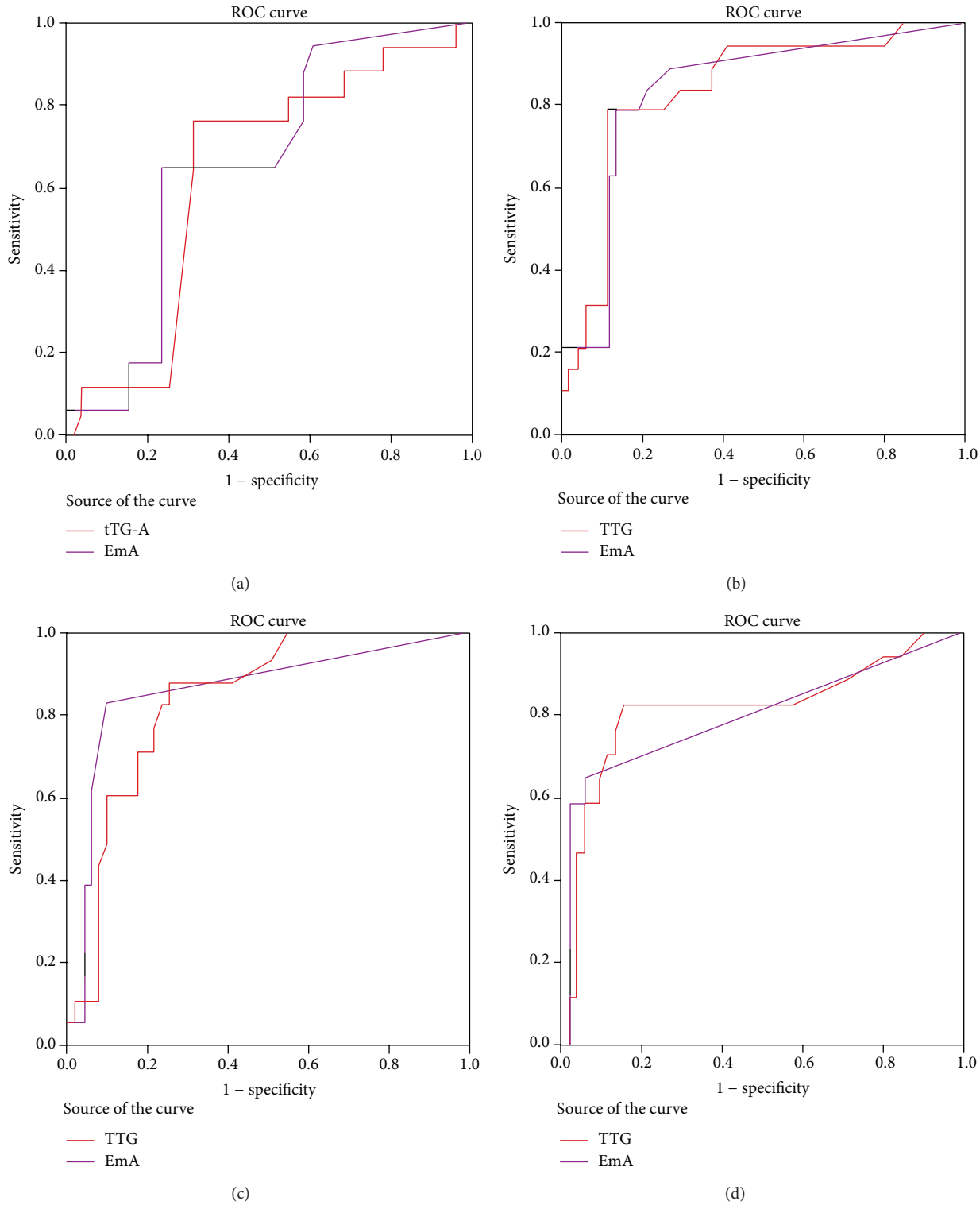

Figure 2: EmA and tTG-A ROC analysis (a) at 6 months after the initiation of GFD, (b) at 1 year after the initiation of GFD, (c) at 2 years after the initiation of GFD, and (d) at 3 years after the initiation of GFD.

serological tests for its accurate and early diagnosis [10]. The only effective treatment for CD is a strict gluten free diet for life [19]. Long term compliance with the GFD is essential to prevent the complications of CD and improve the quality of life [20]. Thus, reliable but also easy applicable markers are needed to monitor patient compliance with these dietary restrictions.

In the present study, which is the first study for celiac disease in Greek adults, the aim was to detect and investigate the specific autoantibodies in the diagnosis, monitoring, and the prognosis of celiac disease.

This study confirms the excellent specificity and sensitivity of EmA (100% and 95.7%, resp.) in CD diagnosis, which is also reported by previous studies [9, 21–27]. We could also recapitulate the high specificity (95.7%) and sensitivity (94.3%) of tTG-A [22, 28–31]. In addition, it was found that EmA predictive value for CD diagnosis was statistically significantly higher compared to tTG-A ($P = 0.043$).

All the three EmA negative patients were relatives of celiac disease patients. At the time of diagnosis two of them had no other clinical symptoms or signs of celiac disease except for mild anaemia which was due to iron deficiency. The third one, however, underwent a thorough endoscopy in order to investigate the cause because of an upper digestive tract bleeding.

Despite the indisputable role of EmA and tTG-A in the diagnosis of CD, the available literature is controversial on their value for assessing compliance with the diet. While some studies have not found the rate of fall of antibody concentration to be a reliable marker of strict adherence to the GFD [32–35], others have found that normalized markers can be useful to confirm GFD, but without concluding which one is the most appropriate [36–40]. In our study fifty-one of the seventeen patients (72.9%) followed a strict GFD while the remaining nineteen (27.1%) had a partial compliance. The serum concentrations of antibodies decreased over time which was inversely correlated with patients' degree of compliance with the diet. Among partially compliant patients, although antibody concentrations also declined, the trends were significantly less pronounced compared with strictly compliant cases.

It was also noticed that, during the first year of a strict compliance with GFD, EmA titres fell more rapidly than tTG-A, whereas in the third year more patients remained having tTG-A positive than EmA despite the fact that they are on a strict GFD. On the other hand, the 19 patients with a partial compliance with GFD presented with a persistence of abnormally elevated antibody concentration that could help in identifying patients with dietary lapses. We propose that EmA can assess the degree of compliance with GFD in the first semester from the beginning of the diet, while both EmA and tTG-A have an equal ability to discriminate between strictly and partially compliant patients in the long term monitoring.

In this study three individuals from the control group as well as three asymptomatic first degree relatives had increased values of tTG-A without having CD. Low levels of tTG-A have been described in a number of conditions unrelated to CD, such as other autoimmune diseases, IBD, infections, tumors, myocardial damage, and liver disorders. These antibodies are not associated with EmA reaction, which explains why EmA has higher reliability for the diagnosis of CD [41–43].

It is well known that CD presents more often among the first degree relatives. In detail, the prevalence of CD among the first degree relatives varies from 2.8% to 8.2% [44–48]. The differences could be partially explained by study methodological differences and the variability of the genetic background of the studied population. In the present study the prevalence of CD among the first degree relatives was 4.2% which is in agreement with the available literature. It is worthwhile mentioning that this prevalence dramatically increases when considering families with two or more cases of CD [49–51].

6. Conclusions

The use of serologic markers in celiac is a noninvasive, easily applicable, direct, and reliable practise than can be used for the diagnosis and monitoring of the disease. More specifically, both EmA and the tTG-A are suitable markers in the diagnostic approach of CD. Regarding the ability to discriminate between strictly and partially compliant patients, EmA can assess the degree of compliance with GFD earlier, while both EmA and tTG-A have equal performance in the long term monitoring. We finally recommend the screening for EmA and tTG-A among the first degree relatives.

Conflict of Interests

Dr. Gerassimos Mantzaris has received advisory board fees from Centocor, MSD, AbbVie, and Danon; lecture fees from Ferring International, MSD, AbbVie, FALK Pharma, and Angelini; Research support from AstraZeneca, AbbVie, MSD, Menarini, and Genesis. The rest of the authors declare no conflict of interests regarding the publication of this paper.

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Research Article

Anti-Transglutaminase 6 Antibodies in Children and Young Adults with Cerebral Palsy

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Objectives. We have previously reported a high prevalence of gluten-related serological markers (GRSM) in children and young adults with cerebral palsy (CP). The majority had no enteropathy to suggest coeliac disease (CD). Antibodies against transglutaminase 6 (anti-TG6) represent a new marker associated with gluten-related neurological dysfunction. The aim of this study was to investigate the prevalence of anti-TG6 antibodies in this group of individuals with an early neurological injury resulting in CP. *Materials and Methods*. Sera from 96 patients with CP and 36 controls were analysed for IgA/IgG class anti-TG6 by ELISA. *Results*. Anti-TG6 antibodies were found in 12/96 (13%) of patients with CP compared to 2/36 (6%) in controls. The tetraplegic subgroup of CP had a significantly higher prevalence of anti-TG6 antibodies 6/17 (35%) compared to the other subgroups and controls. There was no correlation of anti-TG6 autoantibodies with seropositivity to food proteins including gliadin. *Conclusions*. An early brain insult and associated inflammation may predispose to future development of TG6 autoimmunity.

1. Introduction

Patients with cerebral palsy (CP) are characterized by abnormal muscle tone, coordination problems, and delay in motor development leading to difficulties in gait, balance, and involuntary movements. Muscle spasticity is very common. Associated conditions include epilepsy, dysarthria, cognitive impairment, and feeding problems, which may result in difficulties in weight gain and growth [1, 2] particularly for the most severely affected CP groups (tetraplegic and dyskinetic subgroups). The aetiology of the brain damage causing CP is among others mainly hypoxia due to asphyxia leading to ischemic brain injury. Another cause could be intrauterine infection [3–5].

Since coeliac disease (CD) is a common cause of poor growth, we previously analysed our cohort of CP patients for IgA and IgG-antibodies against gliadin and transglutaminase 2 (TG2) and found a high prevalence of seropositive patients. The highest prevalence was for IgG-AGA 36 and 61%, respectively [6, 7] which is of low diagnostic value for CD [8]. The majority of these patients also did not have enteropathy on routine histological or extended immunohistochemical examination of small bowel biopsies [6, 9, 10]. However, a number of patients tested positive for TG2 IgA (7/99) and/or had circulating antibodies to deamidated gliadin peptides (DGP; 7/40 tested), accepted markers in CD diagnosis [6, 10]. The seropositive CP patients had a significantly lower weight, height, and body mass index (BMI) and they were also more severely handicapped, according to gross motor function classification (GMFCS) [11].

Increasing numbers of studies have reported that gluten-related disorders include extraintestinal manifestations, for example, involving skin (dermatitis herpetiformis) and brain (gluten Ataxia) [12–15]. Such manifestations may occur in

the absence of overt gastrointestinal involvement and patients may be seronegative for anti-TG2 IgA autoantibodies, the commonly used diagnostic marker for CD [16].

TG2 is the autoantigen in CD, whilst TG3 is the autoantigen in dermatitis herpetiformis, a blistering skin disease with gluten-induced granular IgA-deposits in the papillary dermis [12]. Another enzyme in the transglutaminase family that is primarily expressed in the central nervous system is transglutaminase 6 (TG6) [17]. We have demonstrated the presence of circulating anti-TG6 antibodies in adults with gluten Ataxia, independent of intestinal involvement [16]. This may suggest a bias of the immune response towards different TG isozymes in extraintestinal manifestations (i.e., TG6 in gluten Ataxia and TG3 in dermatitis herpetiformis).

The aim of this study was to investigate the prevalence of antibodies against TG6 in patients with CP.

2. Material and Methods

2.1. Participants

2.1.1. CP-Group. The study recruited 99 children and young adults with CP living in the Swedish county council of Värmland and Örebro. In Sweden children and young adults with CP are treated at specialized rehabilitation centers where they can obtain specific support such as medical care, physiotherapy, speech, occupational, and psychological therapy until they have finished school. In some cases they are 24 years of age before they are referred to the adult clinic.

There were 46 girls and 53 boys. At the time of enrolment the age ranged from 18 months to 24 years (median age 11 years). There was no comorbidity for CD such as diabetes mellitus or dermatitis herpetiformis at the time of inclusion. The diagnosis of CP subgroups was based on studies by Mutch et al. [18, 19]. A functional assessment of each child was made on the basis of the Gross Motor Function Classification System (GMFCS), graded I–V, where GMFCS V represent the most severe disability [11]. Medical data of the CP-group were reviewed retrospectively in medical files (RS). Thirteen children had percutaneous gastrostomy (PEG) but also received some gluten containing food orally. Eleven children had treatment for gastroesophageal reflux disease (GERD).

2.1.2. Controls. 36 children, aged 2–18 years (19 boys and 17 girls), were included in the serological analysis as controls. These children had been investigated with laboratory analyses for allergy but were found negative. No further clinical information about the children was available.

2.2. Serology. Data on IgG and IgA AGA and TG2, DGP antibodies and HLA testing were available from our previous studies [6, 10] as well as the data of antibodies against dietary proteins [7].

None in the CP-group had IgA deficiency (<0.07 g/L).

2.3. TG6 Antibody ELISA. Detection of antibodies to TG6 by ELISA followed our previously published protocol with minor modifications [16]. Briefly, full-length human TG6 produced in SF9 cells was obtained from Zedira (Darmstadt, Germany). Results with this antigen were comparable to those obtained with recombinant TG6 produced in house in *E. coli* (data not shown) [16]. TG6 was diluted to 2 μg/mL in 20 mM Tris/HCl, 300 mM NaCl, pH 7.6 immediately prior to use and applied to high-capacity protein binding 96-well plates (Immulon 2HB; Thermo Electron, Waltham, MA, USA) overnight at 4°C. All subsequent incubations were conducted at room temperature. Nonspecific binding was blocked by incubation with 3% BSA (immunoassay grade, Sigma 05477) in TBS (20 mM Tris/HCl, pH 7.4, 150 mM NaCl) for 1 h. Patient sera were diluted 1 : 100 in 1% BSA in TBS and any protein aggregates present removed by centrifugation at 10,000 ×g for 5 min prior to being applied to coated plates. All binding steps were carried out for 90 min and followed by five rinses with TBS/0.01% Tween 20. Antibody binding was detected by incubation with peroxidase-conjugated affinity purified anti-human IgA (Jackson ImmunoResearch, West Grove, PA, USA; 109-035-011, diluted 1 : 1000 in 1% BSA/TBS) or anti-human IgG (Dako, Carpinteria, CA, USA; P0214, diluted 1 : 250 in 1% BSA/TBS). The reaction was finally developed for 2 h using 5 mM 5-amino-2-hydroxybenzoic acid/NaOH, pH 6.0, 0.005% H_2O_2, as a peroxidase substrate solution and stopped by addition of an equal volume of 1 M NaOH to each well. After 15 minutes, the absorbance at 490 nm was measured.

All serum samples were analysed in duplicate on wells containing antigen or only BSA, included on the same plate. The BSA only background was subtracted from values for antigen. Units were calculated from a series of standards (0, 1, 3, 10, and 100 U/mL; 1st generation assay) (Zedira) run in parallel. Results are given as the mean of two independent determinations. A measurement >14 U/mL for IgA or >34 U/mL for IgG was considered positive. Thresholds were set as the 98th percentile on a blood donor collective. As anti-BSA antibodies may be prevalent in this patient cohort which has enhanced immunity to dietary components [7] and this may potentially impact on the analysis, we have evaluated all samples also in a commercial assay without background subtraction (Zedira). There was good agreement between the results of the two ELISA methods (supplementary figure).

2.4. Ethics. The study was approved by Regional Ethical Review Board in Uppsala. Participants or parents, as appropriate, gave written informed consent.

2.5. Statistical Analyses. All data were analyzed using the Statistical Package for the Social sciences (SPSS) program, version 15. Differences between groups were evaluated with Pearson's χ^2 test in cross tabulations and when appropriate Fisher's Exact Test. An independent *t*-test was used for body mass index (BMI) (standard deviation, SD), height (SD), and weight (SD), using 2-tailed significance.

$P < 0.05$ was considered significant.

3. Results

3.1. Serological Analysis for Anti-TG6 Autoantibodies. Sera from 96 patients with CP and 36 controls (children from same geographical area) were available for analysis of autoantibodies against TG6. We found elevated levels of anti-TG6 antibodies (IgG and/or IgA) in 12/96 (13%) in the CP-group and 2/36 (6%) in the control group ($P = 0.35$). (Figure 1) However, a positive test for TG6 antibodies was significantly more frequent in the tetraplegic subgroup of CP, 6/17 (35%) compared to the control group 6% ($P = 0.01$). We also found statistical significance when the tetraplegic subgroup was compared to the other CP subgroups ($P = 0.006$) (Figure 2). IgA anti-TG6 antibodies were found in 7/96 (7%) compared to 1/36 (3%) in the control group. ($P = 0.45$) IgG anti-TG6 antibodies were found in 6/96 (6%) compared to 1/36 (3%) in the control group ($P = 0.67$). One child had elevated levels of both IgA and IgG to TG6.

This CP cohort has been tested for CD as reported previously. [6, 10] There was an association between anti-TG6 antibodies and IgA antibodies to TG2 ($P = 0.04$) but not for any of the other gluten-related serological markers analysed (anti-TG2 IgG or AGA IgA/IgG) (Table 1(b)). Five of the twelve patients with CP that tested positive for anti-TG6 antibodies were negative for all gluten-related serological markers. Also, there was no correlation between anti-TG6 antibody titres and the presence of other indicators of CD (Table 1(b)). Eleven of the twelve individuals with TG6 positivity had previously been tested for the coeliac HLA type (DQB1 typing) and 6/11 were positive for HLA-DQ2 and/or HLA-DQ8, and a further 2 carried one-half of the DQ2 heterodimer (DQ7, α5 subunit) which is known to confer susceptibility to CD [21]. A further patient carries the rare HLA-DQ9 which may also confer susceptibility according to recent evidence [22].

3.2. Clinical Data of Patients Testing Positive for Anti-TG6 Antibodies. The clinical data of the 12 CP patients positive for anti-TG6 antibodies are summarized in Table 1(a). The median age was significantly higher in the TG6 positive group, 14.4 years compared to 10.7 years in the TG6 negative group ($P = 0.021$).

The majority was born at term (8/12) and had asphyxia. Five had epilepsy-requiring medication. There was no significant difference in weight ($P = 0.318$) or BMI ($P = 0.987$) between TG6 antibody positive and negative patients. There was, however, a significant difference in height between the 2 groups. The children and young adults with CP positive for anti-TG6 were shorter ($P = 0.021$). As height correlates to the degree of disability this may reflect a more severe disability, consistent with a higher prevalence of anti-TG6 antibodies in the tetraplegic subgroup [1].

Seven of these patients had previously been investigated on clinical grounds using MRI or CT and the results were reviewed as part of this study (NH). Brain malformation was seen in one child and traumatic injury in another two. Developmental malformations or defects as a consequence of ischemia were seen in 3 cases. In one child with Ataxia no significant abnormalities were found (Table 1(a)). As

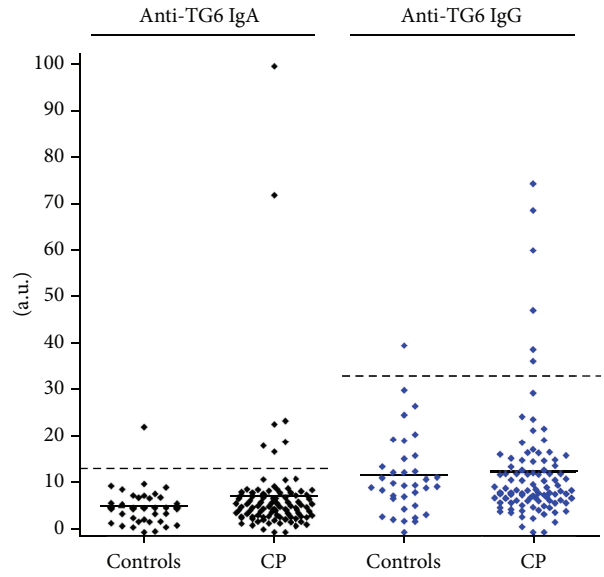

Figure 1: Analysis of serum for antibodies against transglutaminase type 6 (TG6) by ELISA. Relative concentration of antibodies in children ($n = 96$) with cerebral palsy (CP) and controls ($n = 36$) is given in arbitrary units. Bolded line represents the mean titre of the group and dotted line the threshold for a positive test.

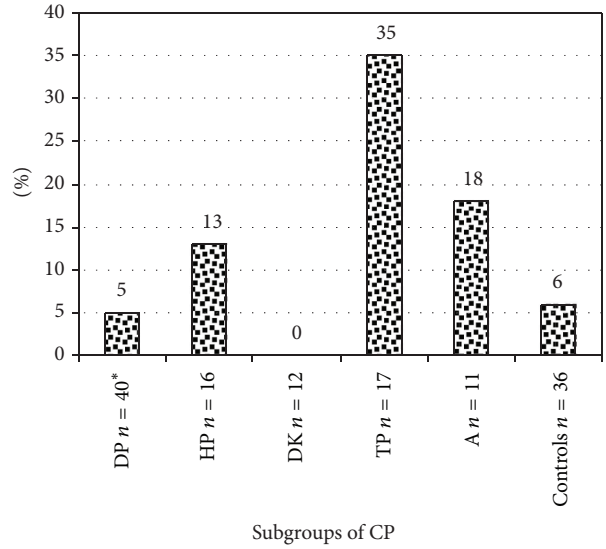

Figure 2: Percentage of patients testing positive for IgA/IgG antibodies to TG6 in different CP subgroups and in a control group. The tetraplegic subgroup compared to the other CP subgroups ($P = 0.006$) and to controls ($P = 0.01$); $^*n = 3$ missing for TG6 antibody analysis. HP: CP-Hemiplegia, DP: CP-Diplegia, TP: CP-Tetraplegia, DK: CP-Dyskinesia, and A: CP-Ataxia.

gastrointestinal dysfunction typically seen in the most severe forms of CP may impact on gut permeability and lead to enhanced immunity to food-derived antigens, we further evaluated whether a correlation existed between TG6 autoantibodies and indicators of feeding difficulties. There was no significant difference for either the group that had treated

Table 1: (a) Clinical data of children with cerebral palsy and autoantibodies to TG6. (b) Summary of gastrointestinal data and laboratory results previously performed [6, 10] for those CP children testing positive for autoantibodies to TG6.

(a)

Patients sex/age (—/yr)	IQ N = normal MR = mental retardation	Diagnosis/subdiagnosis of CP	GMFCS	CP etiology	Neonatal data W = gestational age when born	Brain MRI/CT/US	Epilepsy and treatment
F/17	N	AU/ A	I	Unknown	PN	MRI, normal	—
M/18	MR	TP	V	Asphyxia	PN Asphyxia at birth	US, wide ventricles, small subependymal bleeding	—
M/4	MR	TP	IV-V	Traumatic injury 3 months of age	PN	CT, focal infarct, cortical, subcortical, and basal ganglia damage	Lamotrigine
M/12	N	A	II	Hydrops fetalis	Hydrops fetalis CS; W 28	MRI, cortical and subcortical damage	—
F/18	MR	TP	V	Asphyxia	CS; W 40	CT, focal infarct	—
F/18	N	HF/HP	II	Left cerebral infarction at 1 years of age	PN	MRI and CT, cortical and subcortical damage, and hydrocephalus	—
M/20	MR	TP	V	Asphyxia	CS; W 40	NA	—
F/7	N	HP	I	Asphyxia	CS; W 32	NA	—
F/14	MR	DP	III	Asphyxia	W 26. Asphyxia at birth	NA	Carbamazepine
F/17	MR	HC/ HP	II	Brain malformation at birth	PN	MRI, brain malformation	Valproate
M/20	MR	TP	V	Asphyxia		NA	Valproate
M/9	MR	TP	V	Traumatic injury 1 years of age	PN	CT, focal infarct	Valproate, lamotrigine, and vigabatrin

(b)

Patients sex/age (—/yr)	IgA anti-TG6 cutoff value >14 U/mL	IgG anti-TG6 cutoff value >34 U/mL	IgA AGA	IgG AGA	IgA TG2	IgG TG2	HLA DQ-type	IgA/IgG DGP cutoff >20 U/mL	Small bowel biopsies	GI symptoms
F/17	17.3	—	—	Pos.	—	—	7.8	—	Positive DR staining grade 1	NA
M/18	18.5	—	Pos.	—	Pos.	NA	6.8	—	Positive DR staining grade 2	—
M/4	19.4	—	—	—	—	—	5	NA	NA	—
M/12	23.9	—	—	Pos.	—	Pos.	6	—	NA	Oral dysfunction
F/18	23.1	—	—	Pos.	Pos.	Pos.	2.5	26	IgA deposits and $\alpha\beta/\gamma\delta$ IEL's, DR staining grade 2	C
F/18	72.4	—	—	—	—	—	7	NA	NA	Unspecific abdominal pain
M/20	>100	39.2	—	—	—	—	2.9	NA	NA	GERD, C, D
F/7	—	36.7	—	—	—	—	6.9	NA	NA	—
F/14	—	47.6	—	Pos.	Pos.	Pos.	6.7	32	NA	GERD
F/17	—	60.5	—	—	—	—	2	—	NA	—
M/20	—	69.1	Pos.	—	—	—	NA	—	NA	GERD, PEG, C
M/9	—	74.8	—	Pos.	—	Pos.	6.8	—	NA	PEG, C

Abbreviations: A: Ataxia; HP: Hemiplegia; TP: Tetraplegia; DP: Diplegia; AU: Autism; HC: Hydrocephalus; HF: Heart Failure; GERD: gastroesophageal reflux disease; PN: partus normalis and born term; CS: Caesarean section; C: Constipation; N: normal; MRI: magnetic resonance imaging; CT: computer tomography; US: Ultrasound; PEG: percutaneous endoscopic gastrostomy; GMFCS: Gross Motor Function Classification System, graded I–V; D: Diarrhoea; NA: not applicable; Pos: positive; IEL: intraepithelial lymphocytes.

Table 2: Correlation between indicators of feeding problems and immunity to TG6 and to AGA analysed previously ($n = 99$)* [6].

	AGA positive 41/99* (41%)	AGA negative 58/99* (59%)	P value	TG6 antibody positive 12/96 (12.5%)	TG6 antibody negative 84/96 (87.5%)	P value
Weight	−1.960 SD	−1.00 SD	$P = 0.016$	−1.917 SD	−1.304 SD	$P = 0.318$
BMI	−1.175 SD	−0.136 SD	$P = 0.006$	−0.528 SD	−0.537 SD	$P = 0.987$
PEG $n = 13$	10/13	3/13	$P = 0.005$	2/13	11/13	$P = 0.664$
GERD $n = 11$	6/11	5/11	$P = 0.348$	3/10♦	7/10♦	$P = 0.111$

*90 children age < 18 year were published in the referred paper. Here we have calculated with the whole cohort, $n = 99$, since we have the data available.
♦1 missing for analysis of TG6-antibodies.
SD: weight ± standard deviation (SD), also referred to as weight z-scores, standardized to the Swedish general population by age and sex and based on 3,650 healthy children [20]. GERD: gastroesophageal reflux disease; PEG: percutan endoscopic gastrostomy; AGA: IgA and IgG gliadin antibodies TG6: transglutaminase 6.

GERD or assisted feeding (PEG) with regard to TG6 antibody positivity (Table 2). In contrast, anti-gliadin antibodies previously identified [6] showed a strong association with PEG and also correlated with patient weight and BMI (Table 2).

4. Discussions

The rational for the study was that anti-TG6 antibodies have been described in the context of neurological manifestations in gluten-related disorders and may identify gluten sensitivity in patients serologically negative for anti-TG2 antibodies [23].

There was a significantly higher prevalence of TG6 antibodies in the subgroup of patients with the most severe form of CP but not in the CP group as a whole. Furthermore, a positive correlation of TG6 antibodies with TG2 IgA antibodies but not to other antibodies tested is in keeping with a shared/overlapping mechanistic origin of these autoantibodies as has been suggested [24].

CP is a heterogeneous condition and is likely to have a number of different causes. The most common cause is an ischemic event in the immature brain. Bax et al. showed that magnetic resonance imaging (MRI) abnormalities were detectable in 88% of 351 children with CP [3]. The major findings seen on MRI in the tetraplegic subgroup was white matter damage of immaturity, including periventricular leukomalacia (PVL) (35.1%) and to a lesser extent cortical lesions. The finding of PVL was previously thought to be a typical damage seen in premature-born children. However, this neuroimaging study [3] has shown that white matter damage can be a common finding also among term infants and that in such cases the white matter loss tends to be more extensive. The localisation and severity of the brain lesions depend on the timing of the event in the immature brain and differ between the subgroups of cerebral palsy [3] and so also the clinical outcome of the lesion.

The weight is in general lower in the children with CP compared to normal children and some children with CP require food intake in an alternative way, that is, by a percutaneous gastrostomy (PEG), to gain weight due to their oral and gastrointestinal dysfunction. The most severe forms of CP are in the subgroups of Dyskinesia (DK) and Tetraplegia (TP). These patients also suffer most from gastrointestinal disturbances and have the lowest weight gain of the CP subgroups. In our recently published study from the same cohort of patients, we found mainly IgG-antibodies not only to gluten but also to other food proteins (lactoglobulin and casein) in the DK and TP subgroups of CP who also had the lowest weight [7]. It is likely that these patients have increased intestinal permeability allowing entrance of undigested proteins across the gut barrier into the circulation [25] and therefore have enhanced immunity to food-derived antigens. Such immune reactivity to dietary constituents has also been reported in studies of underweight children from underdeveloped countries [25, 26]. However, we could not find a significant association between lower body weights or the use of PEG and the presence of anti-TG6 autoantibodies; in contrast, AGA antibodies significantly correlated with low weight and assisted feeding (PEG).

There was a significantly higher prevalence of TG6 autoantibodies in the tetraplegic subgroup of patients compared to controls and the other subgroups. In fact there was no antibody response to TG6 in the other subgroup of CP with severe deficiencies, the dyskinetic group. The reason for that is not clear. One possibility could be that the type of brain damage in the tetraplegic subgroup (extensive white matter involvement) may predispose to future development of an immune reaction against TG6. Indeed white matter abnormalities on brain MR imaging are a common finding in patients with CD and headaches [8, 27–30]. The hypothesis of a primary brain insult leading to sensitisation to gluten is supported by a recent study demonstrating increased prevalence of CD in patients with previous head injury [31].

TGs are a family of structurally and functionally related cross-linking enzymes. In mice, TG6 expression is associated with neurogenesis in CNS development and in the mature brain, in neurons in regions associated with motor function including the cerebral cortex and cerebellum [17]. Antibodies isolated from CD patients frequently react with human and mouse neurons. Such antibodies are not the predominant anti-TG2 IgA but either directed to TG6 or cross-reactive between the closely related isozymes, TG2, TG3 and TG6 [32]. Intraventricular injection of such patient-derived

TG-specific immunoglobulins in mice induced Ataxia-like deficits [32]. These data not only indicate that autoantibodies to TG6 could be a marker of brain lesions but that such antibodies may play a part in pathogenesis. The role of TG6 in cerebellar functioning has recently been further highlighted by the identification of mutations in the human gene encoding TG6, causing autosomal dominant spinocerebellar Ataxia [33, 34].

5. Conclusion

This study group of children with CP does not have a higher prevalence of celiac disease than expected in the regional population but they do have more frequent immunoreactivity to gluten and other dietary food components compared to matched controls [6, 7]. Here, we report significantly increased prevalence of anti-TG6 autoantibodies in the tetraplegic subgroup of patients with CP. This antibody response seems not to be correlated with low body weight and the associated immune response to food constituents. The aetiology of anti-TG6 antibodies in this CP subgroup remains unclear, but the results could support the hypothesis of a primary brain insult leading to TG6 autoimmunity.

Abbreviations

A:	CP-Ataxia
AU:	Autism
CP:	Cerebral palsy
CT:	Computer tomography
DP:	CP-Diplegia
DK:	CP-Dyskinesia
GMFCS:	Gross Motor Function Classification System, graded I–V
GERD:	Gastroesophageal reflux
GRSM:	Gluten-related serological markers (IgG/IgA-TG2, IgG/IgA-AGA)
HP:	CP-Hemiplegia
MRI:	Magnetic resonance imaging
PEG:	Percutaneous endoscopic gastrostomy
TP:	CP-Tetraplegia
US:	Ultrasound.

Conflict of Interests

The authors report no potential conflict of interests or commercial or other financial relationships. D. Aeschlimann serves as a scientific advisor/collaborator to Zedira (without financial incentives) but receives royalties from Zedira for patents.

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Research Article

Exploring T Cell Reactivity to Gliadin in Young Children with Newly Diagnosed Celiac Disease

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Class II major histocompatibility molecules confer disease risk in Celiac disease (CD) by presenting gliadin peptides to CD4 T cells in the small intestine. Deamidation of gliadin peptides by tissue transglutaminase creates immunogenic peptides presented by HLA-DQ2 and DQ8 molecules to activate proinflammatory CD4 T cells. Detecting gliadin specific T cell responses from the peripheral blood has been challenging due to low circulating frequencies and heterogeneity in response to gliadin epitopes. We investigated the peripheral T cell responses to alpha and gamma gliadin epitopes in young children with newly diagnosed and untreated CD. Using peptide/MHC recombinant protein constructs, we are able to robustly stimulate CD4 T cell clones previously derived from intestinal biopsies of CD patients. These recombinant proteins and a panel of α- and γ-gliadin peptides were used to assess T cell responses from the peripheral blood. Proliferation assays using peripheral blood mononuclear cells revealed more CD4 T cell responses to α-gliadin than γ-gliadin peptides with a single deamidated α-gliadin peptide able to identify 60% of CD children. We conclude that it is possible to detect T cell responses without a gluten challenge or in vitro stimulus other than antigen, when measuring proliferative responses.

1. Introduction

Celiac disease (CD) is a T cell mediated enteropathy triggered by the ingestion of dietary gluten resulting in villous atrophy and crypt hyperplasia in the small intestine [1]. Specific human leukocyte antigen (HLA) genes are involved in the disease process with restriction primarily limited to HLA-DQ2 (DQA∗05:01, DQB∗02:01 and DQA∗02:01, DQB∗02:02) and DQ8 (DQA∗03:01, DQB∗3:02) [2]. HLA genes encode class II major histocompatibility molecules (MHC), which present antigens to CD4 T cells. There has been remarkable progress in the understanding of the pathogenesis and epitopes involved in the disease process [3–6]. Gliadin, one of the two principle protein components of gluten, contains a number of well-studied T cell epitopes. Deamidation of gliadin peptides by tissue transglutaminase (TTG) type 2 converts glutamine into glutamic acid, resulting in immunogenic T cell epitopes [7].

Despite the current understanding of T cell—peptide—MHC interaction, simple and reliable T cell assays from the peripheral blood to monitor CD activity have been difficult to develop. Peripheral blood biomarkers for CD are hindered by variables such as the type of assay, use of the proper antigen, low circulating frequencies of T cells, and the timing of gluten exposure when the assay is performed. For example, it has been reported that, to obtain sufficient T cells for study without in vitro expansion, CD blood donors on a gluten-free diet need to undergo short-term gluten challenge, to detect IFN-γ T cell responses by enzyme linked immunospot (ELISPOT) assays [8, 9]. However, both gluten-free and gluten-exposed CD patients can have measurable T cell proliferative responses with response rates more

frequent in gluten-exposed patients [10]. Comprehensive epitope mapping studies have identified four immunodominant DQ2 epitopes in treated CD adults (gluten-free diet) followed by a gluten challenge [11]. Such an unbiased epitope mapping project has not been undertaken for children with CD, even though there are several well-studied epitopes in the literature.

In this study, we investigated the peripheral T cell responses to alpha and gamma gliadin epitopes in young children with newly diagnosed and untreated CD. The selected epitopes are known to stimulate T cell clones derived from adult CD patient intestinal biopsies [12, 13]. Recombinant DQ2 and DQ8 proteins with α-gliadin epitopes were created to test T cell clone stimulation without the use of antigen presenting cells, as part of an effort to develop a T cell stimulation assay feasible for large scale, consistent, and rapid assessment of CD activity. We explored the utility of peptide/MHC complexes first in stimulating T cell clones and then peripheral blood mononuclear cells (PBMCs) of newly diagnosed CD children prior to treatment with a gluten-free diet to produce inflammatory cytokines. Subsequently, proliferation assays utilizing a panel of previously described DQ2 and DQ8 peptides on the same CD children provide insight into the possible degenerate nature of α-gliadin peptide binding motifs for HLA-DQ2.

2. Methods

2.1. Subjects and Samples. Subjects were recruited from the Children's Hospital Colorado, and written informed consent was obtained after the nature and possible consequences of the study were explained to individuals. The clinical investigation in this study was conducted in accordance with the Declaration of Helsinki principles, and study approval was provided by the Colorado Multiple Institutional Review Board. Peripheral blood was obtained for T cell assays, TTG antibodies, and HLA genotyping. TTG antibodies were measured from the serum by radioimmunoassay as previously described [14]. HLA-DRB, DQA, and DQB genotyping were performed using linear arrays of immobilized sequence-specific oligonucleotides similar to previously described methodology [15].

2.2. Expression and Purification of Recombinant Protein. The extracellular domains of the HLA-DQ8 α chain (residue 24–204) and β chain (residue 33–221) were coexpressed in S2 drosophila cells (ExpreS2ion Biotechnologies, Denmark). Double deamidated α-gliadin (QQYPSGEGSFQPSQENPQ) was covalently attached to the N-terminus of β chain with a Factor X cleavage site (GGGGSIEGRGSGGGS) between the peptide and the β chain. To stabilize the heterodimer, Fos and Jun leucine zippers were attached to the C-terminus of α chain and β chain via a thrombin cleavage sequence (SSADLVPRGS). Deamidated α-gliadin/DQ8 was extracted from the medium using anti-FLAG M2 (Sigma Aldrich) affinity chromatography. The recombinant protein was purified by Superdex 200 column in buffer containing 10 mMTris, pH 8.0, and 150 mM NaCl. For the α-I-gliadin/DQ2 construct, the extracellular domains of HLA-DQ2 α chain (residue 24–206) and β chain (residue 33–221) were used, expressed in S2 drosophila cells, and purified in a similar manner to the DQ8 recombinant protein construct. The amino acid sequence of α-I gliadin, QLQPFPQPELPY, was covalently attached to the N-terminus of the β chain via TEV cleavage site (GGGGENLYFQGGSGGGS). To stabilize the heterodimer, Fos and Jun leucine zippers were attached via PreScission cleavage site (SSADLEVLFQGP) to the C-termini of α chain and β chain, respectively. The final proteins were confirmed by LC-MS. Diagrams of the two recombinant protein constructs are depicted in Figure 1(a).

2.3. Generation of T Cell Receptor Hybridomas. T cell receptor (TCR) hybridomas, containing the TCR from a DQ2 and DQ8 restricted T cell clone responding to α-gliadin peptides, were created as previously described [16]. The sequences for the $\alpha\beta$ TCR clones in Figure 2 were a kind gift from Ludvig Sollid. The sequences for the $\alpha\beta$ TCR clones in the supplemental Figures (see supplementary material available online at http://dx.doi.org/10.1155/2014/927190) were derived from published information on gene usage [13]. Briefly, a single TCR sequence, α and β chains linked by the PTV1.2A sequence, was cloned into MSCV-based retroviral vectors carrying green fluorescent protein (GFP) (pMIGII) [17], followed by production of replication-incompetent retroviruses encoding TCR sequences. The 5KC hybridoma line lacking TCR α and β chains was used to reconstitute TCRs [18]. The transduced 5KC hybridomas were sorted by GFP expression and TCR expression was confirmed by staining with anti-mouse TCRβ antibody (clone H57-597, BD Biosciences). Alternatively, the expression of plasmids in the pMSCVpuro retroviral vector (Clontech) was transfected to AmphoPack-293 or GP2-293 (VSV-G envelope) packaging cells (Clontech) to produce retrovirus and mouse 5KC cells were spin-infected with retroviral supernatants and cultured with puromycin. TCR expression was confirmed in isolated single clones by staining with anti-mouse CD3 (clone 145-2C11; BD Biosciences) or mouse TCRβ antibody (clone H57-597; BD Biosciences).

2.4. Cytokine Stimulation Assays. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque and resuspended at a density of 10^6/mL in IMDM-C media (IMDM supplemented with 5% heat inactivated human AB serum, 100 μg/mL Pen-Strep, 100 μM MEM NEAA, and 50 μM 2-mercaptoethanol). 2×10^5 PBMCs or T cell hybridomas in 200 μL of media were added to a 96-well tissue culture plate coated with 1 μg/well of recombinant protein and cultured at 37°C in 5% CO_2 overnight. Secreted cytokine was measured in the supernatant by electrochemiluminescence assay (Meso Scale Discovery) for human IFN-γ, IL-2, and by ELISA for IL-17 (R&D Systems). PBMCs in culture without protein (background) were a negative control, while anti-CD3 stimulation (OKT3, eBioscience) was a positive control. DQ antibody SPV-L3 (Abcam, Cambridge, UK), 1a3 (Leinco, St. Louis, MO, USA), or HB-144 (ATCC, Manassas, VA, USA) was added at either 10 μg/mL or at

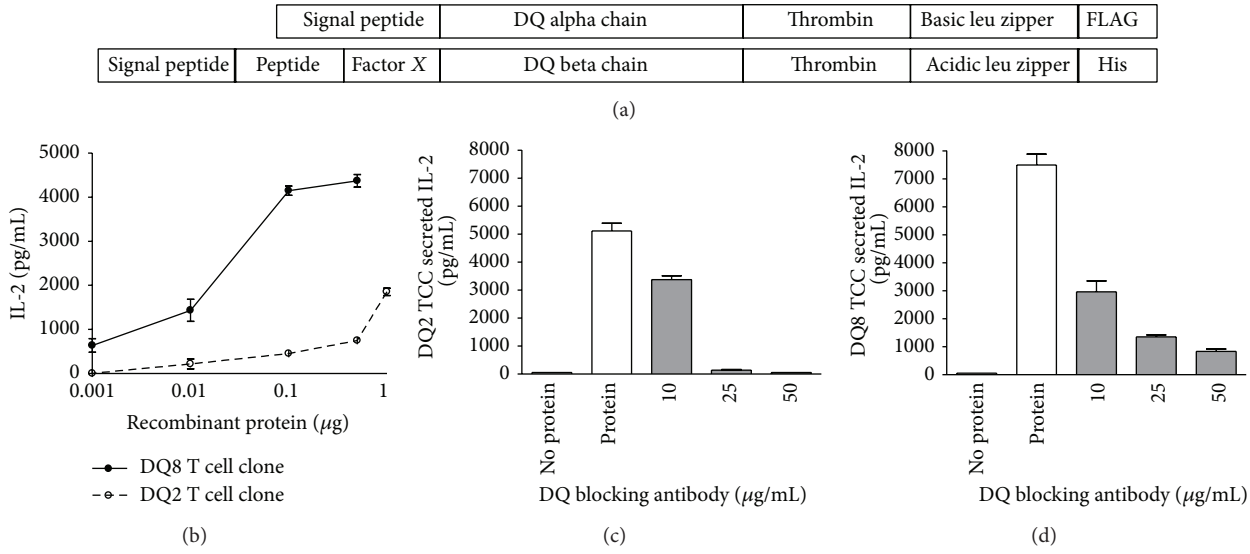

Figure 1: Recombinant DQ8 and DQ2 proteins with gliadin epitopes stimulate T cell clones. (a) Diagrams of the constructs used to produce recombinant protein for deamidated α-gliadin p1E, p9E/DQ8, and α-I gliadin/DQ2. The amino acid sequence of the α-gliadin peptide in DQ8 is QQYPSG**E**GSFQPSQ**E**NPQ and the α-I gliadin peptide (QLQPFPQP**E**LPY) with DQ2. Thrombin, TEV, and PreScission are protease cleavage sites incorporated into the protein constructs. (b) T cell clones restricted to either DQ8 or DQ2 produce IL-2 in response to the deamidated α-gliadin/DQ8 or α-I gliadin/DQ2 recombinant protein, respectively. (c) The DQ2 and (d) DQ8 T cell responses can be blocked in a dose dependent manner with a monoclonal DQ antibody.

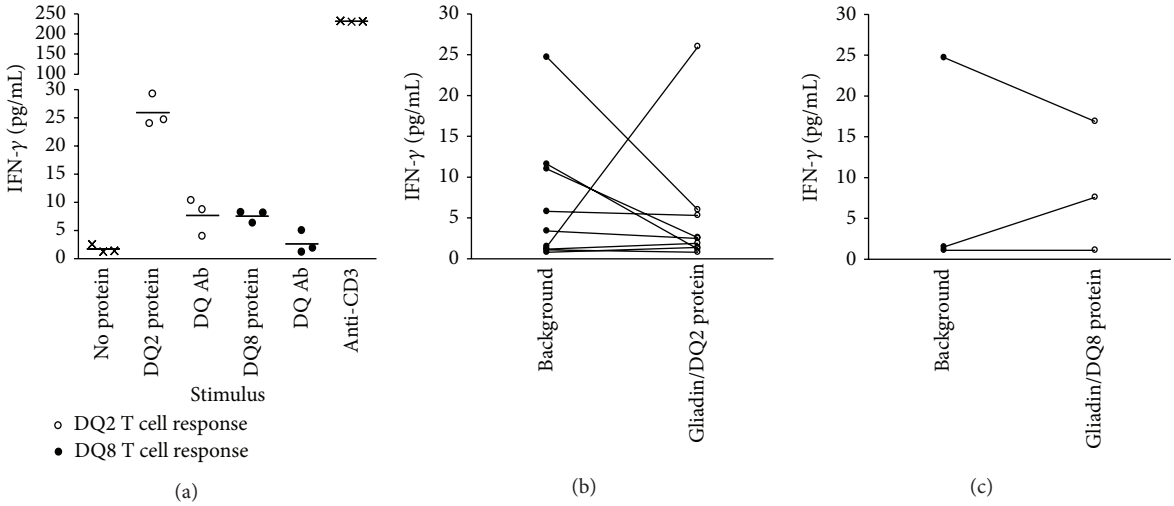

Figure 2: Recombinant peptide/MHC protein stimulates IFN-γ production from bulk unfractionated PBMCs. (a) Stimulation of PBMCs from a single subject in triplicate having both HLA-DQ8 and DQ2 alleles showing response to the recombinant proteins greater than background. The IFN-γ response is DQ restricted as it can be blocked with a monoclonal DQ antibody. An anti-CD3 monoclonal antibody is used to stimulate T cells as a positive control. The α-gliadin p1E, p9E peptide (QQYPSG**E**GSFQPSQ**E**NPQ) is present in the DQ8 recombinant protein, while α-I gliadin (QLQPFPQP**E**LPY) is present in the DQ2 protein. (b) Summative stimulation data from nine subjects all with HLA-DQ2 (DQA$*$05:01, DQB$*$02:01). (c) Data from three subjects having the HLA-DQ8 (DQA$*$03:01, DQB$*$03:02) allele.

varying concentrations for blocking experiments. All study subjects had cytokine stimulation assays performed.

2.5. CFSE Proliferation Assay. Isolated and unfractionated PBMCs were suspended at a density of 10^6/mL in CFSE labeling buffer (1% BSA in PBS). Cells were labeled with 1 μM CFSE (eBioscience) for 10 minutes at 37°C. Labeling was quenched by adding chilled IMDM-C media at 5 times the volume at 0°C; cells were then incubated on ice for 5 minutes. Labeled cells were washed in PBS with 1% human AB serum, resuspended in media, and plated into a 24-well tissue culture plate at 10^6 cells/well in 1 mL of media. Peptides (Genemed Synthesis Inc.) were HPLC purified (>95%), dissolved in PBS at a neutral pH, and used at a concentration of 10 μM. Pentacel vaccine (Sanofi Pasteur) was added at 2 μL per well as a positive control. After seven days of incubation

Table 1: Clinical characteristics, TTG antibody levels, histology, and HLA genotype of study participants.

Case	Age (yrs)	Sex	TTG Ab level*	Histology marsh score	HLA DQ and DR alleles					
					DRB1	DQA1	DQB1	DRB2	DQA2	DQB2
1	4	F	0.145	2	0404	0301	0302	0301	0501	0201
2	5	F	0.130	0	0403	0301	0302	0301	0501	0201
3	5	M	0.064	1	0301	0501	0201	1602	0501	0301
4	5	M	0.178	3b	0301	0501	0201	0301	0501	0201
5	4	F	1.127	3c	0301	0501	0201	0801	0401	0402
6	7	F	0.877	No biopsy	0701	0201	0202	1104	0501	0301
7	7	F	0.608	3c	0301	0501	0201	1301	0103	0603
8	7	M	0.755	3b	0301	0501	0201	0701	0201	0202
9	7	F	0.461	3b	0301	0501	0201	1501	0102	0602
10	12	F	0.624	3b	0701	0201	0202	1101	0501	0301
11	13	M	0.511	3b	0403	0301	0302	0701	0201	0202
12	9	F	0.169	3b	0301	0501	0201	0301	0501	0201

*TTG Ab ≥ 0.05 is elevated.

at 37°C in 5% CO_2, nonadherent cells were harvested and stained for FACS analysis using antibodies to CD4 (RPA-T4, BD Bioscience) and CD8 (RPA-T8, BD Bioscience). FACS analysis was done using a Becton-Dickenson FACS Caliber. Ten of the 12 study participants gave adequate numbers of PBMCs to perform CFSE proliferation assays.

2.6. Statistical Analysis. The percentage of $CD4^+CFSE^{lo}$ after proliferation to a given stimulus was compared with a paired Student's t-test as conditions were matched in the same subject. Response rates between α-gliadin and γ-gliadin peptides were compared with a two-sided Fisher's exact text. For all statistical tests, a two-tailed P value of <0.05 is considered significant. Analyses were performed using GraphPad Prism 4.0 software (La Jolla, CA).

3. Results

3.1. Subjects. Subjects with new-onset Celiac disease ($n = 12$) were recruited from the Children's Hospital Colorado Celiac Disease Center clinics. The study protocol was approved by the Institutional Review Board and written informed consent was obtained from all study participants. The Celiac subjects were young children and adolescents, with a mean age of 7.1 years, known to be TTG antibody positive and not on a gluten-free diet prior to having a small intestine biopsy. At the visit for intestinal biopsy, 11/12 (92%) patients had a biopsy and blood was collected for TTG antibody levels, HLA genotyping, and immune assays. Demographic and clinical characteristics are presented in Table 1. All of the subjects had positive TTG antibody levels and the majority 8/12 (75%) had stage 3 Marsh scores on histologic examination of a small intestine biopsy. HLA typing revealed that 9/12 (75%) subjects had the high risk HLA-DQ2 (DQA1∗05:01, DQB1∗02:01) allele and 3/12 (25%) had HLA-DQ8 (DQA1∗03:01, DQB1∗03:02) in addition to DQ2.

3.2. T Cell Hybridomas Respond Robustly to Recombinant Peptide/MHC Protein. We produced recombinant peptide/MHC protein to known α-gliadin epitopes presented by HLA-DQ2 or DQ8 (Figure 1(a)). The two recombinant proteins, α-I gliadin/DQ2 and deamidated α-gliadin p1E, p9E/DQ8, are bioactive and able to robustly stimulate T cell hybridomas created from CD4 T cells cloned from small intestine lesions of adult CD subjects [12, 13]. The responses of these T cell hybridomas, measured by secreted IL-2, are dose dependent and required minimal amounts of protein (less than 1 μg/well) for stimulation (Figure 1(b), supplemental Figure 1(a)). The T cell responses are restricted to DQ2 or DQ8, depending on the class II molecule of the recombinant protein, as a DQ monoclonal antibody added in culture was able to abrogate IL-2 secretion (Figures 1(c) and 1(d) and supplemental Figures 1(b) and 1(c)).

3.3. Detection of IFN-γ Responses to Recombinant Peptide/MHC Proteins. Having recombinant α-gliadin/DQ proteins able to robustly stimulate T cell hybridomas, we evaluated the ability of the recombinant proteins to stimulate T cell responses from the peripheral blood of newly diagnosed CD subjects. We measured secreted cytokine responses (IFN-γ, IL-2, and IL-17) after culturing bulk unfractionated PBMCs in the presence of the α-I gliadin/DQ2 or α-gliadin p1E, p9E/DQ8 recombinant protein. Figure 2(a) depicts IFN-γ responses to an individual having both the DQ2 (DQA1∗05:01, DQB1∗02:01) and DQ8 (DQA1∗03:01, DQB1∗03:02) alleles, which are identical to that of the recombinant proteins. There are responses to the protein greater than that of background alone and the responses are blocked by a DQ monoclonal antibody, suggesting that the measured responses are due to reactivity to the α-gliadin/DQ proteins. The cumulative data from subjects having corresponding HLA alleles to that of the recombinant protein, however, failed to identify T cell reactivity above background responses (Figures 2(b) and 2(c)). The measured IL-2 and IL-17 responses were not greater than background in any of the subjects (data not shown).

3.4. Proliferation of CD4 T Cells to the α- and γ-Gliadin Peptides. We next examined T cell responses to eleven known α- and γ-gliadin epitopes [12, 19, 20], in contrast to recombinant peptide/MHC protein, previously identified from CD patients. CD4 T cell proliferation was assessed from the

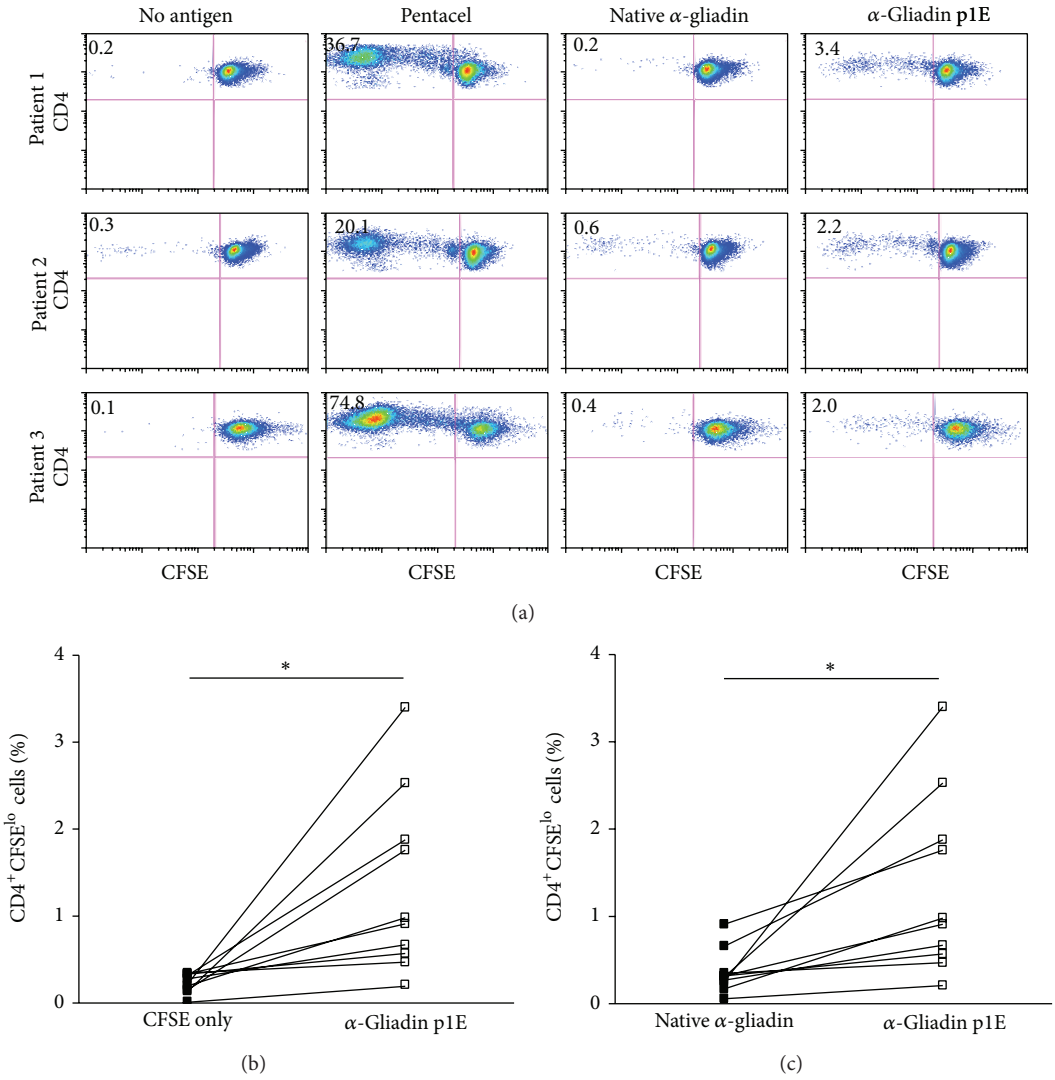

Figure 3: Proliferation of unfractionated PBMCs with α-gliadin peptides. (a) Representative data from three newly diagnosed Celiac subjects with 7-day CFSE proliferation assays. CD4 T cells proliferate in response to the α-gliadin p1E deamidated peptide without the in vitro addition of cytokines. (b) Summary data of proliferative responses comparing CFSE only (no antigen background) to the α-gliadin p1E peptide. (c) Proliferation of native α-gliadin to the α-gliadin p1E deamidated peptide. $^{*}P < 0.01$ using a paired t-test. Pentacel (positive control) is a childhood vaccine containing immunogens directed against diphtheria, tetanus, pertussis, poliomyelitis, and *Haemophilus influenzae* type b.

peripheral blood of newly diagnosed CD subjects. Figure 3 shows the proliferation results after bulk unfractionated PBMCs were labeled with CFSE and cultured for 7 days in the presence of a single gliadin peptide without the addition of any in vitro stimulus, that is, no IL-2, anti-CD3, or anti-CD28. Of the individuals having CFSE proliferation assays performed, there were robust responses to the α-gliadin$_{228-240}$ peptide (SGQGSFQPSQQNP), especially with a deamidated glutamate residue at the pocket 1 position. In all of the subjects, the single deamidated peptide (SG**E**GSFQPSQQNP) resulted in significantly more proliferation as measured by $CD4^{+}CFSE^{lo}$ cells compared to background (no antigen in culture) and the native α-gliadin peptide (Figures 3(b) and 3(c)). Interestingly, none of the subject's PBMC proliferated in response to the double deamidated α-gliadin peptide (α-gliadin p1E, p9E) and few responded (3/10) to the α-I-gliadin peptide, which are the peptides in the DQ8 and DQ2 recombinant protein, respectively (Table 2). Evaluating proliferative responses with a stimulation index ($CD4^{+}CFSE^{lo}$ condition/$CD4^{+}CFSE^{lo}$ background) greater than 3 revealed 12/60 (20%) responses to α-gliadin peptides compared to 3/50 (6%) of γ-gliadin peptides ($P = 0.049$). Overall, there were more proliferative responses to α-gliadin peptides compared to γ-gliadin in young children with newly diagnosed CD (Figure 4). In those children responding to two or more peptides, all three were HLA-DQ2/2 homozygotes (Table 3). Interestingly, the HLA-DQ2/2 children responded to epitopes which have been previously reported in the literature to be

Table 2: Proliferative responses to α- and γ-gliadin epitopes.

Epitope	Amino acid sequence*	Proliferation response**
Native α-gliadin	SGQGSFQPSQQNP	1/10 (10%)
α-Gliadin p1E	SG**E**GSFQPSQQNP	6/10 (60%)
α-Gliadin p9E	SGQGSFQPSQ**E**NP	2/10 (20%)
α-Gliadin p1E, p9E	SG**E**GSFQPSQ**E**NP	0/10 (0%)
α-I-Gliadin	QLQPFPQP**E**LPY	3/10 (30%)
α-II-Gliadin	PQP**E**LPYPQPQL	0/10 (0%)
Native γ-gliadin	FPQQPQQPYPQQPQQ	0/10 (0%)
γ-Gliadin p1E	FP**E**QPQQPYPQQPQQ	1/10 (10%)
γ-Gliadin p9E	FPQQPQQPYP**E**QPQQ	0/10 (0%)
γ-Gliadin p1E, p9E	FP**E**QPQQPYP**E**QPQQ	2/10 (20%)
γ-I-Gliadin	PEQPQQSFP**E**QERP	0/10 (0%)

*Glutamic acid (E) residues in bold are formed by tissue transglutaminase mediated deamidation. Underlined residues form the MHC class II peptide binding register.

**A stimulation index ≥ 3 is considered a response.

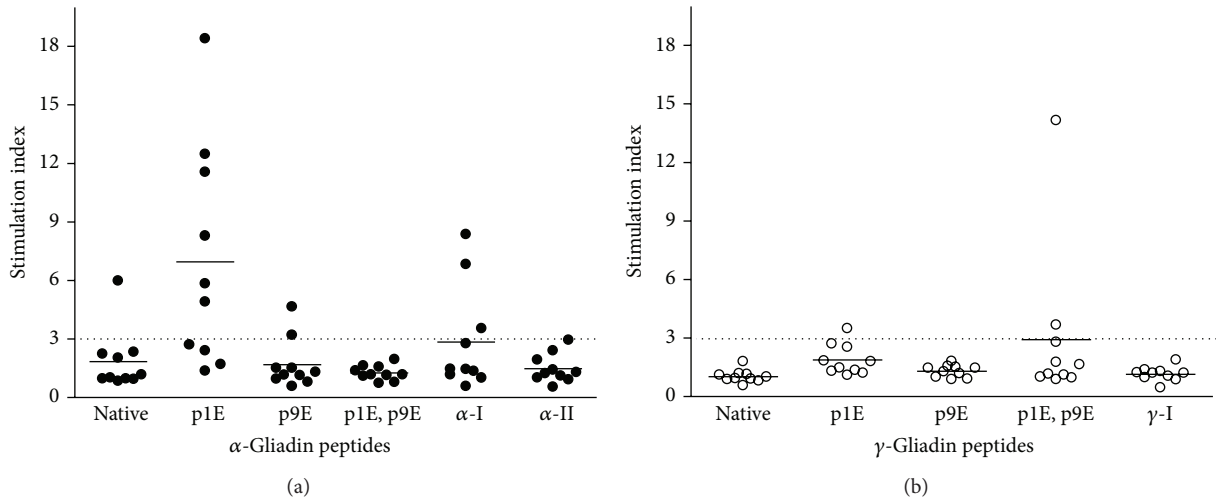

Figure 4: Proliferation of PBMCs from newly diagnosed Celiac patients to α- and γ-gliadin peptides.(a) Proliferative responses to α-gliadin and (b) γ-gliadin epitopes. Celiac subjects proliferate more in response to α-gliadin peptides compared to γ-gliadin, especially the peptide deamidated at pocket 1 in which 6/10 subjects responded. Overall, there are 12/60 responses to α-gliadin peptides compared to 3/50 for γ-gliadin ($P = 0.049$ with a Fisher's exact test). Dotted line is at a stimulation index ($CD4^+CFSE^{lo}$ cells at background/peptide condition) of 3, above which a responder is considered.

DQ8 restricted, suggesting that certain α-gliadin peptides may be presented by either DQ8 or DQ2, particularly after selective deamidation.

4. Discussion

In the present study, we investigated peripheral T cell responses from young children with newly diagnosed CD prior to treatment with a gluten-free diet for two purposes: (1) develop a consistent and rapid assay to monitor peripheral T cell responses and (2) assess T cell responses to a panel of α- and γ-gliadin epitopes. To develop a consistent and rapid assay using limited PBMCs, α-gliadin/DQ2 and DQ8 recombinant protein constructs were produced which have the ability to robustly stimulate six different T cell clones derived from adult CD patients, suggesting the utility of this approach with polyclonal T cells from the peripheral blood. However, stimulation of PBMCs from young untreated children with CD did not uniformly elicit T cell responses. There are several possible reasons for the inability to detect reactivity with these protein constructs including the possibility that CD donors lack T cells that recognize a single DQ2 or DQ8 α-gliadin epitope. Interestingly, the double deamidated α-gliadin epitope covalently linked to recombinant DQ8 protein failed to elicit proliferative T cell responses in our study population, while three individuals with proliferative responses to α-I gliadin did not produce IFN-γ above background levels when stimulated with the recombinant α-I gliadin/DQ2 protein. As these epitopes were identified from adult CD patients, our data supports the heterogeneity of T cell responses in CD and that children

Table 3: Overview of the T cell responses to tested DQ2 and DQ8 gliadin epitopes.

Epitopes		α-gliadin						γ-gliadin				
		DQ8 epitopes				DQ2 epitopes			DQ8 epitopes			
Case	HLA-DQ genotype	Native	p1E	p9E	p1E, p9E	α-I	α-II	γ-I	Native	p1E	p9E	p1E, p9E
2	2/8		■									
3	2/7		■									
4	2/2		■			■						
6	2/7											
7	2/6											
8	2/2	■	■	■		■						■
9	2/6											
10	2/7		■									
11	2/8											
12	2/2		■	■		■				■		■

Peripheral T cell responses as measured by $CD4^+CFSE^{lo}$ proliferated cells for each individual with correlation to HLA-DQ genotype. Black boxes represent a response to the peptide with the SI ≥ 3. Gliadin epitopes are denoted as previously reported in the literature to be presented by HLA-DQ2 (α-I gliadin$_{57–68}$ QLQPFPQPELPY, α-II gliadin$_{62–73}$ PQPELPYPQPQL, and γ-1 gliadin$_{139–152}$ PEQPQQSFPEQERP) or HLA-DQ8 (α-gliadin$_{228–240}$ SGQGSFQPSQQNP and γ-gliadin$_{65–79}$ FPQQPQQPYPQQPQQ with and without deamidation at p1 and p9). Three of the new-onset CD children responding to two or more peptides have the DQ2/2 genotype.

may respond to different epitopes than adults [21]. Second, the donor's T cells may already be maximally stimulated as our patient population had not yet started a gluten-free diet. This is in agreement with studies reporting the need for a short-term gluten challenge in adult CD patients already on a gluten-free diet to elicit robust peripheral T cell responses [8, 9]. However, peripheral T cell responses can exist at disease onset and disappear with a gluten-free diet. Finally, it is possible that measuring secreted IFN-γ may not be the best way to detect responses, and other methods for detecting antigen specific T cell responses to gliadin may be necessary, such as using gliadin/DQ2 fluorescent tetramers [22].

In addition to evaluating peptide/MHC recombinant protein to elicit T cell responses, we utilized proliferation assays with bulk unfractionated PBMCs to assess peptide reactivity. Stimulation of PBMCs from newly diagnosed and untreated CD children is better detected using proliferation assays rather than measurement of secreted cytokine. In our panel of DQ2 and DQ8 restricted antigens, we found that 6/10 children responded to at least one of the peptides tested, and the remainder did not respond to any tested peptide. It is notable that a peptide traditionally considered to be a DQ8 epitope, α-gliadin p1E ^{228}SG**E**GSFQPSQQNP^{240}, showed the greatest ability to stimulate T cells in 60% of children, even though only two children expressed the HLA-DQ8 allele (all of the children had at least one DQ2 allele). Furthermore, there were three subjects that had responses to two or more peptides and all three were DQ2/DQ2 homozygotes (Table 3). The peptide binding grooves of HLA-DQ2 and DQ8 share structural similarity with both molecules capable of anchoring peptides with acidic side chains (glutamic and aspartic acid) at pockets 1 and 9 [23]. With tissue transglutaminase present to deamidate gliadin peptides, it is plausible to hypothesize that certain deamidated epitopes have the potential to be presented by both the DQ2 and DQ8 molecules [24, 25]. There is precedence for this concept as both HLA-DR1 and DR4 class II molecules are capable of presenting the hemagglutinin peptide, HA$_{306–318}$, to T cells [26, 27].

In summary, T cell reactivity in young children with newly diagnosed and untreated CD is heterogeneous but favors reactivity to α-gliadin epitopes more than γ-gliadin. It is unlikely that a single gliadin epitope will elicit T cell responses in all individuals and a short-term gluten challenge may be necessary to detect ample T cell reactivity in the peripheral circulation. However, it is possible to detect T cell responses without a gluten challenge or in vitro stimulus other than antigen, when measuring proliferative responses. A more comprehensive screening of gliadin epitopes in young children with newly diagnosed CD is necessary to identify peripheral blood T cell reactivity, followed by repeat assessments over time to correlate responses to disease activity and treatment with a gluten-free diet. Monitoring peripheral T cell responses to gliadin epitopes present at disease onset, which can disappear with a gluten-free diet, has clinical utility in identifying cases of refractory CD or in assessing the effectiveness of emerging therapies for CD treatment.

Conflict of Interests

The authors E. Liu, K. McDaniel, S. Case, L. Yu, and A. W. Michels have no conflict of interests regarding the publication of this paper. The authors B. Gerhartz, N. Ostermann, G. Fankhauser, V. Hungerford, C. Zou, M. Luyten, and K. J. Seidl are all employees at Novartis and may own Novartis stock.

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Review Article

A Metabolomic Perspective on Coeliac Disease

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Metabolomics is an "omic" science that is now emerging with the purpose of elaborating a comprehensive analysis of the metabolome, which is the complete set of metabolites (i.e., small molecules intermediates) in an organism, tissue, cell, or biofluid. In the past decade, metabolomics has already proved to be useful for the characterization of several pathological conditions and offers promises as a clinical tool. A metabolomics investigation of coeliac disease (CD) revealed that a metabolic fingerprint for CD can be defined, which accounts for three different but complementary components: malabsorption, energy metabolism, and alterations in gut microflora and/or intestinal permeability. In this review, we will discuss the major advancements in metabolomics of CD, in particular with respect to the role of gut microbiome and energy metabolism.

1. Introduction

Coeliac disease (CD, MIM 212750), first described in 1887, is a common complex chronic immune-mediated disorder with a known (gluten) environmental trigger. Recent surveys indicate that it may affect 1 in 105 subjects in the United States [1], 1 in 67 Finnish school children [2], and 1 in 230 in Italian school age children [3], with seroprevalence of about 1% in subjects of white European origin [4, 5].

Coeliac disease has a strong genetic component with multiple contributing genes: the most important and best characterized genetic risk factors are the HLA class II genes DQ2 and/or DQ8 which are located on chromosome 6p21. More than 97% of patients have at least one of the two genes: most patients (>90%) carry the DQ2 gene, while the rest expresses the DQ8 gene. HLA-DQ2 is encoded by the HLA-DQA1*05 allele (α chain) and the HLA-DQB1*02 (β chain) [6, 7]. Common to many other autoimmune disorders, the two alleles are often present in the *cis* conformation on the DR3 haplotype [8]; HLA-DQ2 and HLA-DQ8 are necessary but not sufficient for the development of CD.

Genome wide association studies indicated 39 non-HLA loci to be predisposing to CD [9–11]. Altogether, the nonHLA loci explain only 5% of the risk for CD [6], while the HLA loci account for 35% of the risk [7].

Several of these nonHLA CD susceptibility genes are associated with other diseases/traits [6] such as type 1 diabetes [12, 13], rheumatoid arthritis [14], and systemic lupus erythematosus [15] indicating a possibly shared genetic background with other diseases [7].

The environmental trigger of coeliac disease is gluten, a protein complex formed by gliadin and glutenin, which is

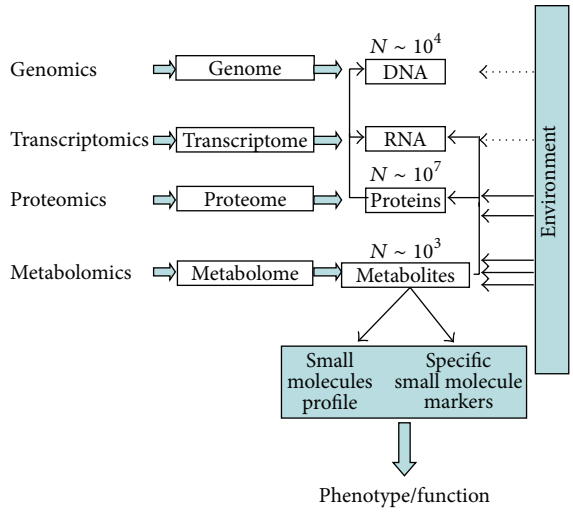

Figure 1: Relationships between the omics sciences.

found in wheat and related grain species like barley and rye and also in processed food where it is used to enhance food texture and as a stabilizing agent.

The active disease component is gliadin [16] with the α-, β-, γ-, and ω-fractions. These fractions are rich in proline and glutamine and resistant to enzymatic digestion; large proline/glutamine-rich peptides accumulate in the smallest intestine, triggering an abnormal innate and adaptive immune response in genetically predisposed subjects.

The response of the adaptive immune system is due to the gliadin-reactive CD4+ T cells; HLA-DQ molecules bind to these peptides which are deamidated by the intestinal brush border enzyme tissue transglutaminase; these complexes interact with the T-cell receptor on T cells leading to T-cell activation with subsequent release of proinflammatory cytokines and the production by B-cells of specific antibodies (anti-tissue transglutaminase and endomysial antibodies) [17, 18].

The role of the innate immune systems in CD is less clear [7]. Increased expression of interleukin-15 has been observed [19]; enhanced intestinal permeability has been also observed [20], induced by zonulin [21], whose release is mediated by gluten activated CXCR-3 [22, 23]. Loss of functionality in the intestinal barrier permits the passage of immunoreactive peptides and other antigens from the gut lumen to the lamina propria, with subsequent triggering of the innate immune system.

2. Metabolomics

The advent of high-throughput techniques led to a rapid expansion of data sets originated from the analysis of gut microbiota and currently several ongoing projects are aimed at the study and definition of the microbiome [24, 25]. In this framework, metabolomics is playing a crucial role.

Since the systematic genome sequencing of the first free-living microbe [26], we have seen the rising of genome-wide expression profiling methods, aimed to understand complex biological systems on a large scale [27]. The fast development of genomics, transcriptomics, proteomics, and the other *omics* disciplines is the consequence of this new scientific paradigm.

In this framework, metabolomics has already proved in the past decade to be a useful complement for the characterization of several physiological and pathological conditions and offers promises as a clinical tool [28]. Metabolomics is based on the analysis of the measured dynamic changes of a living organism in response to genetic modifications or physiological stimuli such as nutrients, drugs treatment, or toxic insults [29].

The metabolome, the complete collection of all metabolites contained in a biological specimen, can be considered the downstream end-product of the complex interaction of genome, transcriptome, proteome, and the environment: it can be regarded as a cascade linking genome to the phenotype [30] (Figure 1). The metabolome, consisting of low-molecular weight chemical intermediates [31], can be considered as an amplified version of gene expression. While changes in gene expression levels (and thus proteins) will have only small effects on metabolic fluxes, they must have large effects on metabolic pathways (and thus metabolites concentrations) [27]. From this point of view, the metabolite space represents the optimal level at which changes in biological systems are analyzed with optimal sensitivity [32] under conditions of negligible effects on the global phenotype [33].

Metabolomics does not rely on the measurement of a single metabolite but considers the spectrum of (possibly) all metabolites as a whole, taking a holistic approach; this offers evident advantages with respect to a targeted search of metabolites; indeed, no assumption is required on the identity of the metabolites that are or may be relevant for the biological phenomenon under investigation.

The main analytical techniques employed in metabolomics (Boxes 1 and 2) are nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). Both MS and NMR methods provide information on the relative and absolute concentrations of different classes of metabolites in a single measurement (see Box 3) and can be also used

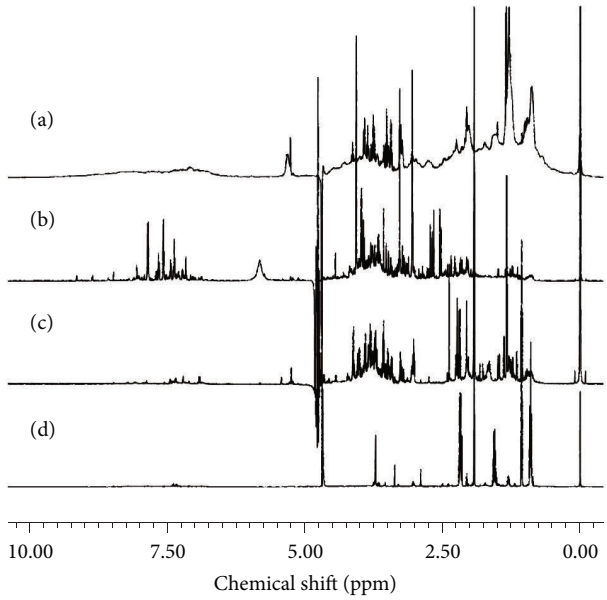

Figure 2: Examples of NMR profiles of (a) serum, (b) urine, (c) saliva, and (d) faecal extract.

to determine metabolite structures providing mechanistic insights.

The most common biological specimens used in metabolomics are serum/plasma and/or urines, firstly, because they can be collected with low invasiveness, and, secondly because, as they contain thousands of metabolites, they are rich in biological information at the systemic level; a number of other biofluids such as saliva [34], tissue extract [35], cerebrospinal fluid [36], bile [37], seminal fluid [38], amniotic fluid [39], synovial fluid [40], exhaled breath condensate [41, 42], and faecal extracts [43] can also be studied. Figure 2 shows typical NMR spectra of four different biofluids (Box 3).

Targeted and untargeted approaches are possible in metabolomics, the former focusing on the analysis of a subset of known compounds or class thereof (targeted MS and NMR) and the latter focusing on the whole array of metabolites within the detection limit of the technique employed (untargeted MS and NMR). Using both approaches, hundreds to thousands of metabolites are measured. Data are usually analyzed following the classical metabolomics pipeline (Box 4), and information is extracted using state-of-the-art statistical tools (Box 5).

Metabolomics has provided significant information on a wide range of pathologies, such as cancer [44], meningitis [45], neurological disorders [46], cardiovascular diseases [47], inborn errors of metabolism [48], and CD [49–51]. The first metabolomics investigation of CD revealed that a metabolic fingerprint for coeliac disease can be defined [49], which accounts for three different but complementary components: malabsorption, energy metabolism, and alterations in gut microflora and/or intestinal permeability.

In this review, we will discuss the major advancements in metabolomics of CD with respect to the role of gut microbiome and energy metabolism.

3. Gut Microbiota and CD

Recent studies [52–56] pointed to the possible role of intestinal microbiota (faecal and duodenal species) in the development of coeliac disease. A summary of the most relevant findings in this research area is reported in Table 1 together with the associated bacteria strains involved.

Nistal et al. [57] compared the differences between gut microorganisms in the upper small intestinal mucosa in adults and in children. A two-time higher number of microbial genera have been identified in adults compared to children, although the dominant genera were very similar: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria,* and *Fusobacteria*. Differences in the amount of bacterial communities between adult and juvenile groups can be also directly connected with the age of investigated subjects.

A comparison between healthy, diseased, and treated coeliac adults showed a lower number of *Streptococcus* spp. and *Prevotella* spp. families in untreated coeliac adults. Interestingly, similar patterns were also observed in CD children, suggesting that these bacterial populations may have been modified by changes in the intestine environment caused by active CD.

In the study by Nadal et al. [58], the bacterial species present in faeces and duodenum of children with active and treated CD were compared with a healthy control group. The ratio of harmless Gram-positive bacteria (*Lactobacillus* + *Bifidobacterium*) to potentially harmful Gram-negative (*Bacteroides*/*Prevotella* + *E. coli*) bacteria was significantly lower in CD patients than in controls, while no distinction was possible between active and inactive CD.

Sánchez et al. [59] applied denaturing gradient gel electrophoresis (DGGE) to analyze intestinal microbiota from biopsy specimens obtained from three groups of children, investigating the composition of *Bacteroides*, *Bifidobacterium*, and Lactic acid bacteria in duodenal biopsies of patients with active and treated coeliac disease. Dysbiosis in *Bacteroides* (the most abundant intestinal bacterial group) was observed, with a significant reduction in coeliac and coeliac treated patients in comparison with the control group. Moreover, it was observed that a treatment with gluten-free diet did not restore the balance of the *Bacteroides* composition. Interestingly, it was observed that the lactic acid bacteria (*Lactobacillus*) and the *Weissella* family were more abundant and diverse in treated coeliac and control patients than in patients with untreated coeliac disease. The authors suggested that some of the changes in duodenal bacterial community could be due to the inflammatory consequences of the active phase of the disease; nonetheless, the influence of different dietary habits could not be discarded.

Biopsies from treated coeliac children were analyzed in a study by di Cagno et al. [63], that also confirmed that a gluten-free diet lasting two or more years is not able to restore completely the microbiota. In addition, a higher diversity of the *Eubacteria* community was observed in the duodenum

Table 1: Most relevant findings, and associated references, for studies linking gut microbiota and CD.

References	Type of sample	Technique	Microbiota phylum/class	Relevant findings
Wacklin et al. (2013) [55]	Mucosa biopsy	PCR-DGGE (real-time polymerase chain reaction, denaturing gradient gel electrophoresis), 16S rRNA sequencing	*Firmicutes Bacteroides Proteobacteria Actinobacteria*	Diversity in mucosal microbiota of celiac disease patients is associated with the symptoms of the disease.
Nistal et al. (2012) [57]	Duodenal biopsies	PCR (polymerase chain reaction)	*Firmicutes Proteobacteria Bacteroidetes Actinobacteria Fusobacteria*	Composition of small intestinal microbiota is similar between adults and children; there is higher number of *Streptococcus* and *Prevotella* in healthy subjects.
Nadal et al. (2007) [58]	Duodenal biopsy	FISH (Fluorescent in situ hybridization), Flow cytometry detection.		In faeces and duodenum of CD children, smaller amount of harmless bacteria (*Lactobacillus* and *Bifidobacterium*) and higher number of harmful bacteria are found (*Bacteroides/Prevotella* + *E. coli*) compared to healthy children.
Sánchez et al. (2010) [59]	Duodenal biopsy	PCR-DGGE	*Bacteroidetes*	Reduced number of intestinal microbiota in CD children but also in treated CD children was noticed. Treatment with GFD does not restore the bacteria composition.
Sánchez et al. (2011) [60]	Faeces samples	PCR-DGGE		Studies were carried out on stools of infants with high/low risk of CD and different types of milk feeding. High-risk infants have higher prevalence of *Bacteroides vulgatus*, whereas low-risk infants have higher population of *B. uniformis, B. ovatus,* and *B. plebeius* considering the subgroup of either breast-fed or formula-fed infants.
Cheng et al. (2013) [61]	Duodenal biopsy	qRT-PCR (quantitative real-time PCR)	*Bacilli Bacteroides Clostridium Proteobacteria*	Overall microbiota composition in the duodenal mucosa is comparable between healthy and CD children, but studied groups differ regarding bacteria subpopulation profile.
Sellitto et al. (2012) [51]	Faeces samples	qPCR (quantitative PCR)	*Bacteroidetes Firmicutes*	Lack of microflora maturation during first 2 years of life in infants at risk of CD. Moreover, there was observed absence of *Bacteroidetes* and abundance of *Firmicutes*.
Sanz et al. (2007) [54]	Faeces samples	PCR-DGGE	*Actinobacteria Firmicutes*	*Lactobacillus* and *Weissella* are more abundant and diverse in treated CD patients and control subjects than in active CD individuals. Composition of lactic bacteria and *Bifidobacterium* differs between celiac children and age-matched controls.
Kaufman and Rousseeuw (2009) [62]	Intestine biopsies	PCR	*Proteobacteria*	There observed no statistical differences in bacteria composition between healthy and CD children. Nevertheless, *Haemophilus* was more common in CD patients and *Neisseria polysaccharea* in control individuals.
di Cagno et al. (2011) [63]	Faeces sample, duodenal biopsy	RAPD (random amplification of polymorphic DNA) -PCR	*Eubacteria*	Higher number of different *Eubacteria* classes was found in duodenum of coeliac children under gluten-free diet than in healthy children.

Table 1: Continued.

References	Type of sample	Technique	Microbiota phylum/class	Relevant findings
Medina et al. (2008) [64]	Faeces sample	PBMC (peripheral blood mononuclear cell) phenotyping and flow cytometric analyses	*Actinobacteria*	Studies regarding interaction between faecal bacteria and immune system response of coeliac disease patients. It appeared that Gram-positive bacteria such as *Lactobacillus* and *Bifidobacterium* may act as inhibitors of inflammation.

of coeliac children under a gluten-free diet with respect to healthy children. Compared to that of duodenal biopsies, the faecal bacterial population was found to be more diverse. PCR-DGGE faecal profiles of *Lactobacillus* and *Bifidobacterium* differ between treated coeliac children and healthy controls. The ratio between *Lactobacillus*/*Bifidobacterium* and *Bacteroides*/*Enterobacteria* was lower in coeliac children under treatment compared to healthy children. Some of the differences could be related to both coeliac disease and dietary variations.

Surprisingly, a study by Ou et al. [65], based on biopsies collected from the distal duodenum/proximal jejunum of 45 children, did not reveal significant differences between the microbiota in the small intestine of diseased and healthy children, although bacteria from the *Haemophilus* family were more abundant in CD patients, while *Neisseria polysaccharea* were more widespread in the control group. However, at the *genus* level, no differences between the two groups were observed. The authors commented that differences at the species level could not be excluded because complete 16 S rDNA were not sequenced. Similar results, pointing to a lack of significant differences in global composition of duodenal microbiota between healthy controls and CD patients, were obtained also by Cheng et al.; on the other hand, a subpopulation profile, containing eight genus-like bacterial groups, was found to distinguish healthy controls from CD patients [61].

The possible effect on microbiota of different types of milk feeding in early life and the link to the risk of CD development were investigated by Sánchez et al. [60]. The study was carried out on stools of breast-fed infants with different genetic risk of CD (low and high); it showed that high-risk infants had a higher prevalence of *Bacteroides vulgatus*, whereas low-risk infants had higher population of *B. uniformis*, *B. ovatus*, and *B. plebeius*. In the study group of formula-fed infants, *B. ovatus* and *B. plebeius* were increased in subjects with lower genetic risk, while *B. vulgatus* had higher prevalence in those subjects with higher genetic risk. The authors concluded that both types of milk feeding in conjunction with HLA-DQ genotype can influence the *Bacteroides* colonization, increasing the risk of coeliac disease onset. Also, the time of exposure to milk feeding was found to be relevant in prompting coeliac disease development [66].

Sellitto et al. [51] reported the impact on the intestinal tract of two different patterns of gluten introduction. A delay in gluten exposure of at least 6 to 12 months was found to have a positive effect on gluten tolerance: it caused a delay in CD autoimmunity onset in infants that were genetically susceptible to CD. Instead, the early exposure to gluten of infants at risk of coeliac disease was found to induce an immune response and led to a more frequent development of CD. Moreover, a lack of gut microflora maturation during the first 2 years of life in infants at risk of CD was also noted. The gut metabolome of the first 6 months of infant's life reflects mainly the milk diet (rich in polysaccharides and other sugars) and is very similar in all infants; once the solid food is introduced, a shift occurs and a group of short-chain fatty acids are found in faeces. By the end of the second year of life, *Bacteroides* are the main bacteria group found in the metabolome of healthy infants. Conversely, in infants with a genetic risk of celiac disease, an overall lack of bacteria of the phylum *Bacteroides* and abundance of *Firmicutes* were observed.

Recent studies [67] suggest that the colonization of gastrointestinal tract is very important in the development of autoimmune disorders and food-related disease. Furthermore, possible interaction between the intestinal bacteria and the mammalian immune system in the direct differentiation of both pro- and anti-inflammatory T-cells population has been suggested [68]. To clarify whether the gut microflora present in the faeces of CD patients is involved in the proinflammatory activity of coeliac disease, *Bifidobacterium* from healthy subjects was co-incubated together with the faecal microflora or the peripheral blood mononuclear cell culture of coeliac subjects [64]. It appeared that certain strains of *Bifidobacterium* are able to suppress and reverse the proinflammatory effect by increasing IL-10 cytokine production. These results may suggest the use of selected strains of *Bifidobacterium* as probiotics for treatment of CD.

It has been also suggested that gluten intolerance may be also triggered by environmental factors like viruses or bacteria showing molecular mimicking with gluten proteins, causing an autoimmune response that may last even after infection [69]. Several studies pointed to infections by human adenovirus [70], hepatitis C virus [71], rotaviruses [72], or *Campylobacter jejuni* [73] that could induce allergic reactions similar to that induced by gluten exposure, causing the onset of CD.

4. Body Composition and Energy Expenditure in CD Patients

Patients with the classic form of coeliac disease are always characterized by weight loss directly connected with

malabsorption and subsequent risk of malnutrition. Often coeliac disease results in a general lack of energy and strength that can create abnormal conditions described as (chronic) fatigue. Appearance of fatigue and fatigue-related problems seems to be more frequent in nontreated coeliac patients than in patients on a gluten-free diet [74].

Body composition, resting metabolic rate (RMR), and substrate oxidation rates were investigated in [75, 76]. The results showed that untreated and treated CD patients had a lower body weight, lower levels of fat-free mass (FFM), and lower fat mass (FM) in comparison to the healthy controls. In [77], the analysis of body composition at the diagnosis time and after one year of treatment with a gluten-free diet was carried out. The analysis showed a significant increase of body weight and FM but only a slight increase of FFM after treatment with gluten-free diet. Additionally, RMR values were higher in CD patients (treated and untreated) than in controls. Moreover, untreated CD patients showed a higher npRQ (nonprotein respiratory quotient); this may indicate that untreated patients oxidize larger amounts of carbohydrate under resting metabolite conditions than treated CD and healthy subjects.

Interestingly ghrelin, one of the hormones responsible for energy balance regulation, is also changed in CD patients. Ghrelin is a 28-amino acid-peptide produced by the enteroendocrine cells of the gastric mucosa and the intestine [78]. Recent studies have shown that ghrelin is able to increase food intake, decrease fat use, and reduce energy expenditure [79]. While serum ghrelin concentration was increased in CD patients, body mass was decreased [80, 81]. Lower levels of circulating ghrelin were found in CD patients after gluten-free treatment in comparison with CD and control subjects [82]. These results suggest that low amounts of ghrelin in the blood may be partially responsible for the slight increase in body weight and FM in CD patients after treatment with a gluten-free diet.

5. Metabolomic Signature of CD

In many cases, the diagnosis of CD is not an easy task, mainly because CD has a variable clinical picture due to its intertwingled genetic, immunological, and environmental components. The presence of the HLA genetic factor, together with a positive biopsy and serological antibodies upon gluten-containing diet, is used to diagnose coeliac disease at any age. In order to better understand the processes underlying the activation and development of coeliac disease, it is important to examine the mechanisms from the early beginning.

To date, a limited number of metabolomics studies of coeliac disease are available, but they clearly show that metabolic differences between healthy individuals and coeliac patients exist. In the first (to our knowledge) metabolomic study on CD, Bertini et al. [49], examined adult healthy controls and coeliac patients by ^{1}H NMR profiling of their serum and urine profiles before and after GFD, showing that a metabolic fingerprint for CD can be defined. This fingerprint was found to be made up by three components, one related to malabsorption, one related to energy metabolism, and the third related to alterations in gut microflora and/or intestinal permeability. Using this metabolic fingerprint, it was possible to make predictions about the coeliac status with a very good accuracy (ca. 84%). One of the most interesting findings was that the metabolic profile of CD patients reverts to normality after 12 months of a strict gluten-free diet; interestingly, a similar behavior was not found in CD patients when analyzing them from a gut microflora prospective [58, 59, 63].

The main observed differences in serum spectra between CD patients and controls were lower levels of several amino acids (asparagine, isoleucine, methionine, proline, and valine), methylamine, pyruvate, creatinine, choline, methylglutarate, lactate, lipids, and glycoproteins and higher levels of glucose and 3-hydroxybutyric acid. Notably, the best discrimination is obtained from CPMG spectra (Carr-Purcell-Meiboom-Gill spin echo sequence) [83], that is, from spectra in which signals arising from large macromolecules such as lipidic components are suppressed [49]. So, although it is known that coeliac patients usually appear to be hypocholesterolemic, lipids do not contribute significantly to the metabonomic signature of coeliac disease. A decrease in the level of pyruvate and lactate and a higher level of glucose in the blood of coeliac patients were observed, probably as a consequence of an impaired glycolysis process. Glycolysis impairment can cause a lowering of pyruvate and lactate levels and an increase of glucose levels in blood. If this metabolic way is reduced, β-oxidation is probably increased. Enhanced β-oxidation and malabsorption can then explain lower levels of lipids in serum [49]. In these conditions, the authors hypothesized an increase of the use of ketonic bodies as a source of energy in coeliac patients, consistently with the higher observed levels of 3-hydroxybutyric acid in blood and acetoacetate in urines [49].

Energy conversion from lipids and catabolism of ketonic bodies are far less efficient than that from glucids. Untreated coeliac subjects often report symptoms of fatigue. In patients on a gluten-free diet, fatigue tends to be reduced and, in fact, it has been proposed that this condition is gluten-related [74]. In [49], the authors found that in CD patients on a gluten-free diet the levels of glucose and 3-hydroxy-butyric acids revert to normality.

Further, the authors found that CD patients are characterized by higher urine levels of some metabolites related to gut microbiota: indoxyl sulfate (IS), meta-[hydroxyphenyl] propionic acid (m-HPPA), and phenylacetylglycine (PAG). M-HPPA mostly originates from gut microflora, being one of the several products of the microbially mediated breakdown of larger plant phenolic compounds such as caffeic acid and its conjugate chlorogenic acids [84]. IS is a harmful uremic toxin produced in the liver from indole through indoxyl. Indole is a subproduct of tryptophan metabolism by intestinal bacteria [85]. Modulation of PAG excretion in urine has been attributed to gut microflora, and increases of PAG have been reported in cases of drug-induced phospholipidosis; nonetheless, the contribution of mammalian and microbial sources to PAG excretion is not yet fully characterized [86]. All these findings are consistent with the hypothesis that in CD patients the gut microflora of the small bowel is

altered or presents peculiar species with their own microbial metabolome.

In a following investigation [50], the same research group highlighted again the existence of a metabolic fingerprint for coeliac disease, confirming most of the previously discussed metabolites with the additional finding of higher levels of *p*-cresolsulfate in the urines of CD patients. Interestingly, *p*-cresolsulfate, a metabolite of bacterial origin, is associated with several gastric-related disease [87], including bowel cancer [88]. In the same study, the analysis of the so-called "potential coeliac patients" (i.e., subjects who have a positive antibody test but no evidence of intestinal damage) showed that the metabolic patterns of overt and potential coeliac patients are similar [50] indicating that CD-related dysmetabolism precedes the intestinal damage. Only a few serum metabolites differentiate between potential and overt CD, and none of these metabolites are related to the energy metabolism [50]. It appears that, as in overt CD patients, glycolysis is somehow impaired also in potential CD patients. Impairment of glycolysis explains both the observed lower lactate levels and the higher glucose levels in blood of potential CD patients. In urine, there are more metabolites that discriminate potential and overt CD. The key differences lie in the concentration of metabolites originating from the gut microflora (m-HPPA, IS, and PAG) which in potential coeliac subjects are similar to those of controls, suggesting a relationship between overt CD, villous atrophy, and bacterial consortia of the host [50].

The authors concluded that, although free from intestinal injury, placing potential CD subjects on a gluten-free diet could be justified because they are experiencing most of the pathological alterations experienced by overt coeliac patients [50]. Figure 3 shows the discrimination between overt CD patients and healthy controls and the statistical prediction of the potential CD patients: almost all potential CD patients are predicted as overt CD. The plot shown in Figure 3(a) was obtained using a training set composed of the serum CPMG spectra of 34 overt CD patients, 34 healthy controls, and 13 (out of the 34) CD patients after 12 months of gluten-free diet. It clearly appears that all but one patient on gluten-free diet were classified as healthy. Similarly, the plot in Figure 3(b) was built using the CPMG spectra of 61 overt CD patients, 51 healthy controls, and 29 potential CD patients. Almost all the potential CD patients fall in the CD group, underlining the affinity between the metabolic fingerprints of these two dissimilar clinical conditions.

Differences between the metabolic profiles of faeces and urine of CD and healthy children using a combination of ^{1}H-NMR and GC-MS/SPME techniques were reported by di Cagno et al. [63]. The analysis allowed the identification of a group of compounds that were significantly changed in the treated coeliac children group. A set of volatile organic compounds and short fatty acids were identified using MS, whereas amino acids were identified using NMR [63]. Faecal and urine samples of treated CD children showed elevated levels of free amino acids (proline, methionine, histidine, and tryptophan) and lowered levels of some short fatty acids (butyric, isocaproic, and propanoic acids) compared to healthy children [63]. The authors suggested that these changes may be associated with intestinal and faecal bacteria modifications that could induce a nonspecific inflammation and a reduction of the absorptive surface of the intestinal mucosa; this may lead to a reduction of the absorption of amino acids which are subsequently lost with stool [63]. By combining microbiology and metabolomics, the authors showed that a gluten-free diet lasting at least two years did not completely restore the microbiota of the CD children. From that work, a broader picture seems to emerge that microbial indices (i.e., the ratio of faecal cell density of lactic

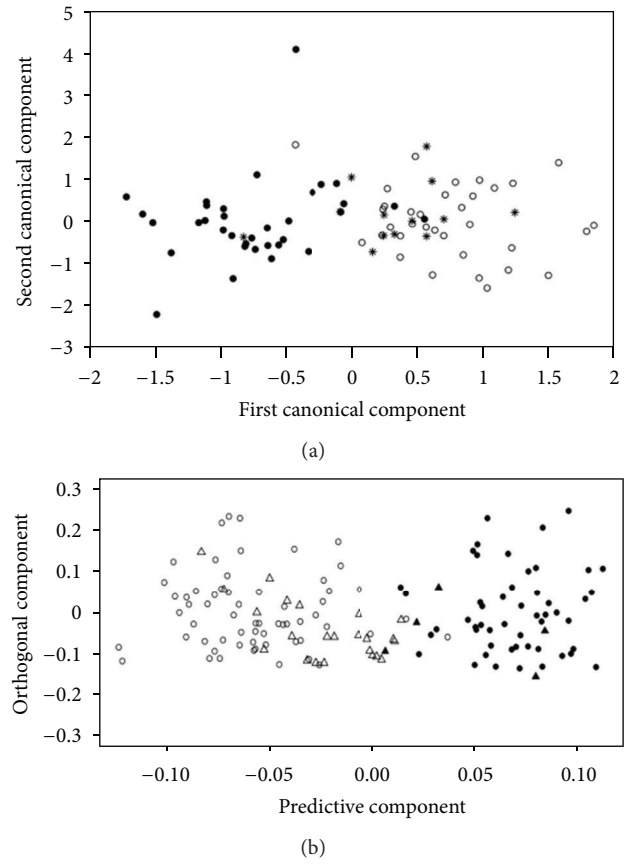

Figure 3: (a) Clustering of CMPG (Carr-Purcell-Meiboom-Gill spin echo sequence) [83] serum spectra of CD patients (filled circles) and controls (open circles). The discriminant model between the two groups was calculated using a combination of partial least square [89] and (regularized) canonical analysis [90] (PLS-RCC) and was validated using cross-validation. The CPMG spectra of 13 (out of the 34) CD patients after 12 months of gluten-free diet were then projected into the discriminant space of the model (stars) and were assigned to the CD or the healthy group applying a support vector machine [91] classifier (SVM). (b) Clustering of overt CD patients (open circles) and healthy controls (filled circles) obtained with CPMG serum spectra. The discriminant model was calculated using orthogonal partial least square [92] (OPLS) and validated using double cross-validation [93]. The CPMG spectra of 29 potential CD patients were then projected in the model (triangles) and filled or not according to the results of an SVM classifier. Adapted with permission from [49, 50]. Copyright (2009 and 2011) American Chemical Society.

acid *bacteria-Bifidobacterium* to *Bacteroides-Enterobacteria*) and the levels of some metabolites (i.e., ethyl-acetate, octyl-acetate, SCFA, and glutamine) are characteristic of CD patients [63].

6. Perspectives

Metabolomics is a rapidly growing discipline bringing together analytical technologies, metabolite pathways evaluation, and information technology. A major advantage is the noninvasive or minimally invasive measurement of potentially useful biomarkers from biofluids such as urine and plasma. A great deal of validation work (both at the analytical and data analysis level) has been carried out to gain full acceptance to metabolomics in routine clinical practice. Challenges for the development of metabolomics still exist, including simplified systems to present data to end-users (such as interpretation of often complex statistical models), the coordination of multiple data streams, and the implementation of quality control programs [94]. We expect that in the next few years it will be clear whether or not metabolomics will take its place as a complementary or even an alternative tool in the clinical setting.

At the present time, only few applications devoted to the investigation of coeliac disease have been presented in the literature, but a complex picture of the interaction between energy metabolism and gut microbiota seems to emerge, providing new hints on the biochemistry of the disease. In our institutions, as a logical complement to the results obtained analyzing overt coeliac and potential coeliac subjects, we are currently applying metabolomics to the biomolecular investigation of a gluten-related condition defined as gluten sensitivity [95]. This condition is still not very well characterized and its pathogenesis is caused by unknown mechanisms; we believe that metabolomics is a useful tool to expand our current limited knowledge of this condition.

Metabolomics-based approaches are expected to enable diagnosis, prognosis, and prediction of response of individuals to treatment. We can expect that metabolomics will provide more accurate and less expensive biomarkers (obtained by means of proper statistical analysis and properly validated) than presently available, which could improve diagnostic accuracy and sensitivity. However, far more research is essential to reach such a goal, and a validation of the results on an epidemiological scale is indeed needed.

Box 1 (MS and metabolomics). The main analytical techniques used in metabolomics are nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) [96]. Both MS and NMR methods provide information on a wide range of metabolites in a single measurement. Furthermore, both can be used to identify the metabolites' structures and to measure the relative and absolute concentrations of the molecules (MS has higher sensitivity but NMR is more reliable for determining concentrations) [97].

Mass spectrometry is a technique to determine extremely accurate mass of molecules in a pure sample or in a mixture. The molecules in a sample are converted to ions by an electron beam; the ions are accelerated by charged plates and then deflected by a magnetic field according to the mass-to-charge ratio of each ion. When the ions reach the detector, the mass-to-charge ratio is registered to provide a spectrum where series of peaks are shown reporting the intensity of each ion generated by the sample. MS is a destructive technique but requires a very low quantity of sample [98]. Over the last few years, its application to mammalian study increased, especially for its high sensitivity, and because it is a major technique for molecular identification [99]. As opposed to NMR, MS usually requires metabolites separation before detection, typically by using gas chromatography (GC) or liquid chromatography (LC). GC-MS is a robust technique for the analysis of volatile and semivolatile compounds suitable for chemical derivatization to increase their volatility [100]. Electron ionization (EI) in GC-MS is quite reproducible [100]. In contrast to GC-MS, LC-MS is especially suitable for the analysis of nonvolatile and/or thermally unstable metabolites. The introduction of UPLC (ultraperformance liquid chromatography) and capillary LC enabled better peak resolution and further increase in sensitivity and speed, and it is now successfully applied to metabolomics studies [101].

Box 2 (NMR and metabolomics). NMR spectroscopy is an analytical technique that exploits the magnetic properties of certain atomic nuclei. It determines the physical and chemical properties of molecules by detecting the magnetically active nuclei. When placed in a magnetic field, an active nucleus (such as ^{1}H or ^{13}C) absorbs electromagnetic radiation at a characteristic frequency and then reemits it. After absorbing electromagnetic radiation in the range of frequencies of ^{1}H (or ^{13}C, or ^{31}P,...), the sample emits all frequences of its active nuclei of that type, which constitute its ^{1}H (or ^{13}C or ^{31}P,...) NMR spectrum. The resonance frequency and the corresponding intensity of each signal are dependent, respectively, on the chemical environment where that particular nucleus is located (i.e., molecular structure) and on the concentration of that molecule.

NMR spectroscopy is a nondestructive and highly reproducible technique and provides detailed information on the molecular structure of both pure compounds and complex mixtures [102]. In a typical biological fluid, all hydrogen-containing molecules will give a ^{1}H-NMR spectrum as long as they are present in concentrations above the detection limit. The NMR spectrum of a biological fluid is therefore the superposition of the spectra of thousands of different small molecules (up to 2500 for urine and up to 200 for serum/plasma) present in the sample at concentrations $>1\,\mu$M [103]. An advantage of NMR is that the biological fluid requires only a mild treatment prior to the analysis.

The main disadvantage of NMR is its relatively low sensitivity. Another disadvantage of the NMR approach is the difficult identification of all metabolites in the samples: ^{1}H-NMR spectra of biological fluids are very complex and often additional two-dimensional NMR experiments may be needed to assign metabolites in biofluids. The development of high-resolution ^{1}H magic angle spinning (MAS) spectra made viable the acquisition of data on small slices of tissue without any pretreatment [104–106].

Box 3 (biofluids and metabolomics). Most biofluids used in metabolomics can be collected noninvasively. The Human Metabolome Database (http://www.hmdb.ca/) lists 16 different biofluids investigated and up to 5000 identified or putative metabolites: amniotic fluid (17), aqueous humor (1), ascites fluid (1), bile fluid (18), blood (4297), breast milk (37), cellular cytoplasm (49), cerebrospinal fluid (436), faeces (0), lymph (1), menses (0), mucus (0), pericardial effusion (1), prostate tissue (13), saliva (70), sebum (0), semen (4), sweat (1), synovial fluid (0), tear fluid (1), urine (3873), and vaginal fluid (0). Of these, 694 have been associated with one or more diseases and pathologies.

Blood, urine, cerebrospinal fluid, and saliva are the richest in metabolites. The Human Serum Metabolome project. [107] (http://www.serummetabolome.ca/) lists 4229 *detectable* metabolites (most of them lipids) obtained by enhanced NMR, MS, and other analytical platforms. NMR was able to measure 1.2% (49/4229) of the human serum metabolome, GC 2.13% (90/4229), ESI-MS/MS (lipid mediator profiling) 2.3% (96/4229), and TLC/GC-FID-MS (general lipidomics) 79.9% (3381/4229, mostly, however, components of the complex lipid fraction) and DFI MS/MS is able to access 3.3% (139/4229) of the serum metabolome. Some of the compounds identified by NMR are urea (6 mM), glucose (5 mM), lactic acid, (1.4 mM), glutamine (0.51 mM), and glycerol (0.43 mM). The least abundant compounds were carnitine (46 μM), acetic acid (42 μM), creatine (37 μM), cysteine (34 μM), propylene glycol (22 μM), and aspartic acid (21 μM), and the lowest concentration reliably detected using NMR was 12.3 μM (for malonic acid) and 14.5 μM (for choline).

The Human Urine Metabolome project [108] (http://www.urinemetabolome.ca/) lists up to 3100 metabolites identified in urine. Human urine contains many classes of compounds excreted as waste products, including organic acids, amino acids, purines, pyrimidines, sugars, sugar alcohols, sugar acids, amines, and other compounds, at a variety of concentrations. Fresh urine is also characterized by the presence of human cells (erythrocytes, leucocytes, urothelial cells, and epithelial cells), bacteria, fungi, sperms, and noncellular components (mucus filaments, cylinders, cylindroids, pseudocylinders, and crystals, urates).

Some of the metabolites identified in saliva using NMR are [109] glucose, propionate, acetate, taurine, glycine, alanine, sucrose, dimethylamine, formate, glycine, lactate, methanol, propionate, propylene glycol, pyruvate, succinate, and taurine.

A large panel of metabolites has been also identified in cerebrospinal fluid by using NMR and GC-MS [36]. Among those obtained by NMR, there are amino acids, sugars, 2-oxoglutarate, 2-oxoisovalerate, 3-hydroxybutyrate, 3-hydroxyisovalerate, xanthine, and pyruvate.

Up to 50 metabolites were identified in faecal extracts via NMR [43]: amino acids, n-butyrate, propionate, n-caproate, 3-(4′-hydroxyphenyl) propionate, 5-aminopentanoate, glucose, 5-N-acetylneuraminate, 5-aminosalicylate, N-acetyl-5-aminosalicylate, deoxycholate, and phenylacetate, many of which are of bacterial origin.

Box 4 (the metabolomics pipeline). The workflow of a metabolomics study is complex and each step has its own criticalities that need to be addressed. The metabolomics workflow can be summarized as follows [96, 110–112].

Biological Question. It includes definition of the biological/biomedical problem to be addressed.

Study Design. It involves power analysis and treatment design.

Data Acquisition. It concerns quality control strategies, experimental setting (platform specific), Sampling, and measurement design.

Data Preprocessing. It is a fundamental step before analysis involving alignment, baseline correction (MR), phasing, alignment, bucketing (NMR), normalization, and scaling.

Metabolite Identification. It includes spectral matching (MS) and peak assignment (NMR).

Statistical Analysis. It includes explorative (i.e., PCA and clustering), predictive (regression, PLS-DA), and univariate analysis and model optimization and validation.

Biological Interpretation. It involves embedding the results within the framework of existing biological knowledge.

Box 5 (statistical analysis of metabolomic data). Metabolomic data are high dimensional in nature. Tens, hundreds, or even thousands of (un) identified metabolites (relative) concentrations are measured by means of NMR or MS platforms, usually on a limited number of samples. Biological information is retrieved from this data by means of univariate and multivariate statistical methods [27, 113, 114]. Multivariate methods make also use of covariances or correlations which reflect the extent of the relationships among the variables, in contrast to univariate methods that focus solely on the mean and variance of a *single* variable.

Commonly used univariate methods are *t*-test and ANOVA [115] together with their corresponding nonparametric versions [116] and with appropriate correction methods for multiple testing [117]. Multivariate methods are a broad category. When the interest centers on predicting or explaining one variable (either a group category like case/control or a continuous response) by the other variables, methods like multiple regression [118] or partial least squares regression and discriminant analysis (PLS-DA) [119] or its extensions like Multilevel PLS-DA [120], Orthogonal PLS-DA [121], and N-way PLS-DA [122] together with a proper optimization and validation of the models [93, 123, 124] are used. In other cases, the interest centers on providing insight into the underlying structure of the complete set of variables and other tools are used. Some examples are principal component analysis (PCA) [125], used to reduce the number of variables when there is correlation present and to explore relations between objects, or cluster analysis [62], used when objects have to be grouped to represent data structure. Hybrid methods like nearest shrunken centroids [126] or simplivariate methods [127] and machine-learning techniques like

artificial neural networks [128], random forest [129], and support vector machines [91] are also used in metabolomics [27, 112, 130].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Prevalence of Thyroid Autoimmunity in Children with Celiac Disease Compared to Healthy 12-Year Olds

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Objectives. Studies have suggested a correlation between untreated celiac disease and risk for other autoimmune diseases. We investigated the prevalence of thyroid autoimmunity in 12-year-old children (i) with symptomatic celiac disease diagnosed and treated with a gluten-free diet, (ii) with screening-detected untreated celiac disease, and (iii) without celiac disease. *Methods*. Blood samples from 12632 children were collected. All celiac disease cases, previously diagnosed and newly screening-detected, were identified. Per case, 4 referents were matched. Blood samples were analyzed for autoantibodies against thyroid peroxidase (TPOAb). The cut-off value for TPO positivity was set to 100 U/mL. *Results*. Altogether, 335 celiac disease cases were found. In the entire celiac disease group, 7.2% (24/335) had elevated titers of TPOAb compared to 2.8% (48/1695) of the referents. Among the previously diagnosed celiac disease cases, 7.5% (7/93, OR 2.8, 95% CI 1.2–6.4) was TPOAb positive and among screening-detected cases, 7.0% (17/242, OR 2.6, 95% CI 1.5–4.6) was TPOAb positive. *Conclusion*. Children with celiac disease showed a higher prevalence of thyroid autoimmunity. We could not confirm the hypothesis that untreated celiac disease is associated with increased risk of developing thyroid autoimmunity. Early initiation of celiac disease treatment might not lower the risk for other autoimmune diseases.

1. Introduction

Celiac disease is one of the most common chronic diseases in childhood, affecting approximately 0.5–3% of the population in the Western world [1–3]. It is characterized by an autoimmune response triggered by gluten and possibly other environmental cofactors, leading to small-intestinal mucosal injury [4]. This in turn leads to malabsorption with a variable clinical expression ranging from no symptoms to severe malnutrition. The disease can have its onset at any age throughout life [5, 6]. The human leukocyte antigen HLA DQ2 or DQ8 haplotype is carried by all celiac disease cases, and the prevalence in the general population has been assumed to be about 30% [7, 8]. The presence of HLA DQ2 and/or DQ8 is necessary but not sufficient for development of the disease. Studies of identical twins and siblings suggest the HLA contribution to be less than 50%, with the remaining part being explained by a combination of non-HLA genes and environmental factors [9].

Autoimmune conditions including thyroid diseases such as Hashimoto thyroiditis and Grave's disease are associated with celiac disease [10–13] with a reported prevalence up to 10 times that in the general population [14–17]. Some studies have suggested that untreated celiac disease and thus also gluten exposure, with subsequent inflammation and mucosal injury, increase the risk for developing other autoimmune diseases such as thyroid diseases and insulin dependent diabetes mellitus. Ventura et al. showed that patients with celiac disease had a high prevalence of both insulin dependent diabetes mellitus autoantibodies and thyroid-related

serum autoantibodies. Moreover, these autoantibodies were supposed to be gluten-dependent, because they disappeared during treatment with a gluten-free diet [12, 18].

These findings raise questions as to whether abnormal immune responses, at the level of the gut mucosa when exposed to environmental antigens, play a role in systemic autoimmune disease or if these associations instead reflect an underlying joint genetic predisposition. If the duration of gluten exposure is positively correlated to development of autoimmune disease, and if early detection and initiated treatment of the disease reduces the risk for development of autoimmune disease, this would point in the direction of a general celiac disease screening at as early an age as possible.

The aim of our study was to investigate the prevalence of thyroid autoimmunity in 12-year-old children with celiac disease compared to sex-matched referents and to investigate if early introduction of a gluten-free diet in children with celiac disease reduces the risk for developing autoimmune thyroid disease. The hypothesis is that previously diagnosed cases with a consequentially reduced exposure (after the time of the diagnosis) can be expected to display a lower incidence of TPOAbs than screening-detected cases (with ongoing exposure).

2. Materials and Methods

2.1. A Population-Based Celiac Disease Screening Study. A two-phased population-based cross-sectional multicenter screening study for celiac disease in 12-year olds was performed in 2005-2006 and 2009-2010 representing two birth cohorts (children born in 1993 and 1997, resp.) [3, 19]. The study was entitled the ETICS study (Exploring the Iceberg of Celiacs in Sweden) and was part of the PreventCD European project [3, 20]. Both screening efforts covered the same geographical areas and followed a similar protocol, including collaboration with school health services. Families gave their signed informed consent before being enrolled. The study was approved by the Regional Ethical Review Board of Umeå University, Umeå, Sweden.

In total, 12632 children (69% of those invited) participated, with similar sex ratios in both birth cohorts (48% and 49% girls in the 1993 and 1997 groups, resp.). Details of the celiac disease screening strategy and descriptions of both cohorts have been published previously [3, 19]. In brief, blood samples from all participating children were analyzed for antihuman tissue transglutaminase and if borderline values were obtained also for endomysial antibodies (both of isotype IgA). Children with values above a predefined cut-off were referred to the closest pediatric clinic for a small intestinal biopsy, which represents the gold standard for diagnosis [21, 22]. Criteria for diagnosis were Marsh 3a–c enteropathy or the combination of Marsh 1-2 enteropathy, HLA-DQ2/DQ8 haplotype, and symptoms and/or signs compatible with celiac disease [22].Genotyping for HLA alleles encoding for HLA-DQ2/DQ8 was performed by oligonucleotide probe hybridization and was verified in all screening-detected cases. For those who reported clinically detected celiac disease, diagnosis was confirmed by review of histology and serological markers from the National Swedish Childhood Celiac Disease Register [23] and/or medical records.

2.2. A Nested Case-Referent Study on Thyroid Autoimmunity. A case-referent design nested within the ETICS study was used to evaluate the risk of thyroid autoimmunity related to treated (i.e., the previously diagnosed celiac disease cases) and untreated celiac disease, respectively. The mean age at diagnosis among the previously diagnosed celiac disease cases was 4.7 and the median was 2.75 years. They had thus been on a gluten-free diet for approximately 7 to 9 years since the screening was performed at 12 years of age. The total number of celiac disease cases from both screening efforts, including previously diagnosed cases and newly screening-detected cases, together formed our celiac disease case group. This cohort was used as a basis for screening of thyroid autoimmunity, defined as significant titers of TPOAb. The blood samples drawn for the TPOAbs were taken at 12 years of age, that is, at the time of the screening. Autoantibodies of IgG type directed against thyroid peroxidase (TPOAb) that were measured in blood samples were used as an indicator of thyroid autoimmunity and expressed as arbitrary units per milliliter (U/mL). The cut-off value for TPO positivity was set to 100 U/mL (Varelisa TPO Antibodies, Phadia GmbH, Freiburg, Germany).

Four referents were selected for each celiac disease case. Referents, matched for sex, were randomly selected from all cohort members free of celiac disease at the time of diagnosis. In this paper we included cases with a confirmed celiac disease diagnosis, obtained either through the ETICS screening ($n = 242$) or ahead of the screening ($n = 93$). We therefore excluded 90 children, either because of incorrect information about the existing celiac disease diagnosis ($n = 36$ children) or because of no biopsy verifying a celiac disease diagnosis ($n = 54$ children). This resulted in 213 cases and 1150 referents from the first phase (a rate of 5.4 referents per case) and 122 cases and 545 referents from the second phase (a rate of 4.5 referents per case) for the analyses in this paper.

2.3. Statistical Analysis. The relation between celiac disease and TPOAb positivity was analyzed with logistic regression using the nonceliac disease children as the reference group. Results are presented as odds ratios (OR) with 95% confidence intervals (CI). Microsoft Access was used for data handling and Stata 10 for statistical analysis (StataCorp LP, College Station, TX). Statistical significance was accepted at $P < 0.05$ corresponding to a CI not including 1.

3. Results

3.1. Study Population Characteristics. In total, among the 12632 children, we identified 335 celiac disease cases whereof 93 had previously diagnosed celiac disease and 242 were detected within the study (Figure 1). Details regarding the prevalence of celiac disease have been published elsewhere, but in short, the screening procedure revealed a total celiac disease prevalence of 29/1 000 in the 1993 cohort, including

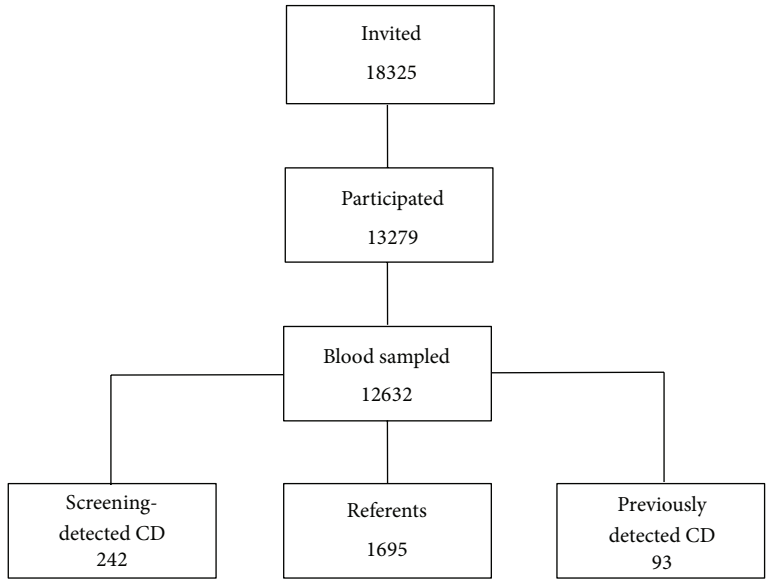

Figure 1: Flowchart depicting the screening procedure. Cross-sectional screenings performed in 12-year olds across Sweden to investigate the total prevalence of celiac disease (CD), including both clinically- and screening-detected cases. Numbers of children are given in the boxes.

Table 1: Risk for thyroid peroxidase antibody (TPO) positivity in 12-year olds when comparing treated and untreated celiac disease (CD) cases with non-CD children through a case-referent study nested within the ETICS[1] study.

Groups	TPO[2]				OR[3]	95% CI[3]
	Positive (n = 72)		Negative (n = 1958)			
	n	%[4]	n	%		
Non-CD[5]	48	2.8	1647	97.2	1.0	—
Previously diagnosed CD	7	7.5	86	92.5	2.8	1.2–6.4
Screening-detected CD	17	7.0	225	93.0	2.6	1.5–4.6

[1]Exploring the Iceberg of celiacs in Sweden (ETICS).
[2]The thyroid peroxidase (TPO) cut-off used was 100 U/mL.
[3]Logistic regression was used to estimate odds ratio (OR) with 95% confidence interval (CI).
[4]Row percentages.
[5]Children not diagnosed with celiac disease (CD).

both previously and screening-detected cases, and 22/1 000 in the 1997 cohort [3, 19]. The proportions of children with previously diagnosed celiac disease and those with screening-detected disease were similar in the cohorts. The mean age at diagnosis among the previously diagnosed children was 4.7 years (SD 4.0) and the median was 2.75 years.

3.2. Thyroid Autoimmunity. In the celiac disease group, 7.2% (24/335) had elevated titers of TPOAb compared to 2.8% (48/1695) of the referents. Among the previously diagnosed celiac disease cases 7.5% (7/93, OR 2.8, 95% CI 1.2–6.4) was TPOAb positive and among the screening-detected cases 7.0% (17/242, OR 2.6, 95% CI 1.5–4.6) was TPOAb positive (Table 1).

4. Discussion

This study demonstrates an increased prevalence of thyroid autoimmunity among 12-year-old children with celiac disease compared to healthy controls. We found the prevalence of thyroid autoimmunity to be almost three times higher than in the age- and sex-matched control group of children without celiac disease. We did not find any difference in the prevalence of thyroid autoantibodies in the group of children with previously diagnosed celiac disease compared to the screening-detected cases. Our previous pilot screening studies, performed on the same birth cohorts, showed that 2.5-year olds have approximately the same prevalence of undiagnosed celiac disease as found in the ETICS study [24]. This supports the theory that not all cases detected through screening are of recent onset and these may constitute a group of children who have had untreated celiac disease since early childhood [24, 25]. The previously diagnosed cases had already been treated for several years with a gluten-free diet. The mean age at diagnosis among the previously diagnosed celiac disease cases was 4.7 years (SD 4.0) and the median age was 2.75 years; they had thus been on a gluten-free diet for approximately 9 years since the screening was performed at 12 years of age. Adherence to a gluten-free diet among children

is generally good [26–28]. In our cohort, 83 out of the 93 previously diagnosed cases had normal tTG at the time of the screening as a measure of very good compliance and only 10 out of 93 had slightly raised tTG (range 4.1 to 29.95) above normal. According to our study results, a gluten-free diet does not seem to be protective against the development of thyroid autoimmunity.

The results are in accordance with those of Sategna Guidetti et al. who observed that many celiac patients developed autoimmune disorders despite strict adherence to a gluten-free diet [29]. Viljamaa et al. also investigated the prevalence of autoimmune disease associated with celiac disease among both adults and children. They found that the duration of gluten exposure did not seem to be of crucial importance regarding development of autoimmune diseases. One-third of the patients in their study developed associated autoimmune diseases despite being on a gluten-free diet [16]. In fact, in the latter study the development of autoimmune disease decreased with duration of gluten exposure. One can hypothesize that these patients, who were diagnosed with celiac disease at a later age, might be generally less susceptible to developing autoimmune diseases. This is also supported by Cosnes et al. who showed that a late diagnosis of celiac disease was associated with a decreased risk of autoimmunity [26]. The above-mentioned findings might support a common genetic susceptibility to the autoimmune conditions. It is important to bear in mind that autoimmune diseases develop at different ages; for example, type 1 diabetes mellitus often develops during childhood, while development of autoimmune thyroiditis increases with increasing age and has its peak incidence in the fifth decade of life [30, 31]. It is thus important to analyze the incidence of autoimmune diseases in relation to both age and compliance with a gluten-free diet. Cosnes et al. found that the risk of development of an autoimmune disease in celiac patients was increased in patients with a family history of autoimmune disease when celiac disease was diagnosed early in childhood or adolescence compared to adulthood [26]. The fact that family history was a strong contributing factor to the development of autoimmune disease also favors a linkage disequilibrium between the genes responsible for celiac disease and those responsible for the coexpressed autoimmune disease [32].

Contrary to the abovementioned findings, Ventura et al. showed that patients with celiac disease had a high prevalence of both insulin dependent diabetes mellitus autoantibodies and thyroid-related serum autoantibodies. These autoantibodies were supposed to be gluten-dependent, since they disappeared during treatment with a gluten-free diet [12]. They also observed that the prevalence of autoimmune disorders in children in whom celiac disease was diagnosed before the age of two years, and who were treated with a gluten-free diet, was comparable to that of controls [18]. In our study, the distribution of TPO positivity at 12 years of age among the previously diagnosed celiac disease cases was equal among those diagnosed before 2 years of age and after 2 years of age. It was also equally distributed regardless of the degree of mucosal damage in the entire celiac disease cohort.

Sategna Guidetti et al. hypothesized that gluten ingestion plays a central role in modifying the immunological response early in life [29]. As mentioned above, we could not verify this theory in our study since we did not find any difference in the prevalence of TPOAb among the screening-detected, and thus untreated, celiac disease cases and the previously diagnosed celiac disease cases. However, some of the previously diagnosed cases in our study were diagnosed at a later age than before the age of two. If the hypothesis of Sategna Guidetti et al. is true and this is a crucial age, the opportunity to modify the immunological response might have disappeared after that age.

The present trial was based on a nationwide, contemporary study on celiac disease in 12632 children/almost 13000 children. Yet, it has some limitations that merit consideration. First, celiac disease was defined pathologically as Marsh 1–3c. The inclusion of Marsh 1 in the diagnosis is currently an unsettled issue in the scientific community. We chose to include Marsh 1 in accordance with previous publications [3, 19] and research showing that patients with low-grade inflammation still benefit from a gluten-free diet [33]. Second, albeit the screening study included 12632 children, power nevertheless constitutes a limitation of the current study, as diagnosis of concomitant celiac disease and elevated TPOAb was relatively rare in our cohorts. Thus, the lack of a significant difference in TPOAb between the screening-detected and previously detected CD cases could possibly be due to a lack of power. The third limitation of the present study is that we only analyzed TPO markers as an indicator of autoimmune disease. We did not investigate how many of the children developed clinical autoimmune thyroiditis, defined as TPOAb positivity in combination with goiter and/or hypothyroidism. Although the clinical significance of these antibodies in celiac disease is still unclear, there is probably a higher propensity for thyroid autoimmunity in children with positive TPO markers. In most cases the immune response to the target cells progressively destroys the endocrine gland, and hypofunction is the main clinical manifestation [34]. The presence of TPOAb in serum is an independent risk factor for the development of hypothyroidism in patients with subclinical hypothyroidism [35, 36]. The Whickham study demonstrated that the presence of antithyroid microsomal (TPO is the antigen involved in the "microsomal" response) antibodies, or elevated serum TSH alone, was associated with a significant increased risk of developing hypothyroidism at 20 years of age [36]. A longitudinal followup would, therefore, seem necessary in patients with positive autoimmune thyroid serology but who are currently euthyroid.

5. Conclusion

Thus far, we can conclude that having celiac disease as a 12-year old increases the risk of also having thyroid autoimmunity almost threefold compared to healthy children. Our findings do not support a general screening for celiac disease on the basis of trying to protect against thyroid disease through earlier diagnosis of celiac disease and initiation of treatment with a gluten-free diet.

Abbreviations

tTG: Transglutaminase
EMA: Endomysial antibodies
TPOAb: Thyroid peroxidase antibodies
OR: Odds ratio
SD: Standard deviation
CI: Confidence interval.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Oral Signs and HLA-DQB1*02 Haplotypes in the Celiac Paediatric Patient: A Preliminary Study

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Celiac disease (CD) diagnosis can be extremely challenging in the case of atypical patterns. In this context, oral signs seem to play a decisive role in arousing suspicion of these forms of the disease. At the same time, the different expressions of the HLA-DQB1*02 allele apparently seem to facilitate the interpretation of signs and highlighted symptoms. The aim of this work was to verify whether it is possible to identify a correlation between the development of oral signs and different DQ2 haplotypes in celiac pediatric patients. 44 celiac patients with a medium age of 9.9 were studied. Oral examinations were performed in order to identify recurrent aphthous stomatitis (RAS) and dental enamel defects (DED). The diagnosis of DED resulted as being related to allele expression (P value = 0.042) while it was impossible to find a similar correlation with RAS. When both oral signs were considered, there was an increase in correlation with HLA-DQB1*02 expression (P value = 0.018). The obtained results identified both the fundamental role that dentists can play in early diagnosis of CD, as well as the possible role of HLA haplotype analysis in arousing suspicion of atypical forms of the disease.

1. Introduction

Celiac disease (CD) is a complex pathologic condition involving the small intestine mucosa resulting from intolerance to gluten assumption [1]. More specifically, it is considered a genetic and autoimmune condition that can affect patients of any age and gender with a great variability of symptoms and clinical signs. Although the final diagnosis of CD is always based on a biopsy with the detection of severe villous atrophy coupled with crypt hyperplasia, the diagnostic pathway leading to this conclusion can often be long and winding [1, 2]. This pathology, initially considered as typical of the European population, is nowadays distributed worldwide. Epidemiological analysis has reported a prevalence of celiac disease as varying greatly between the western and the eastern parts of the world, so that CD is today considered as the most common genetic disorder in the west with a prevalence of 1%, while it is relatively unknown in Asian countries [1, 3]. This irregular distribution in different countries has been related to genetic and alimentary factors. CD is actually an intestinal enteropathy whose symptoms are determined by the ingestion of gliadin in genetically predisposed patients [1]. Gliadin is a prolamin, a class of peptides highly resistant to gastrointestinal enzymes, which causes histological changes in the small intestine mucosa of the celiac patient, leading to a malabsorption syndrome. This abnormal response of the intestinal mucosa is linked to a specific genetic predisposition; almost all the patients affected by CD carry a HLA-DQ2 molecule which is highly frequent in the west and absent in Asian countries. Infections represent another environmental factor identified in the understanding of the CD pathogenesis: the presence of different virus has been described by several authors as a possible cause of pathology development after gluten introduction in the diet [1, 4, 5].

The pathology can be clinically distinguished in different forms, namely, classical, atypical, subclinical, and latent. The classical form is characterized by the typical gastroenterological signs related to the response of the intestinal mucosa after exposure to gliadin. Atypical forms are described with several symptoms and signs in various districts. The oral cavity is

an area which is highly affected by extraintestinal signs of the celiac disease, so that lesions in the oral mucosa or defects in dental enamel may often be the only presenting features of the atypical pattern [6–9]. The HLA haplotype was shown to have a strong effect on the distribution of typical and atypical signs. A previous work by Erriu et al. in 2011 related the distribution of recurrent aphthous stomatitis (RAS) and dental enamel defects (DED) in the oral cavity, in both child and adult celiac patients, to the presence or absence of the HLA-DQB1*02 allele [10]. Enamel defects in children were also analysed in another study in 2010 by Majorana et al., who reported the absence of correlation between DED formation and the expression of HLA-DR and -DQ alleles [11].

Based on the correlation detected in the previous 2011 paper between the HLA-DQB1*02 haplotype and oral signs in patients of all ages, the aim of this work was to verify whether the same evidence can be confirmed in pediatric patients.

2. Material and Methods

2.1. Subjects. 44 celiac patients (16 males and 28 females) were analysed, with a medium age of 9.9 years (range 6–16 years), all typed for anti-gluten antibodies (AGA, both IgA and IgG) and the anti-endomysial antibody (EMA). The pathology had been diagnosed for each patient after small bowel biopsy with positivity for enteropathy with Marsh type 3 villous atrophy. Patients with type 1 or 2 intestinal damage were excluded. Furthermore, all patients responded to a gluten-free diet, as evaluated during further regular annual followups. Anamnestic and diagnostic case studies were compiled for each patient to indicate the past or current presence of celiac oral signs, such as RAS and DED [9, 12, 13]. DED were graded from 0 to IV according to Aine's classification [14–16] while RAS linked to CD was determined by the investigation of past experience of aphthous lesions, described by the patients as the contemporaneous presence of one or more ulcers recurring at least twice a month in the period preceding the gluten-free diet and which were not reported 1 month after the start of the diet. The presence of these lesions was verified by evaluating the medical records of the patient's first admission.

An agreement form explaining the aim and the characteristics of the study was read and signed by the participants' parents, and an identifying code was assigned to each subject according to Italian privacy laws. The research was conducted in accordance with Declaration of Helsinki research ethics. Oral brushing was subsequently carried out on all patients, obtaining a sample for DNA extraction allowing the determination of the HLA-DQB1 haplotype using a conventional polymerase chain reaction (PCR) method [17].

All clinical data and samples were obtained from routine testing and visits carried out at the hospital where CD diagnosis had been performed. No new visits or sampling were carried out on patients in order to perform this study.

2.2. PCR Technique and Determination of HLA-DQB1 Genotype. The kit constituted of a preformed MIX, and eight couples of primers were used in order to perform the molecular analysis. Positivity or negativity of amplification for each couple allowed the HLA-DQB1 genotype to be established [9, 12, 13]. This set of primers can positively identify the HLA-DQB1 alleles corresponding to the serologically defined series HLA-DQ2, DQ3, DQ4, DQ5, DQ6, DQ7, DQ8, and DQ9; thus, all combinations of DQB1 can be readily identified. DQ4, DQ5, and DQ6 were uniquely identified, whereas DQ2 specificity was amplified by three primer mixes, DQ7 and DQ9 specificities were amplified by two primer mixes, and DQ3 and DQ8 specificities were amplified by four primer mixes.

On the contrary, on examining the eight primer mixes with the corresponding amplified DQB1 alleles, the first primer mix amplified allele group DQB1*05, the second amplified allele group DQB1*06, the third, the fourth, and the sixth amplified allele group DQB1*02, the fourth, the fifth, the sixth, and the seventh amplified allele group DQB1*03, and the eighth amplified allele group DQB1*04.

For all the alleles, the reaction was performed in 10.08 μL reaction volumes using the mixture according to the manufacturer's instructions. The mixture contained 3 μL of master mix, 5 μL of DNAsi-RNAsi free water, 0.08 μL of Taq polymerase, and 2 μL of DNA suspension, and this was put into a tube containing the lyophilized primer pair. The thermocycler profile was performed as follows: an initial denaturation at 94°C for 2 min, 10 cycles consisting of 94°C for 10 sec and 65°C for 1 min, and finally 20 cycles consisting of 94°C for 10 sec, 61°C for 1 min, and 72°C for 30 sec. PCR products were analysed by electrophoresis on an agarose gel.

2.3. Statistical Analysis. Descriptive statistics, Fisher's exact test, and binary logistic regression analysis were performed. To test the relation between the HLA haplotype and the presence of oral signs, a logit regression model was used where the outcome variable represents the presence (or the absence) of the specific oral sign to be tested (DED, RAS, or both). A variable was then coded with values 0, 1, or 2 if the individual carried no, one, or two copies of the HLA-DQB1*02 allele, respectively. Values of $P < 0.05$ were considered as significant.

3. Results

According to the clinical evaluation, the result of the patients affected by RAS was 18.2% (8 persons), while a DED was diagnosed for 38.6% of the patients (17 persons), with 52.3% (23 persons) presenting one or more oral signs (Figure 1).

HLA-DQB1*02 distribution showed similarities with the previous work by Erriu et al. [10] (Table 1, Figure 1). The percentage of patients carrying two copies of the alleles was 38.6%, and 40.9% showed heterozygosis while only 20.5% did not carry the allele.

DED diagnosis resulted as being related to the presence or absence of the allele (P value = 0.042). On the contrary, it was not possible to find a similar correlation with RAS (P value = 0.084). When considering both oral signs, correlation with HLA-DQB1*02 expression increased with a highly significant P value (P value = 0.018) (Table 2, Figure 2).

Table 1

	HLA-DQB1*02 homozygosis	HLA-DQB1*02 heterozygosis	HLA-DQB1*02 absence	Trials
Previous study [10]	33	47	18	98
Current study	17	18	9	44
P value	0.574	0.471	0.819	

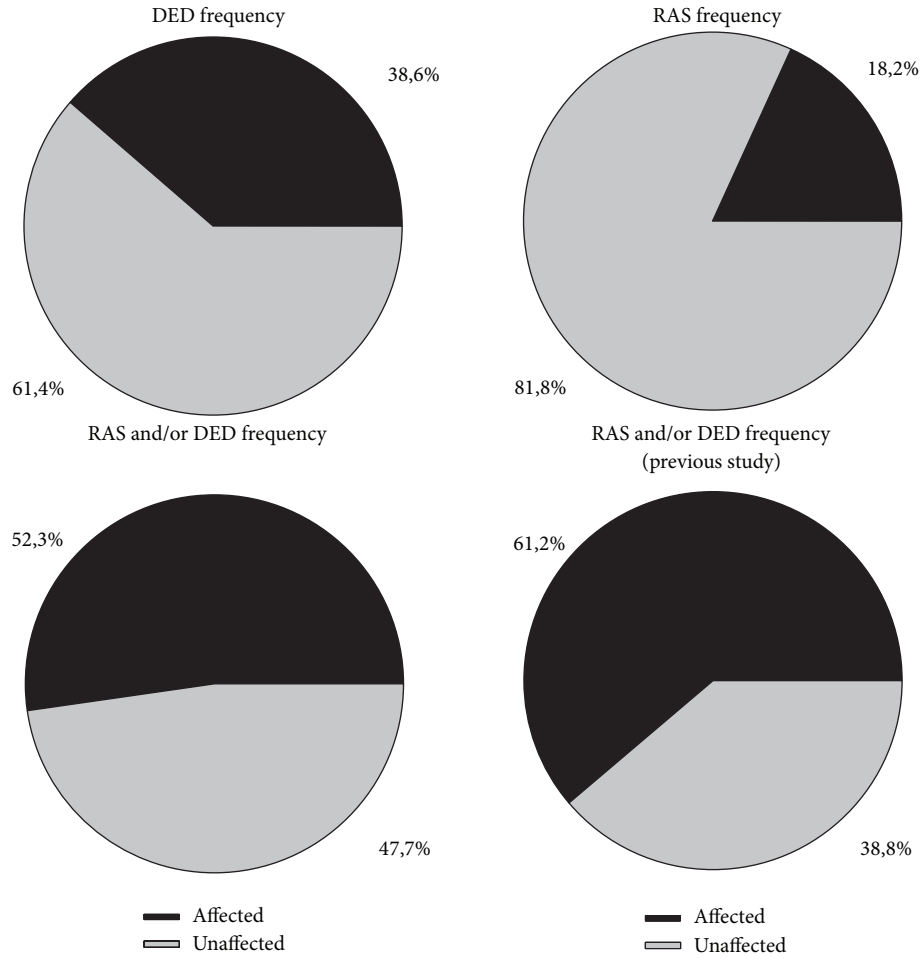

Figure 1

4. Discussion

Clinical manifestations of CD are variable, depending on the form of the disease. Classical CD is normally diagnosed at a young age, according to the easy recognition of the characteristic signs and symptoms. On the contrary, atypical forms can, in some cases, be hard to detect, making early diagnosis difficult to perform [18]. The awareness of the complications of the late diagnosis of celiac disease makes it necessary to establish guidelines allowing the clinician to suspect the disease early, even in the case of atypical forms. For this reason, extraintestinal symptoms have recently assumed increasing importance in order to arouse suspicion of CD. Oral signs have been described by several authors as diagnostic elements of frequent detection in the course of atypical disease [19]. DED and RAS frequency appears to be variable from study to study, in relation to the age of the patients, the geographical area, and environmental factors. A study by Bucci et al. in 2006 looked for a difference in the distribution of DED and RAS in 72 celiac patients compared with 162 healthy subjects. They described a prevalence of 20% of DED in celiac patients (against the 5.6% of the controls) while no statistical differences were found for RAS. In this study, 33.3% of the celiac patients showed oral ulcers against 23.4% of the controls [20]. In 2010, Costacurta et al. reported a frequency of 33.3% of DED and 8.3% of RAS in an Italian population of 300 celiac patients with a mean age of 8.16 years compared with a cohort of 300 healthy subjects [21]. In this study the authors described a statistically relevant difference between the two groups. A review published in 2011 by Rashid et al. described the prevalence of DED in patients with permanent teeth, as ranging between 9.5% and 95.9% (mean 51.1%) [9, 22]. In the same study, RAS frequency was based on a Canadian study which described patients with

Table 2

Copies of HLA-DQB1*02	*N* patients	*N* affected	*N* unaffected	Affected %	Unaffected in %	OR (CI 95%) *P* value
			DED			
0	9	8	1	88.9%	11.1%	0.40 (0.17–0.97) **0.042**
1	18	3	15	16.7%	83.3%	
2	17	6	11	35.3%	64.7%	
			RAS			
0	9	3	6	33.3%	66.7%	0.38 (0.13–1.14) **0.084**
1	18	4	14	22.2%	77.8%	
2	17	1	16	5.9%	94.1%	
			DED and/or RAS			
0	9	9	0	100.0%	0.0%	0.33 (0.13–0.82) **0.018**
1	18	7	11	38.9%	61.1%	
2	17	7	10	41.2%	58.8%	

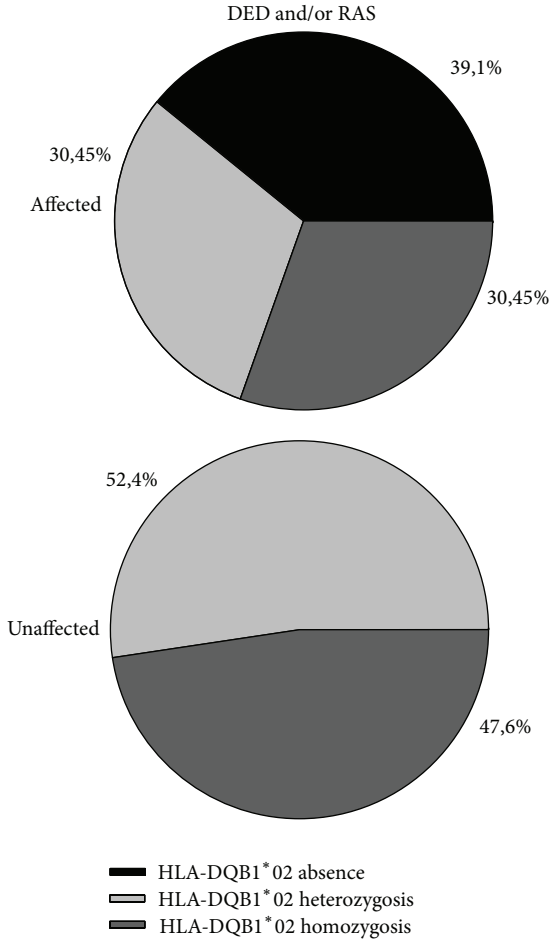

Figure 2

this sign, aged less than 16 years old, in 16% of the cases, while adult patients had reported recurrent oral ulcers in 26% of the cases [23, 24]. In 2008 Campisi et al. analysed the frequency of RAS in a group of 269 Italian celiac patients aged from 3 to 17 compared with a control group of 575 clinically healthy subjects. Their analysis identified a RAS frequency of 22.7% in the patients with CD against the 7.1% of the control group [25]. In 2011 Erriu et al. described the frequency of DED and RAS in a population of 98 celiac patients. In this work DED were identified in 28.6% of the cases and RAS in 38.8% of the patients [10]. In 2012 Yaşar et al. studied the prevalence of RAS in a cohort of 82 patients in Istanbul and identified the patients affected by RAS before esophagogastroduodenoscopy. Their results showed that the prevalence of CD in the RAS population did not significantly differ from that of the unaffected matched population [26]. In the same year El-Hodhod et al. performed a similar study, evaluating 140 Egyptian patients with DED, aged 4–12, while the control group was represented by 720 healthy children. From their analysis, celiac disease was diagnosed in 25% of the patients with DED, against the 0.97% of the control group [27].

The prevalence of oral signs in this paper resulted as being similar to that highlighted in the literature. In fact, DED were identified in 38.6% of the cases while RAS was observed in 18.2% of the patients. In comparison with the previous work performed in 2011, it was possible to identify a reduction of the cases of RAS [10]. This could be explained by the fact that some cases of RAS could have passed unobserved due to the gluten-free diet started in early childhood.

This high variability in the prevalence of the oral signs was analysed in 2011 in the study by Erriu et al. which showed a statistically significant correlation between oral manifestations and HLA expression [10]. In the present study this correlation resulted statistically confirmed in the younger patients. The HLA-DQB1*02 allele has a fundamental influence on the pathogenesis of CD. The study by Jores et al. showed a positive association between an increasing frequency of DQB1*0201 allele homozygosis and the severity of intestinal damage [28]. On the contrary, an only partially explained negative association does seem to exist in the oral cavity. The hypothesis elaborated in 2011 has not yet been adequately detailed in order to reach a conclusive observation. Both the histological diversity of oral and intestinal mucosa, such as the different oral bacterial flora, as well as a reaction

related to additional stimuli typical of the oral cavity, could all be possible responses to the phenomena described [10].

5. Conclusions

The need of an early diagnosis for all the forms of CD is still an open challenge. This study has shown how the role of dentists in identifying atypical patterns could be fundamental. In particular, the presence of oral signs could be very important for detection in the youngest patients, avoiding the several complications related to CD in adulthood.

Authors' Contribution

M. Erriu and G. M. Abbate equally contributed to this work.

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