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Anne Lammi

Gliadin-specific immune responses in the development and prediction of celiac disease in children

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ANNE LAMMI

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ABSTRACT

Celiac disease is a chronic autoimmune enteropathy caused by dietary gluten, a protein rich of gliadin peptides found in wheat and related cereals, in genetically predisposed individuals. Celiac disease can be established in all age groups. The typical symptoms of celiac disease are diarhoea, runny stools, abdominal distension, weight loss and growth disturbance and delayed puberty in children. As a consequence of the malabsorption anemia and ostheoporosis may follow. It is known that some of the affected patients may have unspecific symptoms, or they may be even asymptomatic. The primary screening of celiac disease is mainly based on IgA class tissue transglutaminase autoantibody (tTGA) measurement and the diagnosis is confirmed by the identification of typical histological changes, crypt hyperplasia and villous atrophy, in the small intestinal mucosa by duodenal biopsy. Although serological screening has a central role in the diagnosis of celiac disease, it is known that the T-cell mediated inflammatory process of the intestinal mucosa has already begun when celiac disease-associated antibodies appear. The aim of this thesis was to analyze, by using immunological methods, whether gliadin-specific immune responses can be detected prior to the damage of the intestinal mucosa and the development of the clinical disease.

Peripheral blood gliadin-specific CD4+ T-cell responses were detected in over half of the children with newly diagnosed celiac disease in study I. In children with celiac disease T-cell responses were detected more often to deamidated gliadin (gTG) than to native gliadin whereas the responses to deamidated and native gliadin did not differ in healthy controls.

The detection of antibodies to synthetic deamidated gliadin peptide (anti-DGP) can be used in diagnosis of celiac disease in young children. In study II we demonstrated that the time-resolved immunofluorometric (TR-IFMA) assay for detecting anti-DGP performed with both a high sensitivity and specificity in the diagnosis of celiac disease in pediatric patients.

In study III newborn children were screened for HLA-DQ2, encoded by HLA-DQA1*05 and -DQB1*02 alleles and were followed prospectively by screening for tTGA and anti-DGP antibodies and peripheral blood gliadin-specific T-cell responses until 3 or 4 years of age. The cumulative incidence of tTGA seropositivity until the age of 4 years was 6.5% and the final incidence of celiac disease was 3.1%. All except one of the children in the follow-up study were diagnosed through the serological screening without previous clinical suspicion of celiac disease. We demonstrated both retrospectively (II) and prospectively (III) that in a majority of the children with celiac disease specifically IgG anti-DGP antibodies appeared a median one year earlier than tTGA seropositivity. In the prospective follow-up (III) peripheral blood gliadin-specific T-cell responses were rela-

tively common at the age of 9 and 12 months but the proportion of the positive responses decreased during the follow-up to the similar lever detected in slightly older healthy control children in study I. The common appearance of T-cell responses to gTG may be related with an early immunization to dietary gluten, which in most cases does not lead either to the development of celiac disease-related antibodies or the clinical onset of the celiac disease.

In conclusion, the results of this work indicate that detection of celiac disease earlier than with current approaches may be possible by monitoring tTGA and anti-DGP antibodies frequently in genetically susceptible children whereas the monitoring of peripheral blood gliadin-specific T-cell responses does not enhance early diagnosis. Moreover, anti-DGP antibodies may be the first marker of celiac disease in a majority of pediatric patients.

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Gliadiinispesifisen immunivasteen rooli keliakian kehittymisessä ja sen ennustamisessa lapsilla
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TIIVISTELMÄ

Keliakia on ravinnon gliadiinin aiheuttama krooninen, autoimmuuni suolistosairaus geneettisesti alttiilla henkilöillä. Keliakiaa esiintyy kaikissa ikäryhmissä. Sen tyypillisiä oireita ovat mm. ripuli, löysät ulosteet, ilmavaivat, painon lasku ja lapsilla kasvun hidastuminen tai puberteetin viivästyminen. Suoliston imeytymishäiriön seurauksena voi ilmaantua anemiaa ja osteoporoosia. Keliakian seulonta perustuu pääosin seerumin IgA luokan kudostransglutaminaasivasta-aineiden määritykseen, ja diagnoosi varmistetaan ohutsuolen biopsialla, jossa keliakialle tyypilliset muutokset ovat villusatrofia ja kryptahyperplasia. Vasta-aineiden seulonnalla on keskeinen merkitys keliakian diagnosoinnissa mutta toisaalta tiedetään, että T-soluvälitteinen ohutsuolen tulehdusreaktio on jo käynnistynyt keliakialle spesifisten vasta-aineiden ilmaantuessa. Lisäksi tiedetään, että osalla sekä aikuis- että lapsipotilaista oireet voivat olla epäspesifisiä, kuten niveloireita tai ataksiaa ja osa potilaista voi olla täysin oireettomia. Keliakian toistaiseksi ainoa hoitomuoto on elinikäinen gluteeniton ruokavalio. Tämän väitöstutkimuksen tarkoituksena oli selvittää, voidaanko immunivastetta havaita jo ennen ohutsuolen tuhoutumista ja kliinisen taudin puhkeamista immunologisilla testeillä.

Osatyössä I perifeerisen veren gliadiinispesifisiä T-soluvasteita havaittiin hieman yli puolella lapsista, joilla oli juuri diagnosoitu keliakia. Keliakiaan sairastuneilla lapsilla T-soluvasteita esiintyi merkitsevästi enemmän deamidoitua gliadiinia kohtaan, kun taas terveillä mutta keliakialle geneettisesti alttiilla kontrollilapsilla T-soluvasteet olivat yhtä yleisiä deamidoitua ja natiivia gliadiinia kohtaan.

Erityisesti pienten lasten keliakiadiagnostiikassa voidaan käyttää vasta-ainemääritystä spesifille synteettiselle deamidoidulle gliadiinipeptidille. Osatyössä II osoitimme immunofluorometriaan perustuvan (TR-IFMA) gliadiinipeptidivasta-aineita mittaavan testin olevan sekä hyvin sensitiivinen että spesifinen lasten keliakiadiagnostiikassa.

Osatyössä III vastasyntyneistä seulottiin HLA-DQB1*02 ja -DQA1*05 alleelien suhteen positiiviset lapset, joita seurattiin 3:een tai 4:ään ikävuoteen asti määrittämällä transglutaminaasi- ja gliadiinipeptidivasta-aineita sekä perifeerisen veren gliadiinispesifisiä T-soluvasteita. Kumulatiivinen transglutaminaasivasta-ainepositiivisuus 4:ään ikävuoteen mennessä oli 6,5 % ja keliakian ilmaantuvuus 3,1 %. Yhtä lasta lukuun ottamatta kaikki keliakiaan sairastuneet lapset diagnosoitiin vasta-aineseurannan perusteella ilman edeltävää kliinistä keliakiaepäilyä. Osoitimme sekä retrospektiivisesti (II) että prospektiivisesti (III), että suurimmalla osalla keliakiaan sairastuneista lapsista erityisesti IgG luokan gliadiinipeptidivasta-aineet ilmaantuivat keskimäärin vuotta aiemmin kuin transglutaminaasivasta-ainepositiivisuus. Prospektiivisessa seurannassa (III) perifeerisen veren gliadiinispesifiset T-soluvasteet olivat suhteellisen yleisiä 9 ja 12 kuukauden iässä, mutta positiivisten vasteiden määrä laski seurannan myötä samalle tasolle kuin hieman

vanhemmilla kontrollilapsilla osatyössä I. Gliadiinispesifisten T-soluvasteiden yleisyys voi liittyä varhaiseen immunisaatioon gluteenille, joka suurimmalla osalla lapsista ei johda keliakialle spesifisten vasta-aineiden ilmaantumiseen eikä kliinisen taudin puhkeamiseen.

Tämän tutkimuksen perusteella keliakian varhaisempi havaitseminen voisi olla mahdollista seuraamalla säännöllisesti transglutaminaasi- ja gliadiinipeptidivasta-aineita keliakialle geneettisesti alttiilla lapsilla. Sen sijaan perifeerisen veren gliadiinispesifisten T-soluvasteiden seurannalla ei ole vaikutusta keliakian varhaisempaan toteamiseen. Osoitimme, että gliadiinipeptidivasta-aineet voivat olla ensimmäinen todettavissa oleva immunologinen merkkiaine suurella osalla keliakiaan sairastuneista lapsipotilaista.

Yleinen suomalainen asiasanasto: keliakia, immuunivaste, autovasta-aineet, gliadiinit, lymfosyytit

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List of original publications

This dissertation is based on the following original publications:

I Lammi A, Arikoski P, Vaarala O, Kinnunen T, Ilonen J. Increased peripheral blood CD4+ T cell responses to deamidated but not to native gliadin in children with coeliac disease. Clin Exp Immunol. 2012;168:207-214.

II Lammi A, Arikoski P, Simell S, Kinnunen T, Simell V, Paavanen-Huhtala S, Hinkkanen A, Veijola R, Knip M, Toppari J, Vaarala O, Simell O, Ilonen J. Antibodies to Deamidated Gliadin Peptide in Diagnosis of Celiac Disease in Children. J Pediatr Gastroenterol Nutr. 2015;60:626-31.

III Lammi A, Arikoski P, Hakulinen A, Schwab U, Uusitupa M, Heinonen S, Savilahti E, Kinnunen T, Ilonen J. Development of gliadin-specific immune responses in children with HLA-associated genetic risk for celiac disease. Scand J Gastroenterol. 2015 Jul 10:1-10.

In addition, some unpublished data is presented.

Contents

Abbreviations

AGA	antibodies against gliadin
anti-DGP	antibodies against deamidated gliadin peptide
APC	allophycocyanin / antigen presenting cell
ARA	anti-reticulin antibodies
AU	arbitrary unit
CD	cluster of differentiation
CDI	cell division index
CFSE	carboxyfluorescein diacetate succinimidyl ester
CTLA-4	cytotoxic T-lymphocyte associated protein 4
DIPP	Finnish Type 1 Diabetes Prediction and Prevention study
ELISA	enzyme-linked immunosorbent assay
EmA	endomysial antibodies
FACS	fluorescence-activated cell-sorting
FINDIA	Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes
FOXP3	forkhead box P3 transcription factor
GI	gastrointestinal
gTG	deamidated gliadin
HLA	human leucocyte antigen
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MMP	matrix metalloproteinase
NK	natural killer
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin
PerCP-Cy5.5	peridinin chlorophyllcyanin
PHA	purified phytohaemagglutinin
ROC	receiver operating characteristics
T1D	type 1 diabetes
TCR	T cell receptor
TG2	type 2 tissue transglutaminase
TG3	type 3 tissue transglutaminase
Th	T helper cell
TNF	tumor necrosis factor
TR-IFMA	time-resolved immunofluorometric assay
Tr1	T regulatory cell type 1
Treg	regulatory T cell
TT	tetanus toxoid
tTG	tissue transglutaminase
tTGA	tissue transglutaminase antibodies

1 Introduction

Celiac disease, first described in 1888 by Samuel Gee, has been historically characterized as severe diarrhoea associated with weight loss and growth disturbance in infancy caused by an intolerance to dietary gliadin, a subset of gluten proteins (1). Nowadays, the classical clinical presentation has become rare and celiac disease manifests currently with more mild gastrointestinal (GI) symptoms and is observed in all age groups (2-5). The most common extraintestinal manifestation of celiac disease is dermatitis herpetiformis, a blistering skin disease (6,7). Other extraintestinal manifestations, such as gluten-sensitive ataxia and mental disorders, have also been described (8,9). A lifelong gluten-free diet is currently the only essential treatment for all forms of celiac disease.

Celiac disease has a strong HLA-associated genetic predisposition as approximately 90% of celiac disease patients express the HLA-DQ2 molecule and the rest of the patients express the HLA-DQ8 molecule (10,11). Although the expression of the HLA-DQ2 or -DQ8 molecules is necessary for the development of celiac disease, this alone is not sufficient and environmental factors also play a central role in disease pathogenesis. Dietary gluten is the major environmental factor known to be necessary for the development of celiac disease. Several non-dietary environmental factors such as neonatal infections and the season of birth have been suggested to increase the risk for celiac disease but none of them have been confirmed yet (12,13).

Celiac disease is thought to be a T-cell mediated autoimmune disease, in which immunogenic gliadin peptides, after passing the mucosal layer in the intestinal mucosa, can activate the adaptive immune response. These gliadin peptides are presented to CD4+ T cells in the context of the HLA-DQ2 or -DQ8 molecules on antigen presenting cells (APCs). CD4+ T cells recognizing gliadin epitopes were first isolated from small intestine biopsies of patients with celiac disease (10,11). T cells specific to deamidated gliadin have also been demonstrated in the circulation of adult patients with active disease, most commonly after oral gluten challenge (14-16). Gliadin-specific T-cell responses have been studied far less widely in children with celiac disease, but the small intestinal T-cell response observed in young individuals appears to target a more diverse array of gliadin epitopes compared to that in adults (17,18). The appearance of peripheral blood CD4+ T cells specific to gliadin is a non-invasive tool for detecting the immune response and may allow the earlier detection of the disease process.

Serological screening has a central role in the diagnosis of celiac disease. Antibody responses to gluten components and to self-tissue structures have been characterized and used in the diagnosis of celiac disease ever since anti-gliadin antibodies (AGA) were first described in 1958 (19,20). Nowadays, AGA assays are no more recommended for celiac disease diagnostics, as their specificity is relatively poor (21). The antireticulin antibodies (ARA), against the reticular fibers of endomysium were discovered in 1977 and used widely in the diagnosis of celiac disease (22-24). The ARA assay is also no longer recommended, as nowadays more sensitive and specific tests have been developed (21,25,26). Endomysial antibodies (EmA), in turn, were discovered in 1983. They perform with high specificity in the diagnosis of celiac disease, but the method is laborious and the interpretation of its results somewhat subjective (27). In 1997, tissue transglutaminase (tTG) was discovered to be the autoantigen targeted by EmA (28) and since then, the screening of celiac disease has been based on the measurement of immunoglobulin (Ig) class A tissue transglutaminase autoantibody (tTGA) (21). Assays detecting IgA and IgG antibodies to deamidated gliadin peptides (anti-DGP) have been developed most recently and it has been demonstrated that they may appear as a first marker of the celiac disease (29,30). The diagnosis of suspected celiac disease is currently confirmed by the typical histolog-

ical changes in the small intestine, crypt hyperplasia and villous atrophy with increased number of intraepithelial lymphocytes (IELs), detected by duodenal biopsy (31). However, recently proposed new criteria by The European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) has underlined the significance of serological testing in the diagnosis of celiac disease in children and the need for duodenal biopsy is being evaluated critically (21).

The aim of the present study was 1) to analyze the development of gliadin-specific immune responses in children who are genetically at high risk for the development of celiac disease and 2) to evaluate whether these responses can be detected before the clinical onset of the disease. To this end, serum tTGA and anti-DGP antibodies as well as peripheral blood gliadin-specific T-cell responses were assessed.

2 Review of the literature

2.1 CLINICAL FEATURES OF CELIAC DISEASE

2.1.1 Classical gastrointestinal celiac disease

Celiac disease is a chronic small intestinal immune-mediated enteropathy caused by dietary gluten in genetically predisposed individuals. Celiac disease was historically characterized as severe diarrhoea associated with growth disturbance in infancy (2). This classical presentation of celiac disease has become rare and nowadays the disease often manifests with milder GI symptoms, such as chronic diarrhoea, abdominal pain and distension both in adults and in children (3,32-34). Moreover, in pediatric patients the proportion of individuals with minor or atypical symptoms has increased (35,36). Undiagnosed celiac disease may also cause malabsorption and deficiencies of nutrients leading to various chronic symptoms such as general fatigue, anemia and osteoporosis.

2.1.2 Extraintestinal manifestations and complications

Dermatitis herpetiformis, a blistering skin disease, has been considered as a variant form of celiac disease with the same genetic background (6,7,37). Although only a minority of these patients have GI symptoms, up to 60–80% of them have pathological changes in the intestinal mucosa (38,39). The diagnosis of dermatitis herpetiformis is based on the demonstration of granular IgA deposits in the papillary dermis by a direct immunofluorescence method (7,40). A gluten-free diet is also the essential treatment in dermatitis herpetiformis (41,42). Dermatitis herpetiformis rarely affects children.

Several neurological symptoms associated with the appearance of celiac disease antibodies have also been described (43). It has been suggested that tissue transglutaminase autoantibodies, specifically, may play a central pathogenic role in the extraintestinal manifestations associated with celiac disease (8,44). The most common neurological manifestation of gluten sensitivity is ataxia (8,45). Other neurological symptoms such as polyneuropathy and early-onset dementia have also been described (46,47). Undiagnosed celiac disease has been shown to be associated with mental and behavioural disorders, such as depression during adolescence (9). Several reproductive problems, such as infertility and increased risk of miscarriage, have also been associated with celiac disease (48). In addition to these, other non-classical symptoms, such as dental enamel defects, aphthous ulcer, arthritis and abnormalities in liver function tests, and even severe liver disease, have been demonstrated (49-53). It has also been suggested that calcium malabsorption due to celiac disease may lead to decreased bone mineral density and increased fracture risk but these results are currently conflicting (54-57).

The association of celiac disease with cancer was established in the 1930s. An increased risk for lymphomas and GI cancers has been reported in several studies, albeit with great variation. Early studies reported a 50- to 100-fold risk for such malignancies but subsequent later studies have estimated the risk to be 3- to 6-fold higher (58-60). The increased risk for lymphomas and GI cancers is strongly related to refractory celiac disease, which manifests as persistent or recurrent malabsorptive symptoms and villous atrophy despite of strict adherence to a gluten-free diet (61). The overall risk for lymphomas and GI cancers in patients following a strict gluten-free diet is increased only early after diagnosis and later on it decreases to a more moderate level (62,63). In all, due to improved diagnostics, the risk of complications of celiac disease has decreased in recent times. However, there are still undiagnosed patients who are at risk of long-term complications (64).

2.1.3 Prevalence of screening-detected celiac disease

The prevalence of screening-detected celiac disease has been reported to be as high as 10–20% in studies of subjects with a family history of the disease (65-68). Moreover, screening done in patients with related autoimmune diseases, in particular type 1 diabetes (T1D), has revealed that there is a large number of cases of silent celiac disease (69-73). The co-morbidity with T1D has been partially explained with the same predisposing genetic background for these diseases but the actual mechanism still remains unresolved. Celiac disease has been shown to be overrepresented not only among patients with autoimmune disorders but also in patients with some chromosomal disorders, such as Down's syndrome (74-78). The frequency of celiac disease is also remarkably higher among patients with selective IgA deficiency than in the general population, but this may also be related to the associations of both diseases with the same HLA haplotype (79-81).

2.2 EPIDEMIOLOGY OF CELIAC DISEASE

Both the prevalence and the risk of celiac disease show extensive geographical variation (82-87). The prevalence of celiac disease in the general population in Western Europe and in North America is around 1% (88-92). According to population-based studies it seems that there are still some countries, such as Japan, China and some African countries, where celiac disease is almost non-existent and only a few cases have been described (92,93). Screening studies done in Finland have demonstrated a 1% prevalence of celiac disease in children and even a 2% prevalence in adults (35,94). Moreover, the prevalence of celiac disease has increased both in Finland and in North America over the last decades (89,94,95). Interestingly, a similar trend has been reported in the prevalence of allergic diseases and T1D (94,96-99).

While the prevalence of celiac disease has increased over the last decades, the reasons for this are still under investigation (89,95). In addition to the improved diagnostics also the true prevalence of celiac disease has increased. Environmental factors, such as the so-called hygiene hypothesis, have been suggested to be one explanation, similar to what has previously been suggested for the increase in allergy and atopy (70,100,101). A strong increase in the incidence of celiac disease was discovered in Sweden in the 1980s, and this offered a great opportunity to analyze the possible environmental factors involved (102,103). Based on these epidemiological studies, changes in infant feeding recommendations have been suggested to be one key explanation for the increased prevalence of celiac disease (see 2.3.2.1).

Screening studies in children have shown that the risk for celiac disease has increased more in girls compared to boys (104-106). However, conflicting results on this issue have been reported in one Swedish study, in which the prevalence of screening-detected celiac disease was equal in girls and boys (107). The prevalence of celiac disease in adult patients has been shown to be three-fold higher in women compared to men (108). It is well known that autoimmune diseases are generally more common in women (109), with a protective effect of androgens hypothesized as an explanation (109,110). However, the higher frequency of celiac disease in prepubertal girls suggests that the higher frequency of celiac disease cannot solely be explained by the sex-hormone theory.

2.3. PATHOGENESIS OF CELIAC DISEASE

2.3.1 Genetics of celiac disease

2.3.1.1 HLA genes

The heredity of celiac disease was first suggested in 1951 (111) by the higher prevalence of celiac disease in family members of celiac disease patients. The prevalence in first-degree relatives is around 20% (65,66,112,113). Importantly, the prevalence of celiac disease in monozygotic twins has been reported to be around 70–80% (66,114), which is much higher than the prevalence of the disease in heterozygous twins. This specifically demonstrates that genetic factors have a central role in the pathogenesis of celiac disease.

Celiac disease is known to have one of the strongest human leucocyte antigen (HLA) associations of all autoimmune diseases. The HLA-associated genetic risk comprises around 30–50% of the entire genetic risk for the development of celiac disease (115-119). HLA-genes, which encode the HLA class I and class II molecules, are located on chromosome 6p22 (120,121). HLA class I and II molecules are membrane proteins, both of which have extracellular domains that form a cleft in which a peptide fragment is bound. However, they differ slightly in their structures, as the outer domains of HLA class I molecules are formed by α-1 and α-2 chains whereas HLA class II molecules are heterodimers formed by α- and β-chains. HLA class I molecules are expressed in most of human cells whereas HLA class II molecules are expressed predominantly in APCs.

Association of alleles in the HLA complex with celiac disease was first described in 1972 using serological methods (122). HLA-association with celiac disease was first reported with the HLA class I B8 and HLA class II DR3 alleles (123,124) A more specific HLA-association was established in 1989 when Sollid and co-workers found an association between celiac disease and the combination of alleles encoding the α-chain DQA1*05 and the β-chain DQB1*02 of the HLA class II DQ2 heterodimer (125).

More than 90% of celiac disease patients express one or two copies of HLA-DQ2 encoded by alleles DQA1*05 and DQB1*02 (10,126). In patients with the HLA-DR3-DQ2 haplotype DQA1*05 and DQB1*02 alleles are located on the same chromosome in the so-called cis-position, whereas in patients carrying both the HLA-DR7-DQ2 an HLA-DR5-DQ7 haplotypes the alleles are located on the opposite chromosomes in the so-called trans-position (Figure 1.) (125,126). The highest risk for celiac disease has been reported in DR3-DQ2 homozygous and DR3-DQ2 and DR7-DQ2 heterozygous individuals, which may be explained by a gene dose effect of the DQB1*02 allele (87,127,128). The HLA-DQ2 molecule may also be encoded by the alleles DQA1*0201 and DQB1*0202. The expression of this molecule is associated with lower risk of celiac disease than HLA-DQ2 encoded by DQA1*05, possibly due to the replacement of a tyrosine by phenylalanine residue in DQα at position 22, which has been described to be important for the peptide binding of gluten epitopes in the context of HLA-DQ2 (129,130). The remaining 10% of celiac disease patients, who do not express HLA-DQ2, express the HLA-DQ8 molecule, encoded by the DQA1*03 and DQB1*0302 alleles. The risk for celiac disease in these patients, however, is considerably lower than in those expressing HLA-DQ2 (11,126,129,131). In very rare cases, subjects with celiac disease carry either the DQA1*0501 or DQB1*02 allele alone (132).

Individuals who carry HLA-DR3-DQ2 and/or HLA-DR4-DQ8 are also predisposed to T1D and, therefore, the link between celiac disease and T1D is remarkably strong. Approximately 5–20% of patients with type T1D develop celiac disease during their lifetimes. Celiac disease is also associated with an increased risk for other autoimmune diseases; approximately 15–20% of patients with celiac disease are already affected by other autoimmune diseases or will develop those at a later stage in life (133,134).

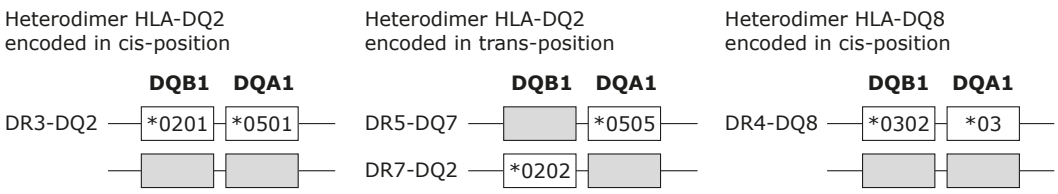

Figure 1. The HLA-DQ2 (DQA1*05–DQB1*02) heterodimer is encoded in cis position in individuals with the DR3–DQ2 haplotype an in trans position in individuals heterozygous for the DR5–DQ7 and DR7–DQ2 haplotypes. The HLA-DQ8 (DQA1*03–DQB1*0302) heterodimer is encoded in cis position in individuals with the DR4-DQ8 haplotype.

2.3.1.2 Non-HLA genes

The prevalence of HLA-DQ2 or -DQ8 expression is around 20–30% in the general population but approximately only around 3% of these individuals develop celiac disease during their lifetimes (135). Therefore, other genetic and environmental factors clearly also play a role. A number of non-HLA gene regions have been shown to be associated to celiac disease in multiple genome wide association studies but it has been estimated that all the non-HLA risk genes together explain only approximately 5% of the entire risk (116-119,136). The majority of the non-HLA genes identified in these studies are immune-related genes and many of them are associated with T and B cell function (137). The genomic region containing the CTLA-4 gene on chromosome 2q33 has been reported to associate with autoimmunity in general, and the CTLA-4 polymorphism is one of the most studied non-HLA gene association also in celiac disease (138,139). Although several studies have reported a linkage between polymorphisms in the CTLA-4 gene and celiac disease, conflicting results have also been reported (140-144). In addition to the CTLA-4 locus, the involvement of several other gene loci has also been studied. Chromosomes 5 and 19 are of major interest: both the 5q31-33 and 19p.13.1 genetic regions have been reported to have a linkage to celiac disease (145-151). There are also some promising results for an association with chromosome 9p21-p31 (148,149,151). Many of the identified non-HLA celiac disease risk loci are also associated with other immune-related diseases, in particular T1D and rheumatoid arthritis (152-154). The similar genetic background among these autoimmune diseases indicates that parts of their pathogenic pathways are common. In all, it seems that none of the non-HLA genes identified to date have a central role in the genetic risk by themselves but their combined genetic effect has been suggested to have a significant impact.

2.3.2 Environmental factors

2.3.2.1 Dietary gluten and gluten introduction

Dietary gluten is the major environmental factor affecting celiac disease. Gluten is a protein found in wheat, barley and rye, that is rich in proline and glutamine residues (155). Wheat gluten contains two main fractions: soluble gliadin storage proteins and insoluble glutenin proteins (Figure 2.) (155). Although gliadin originally refers to the soluble proline fraction of wheat, the name is commonly also used for the similar fractions, hordein and secalin, in barley and rye, respectively. Gliadins are monomers and classified according to their different primary structures into the α, γ- and ω-type gliadins, whereas glutenins are polymers with high or low molecular weight (155). Oat has been deemed safe in the diet of most celiac disease patients probably since instead of gluten it contains avenin, a protein with a different prolamin structure (156).

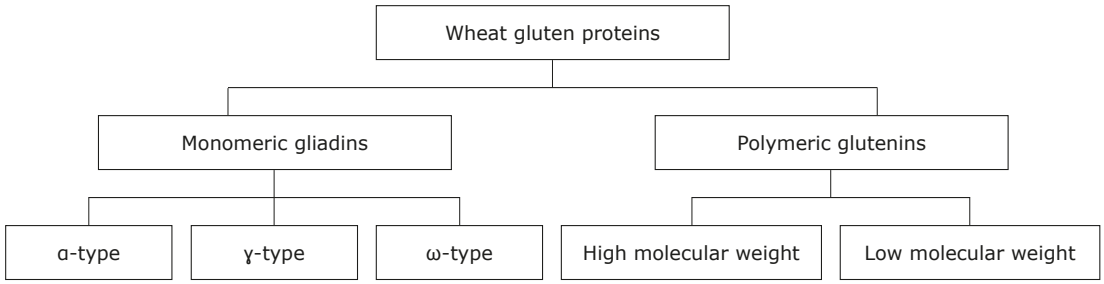

Figure 2. Classification of wheat gluten proteins.

Wheat gluten was identified as a trigger of celiac disease already in the early 1950s by Dicke et al. (157). The age at which gluten was introduced was suggested to play a central role in the development of celiac disease much later, in the late 1980s (158,159). Since then, both the timing of gluten introduction and breastfeeding has been suspected to be associated with the increased risk for development of celiac disease in several studies (160-163). Multiple studies have tried to determine whether breastfeeding itself affects the risk for celiac disease and whether the duration of breastfeeding decreases the risk of celiac disease. Most of these studies have been based on retrospective analysis and thus lack a proper case-control setting. The largest case-control study so far was performed in Sweden by Peters and co-workers (164). They reported that on-going breastfeeding during gluten introduction at infancy has a protective effect on future celiac disease in children under 2 years of age but not in older children between 2 and 15 years (Peters et al. 2001). Although the protective effect of breastfeeding for celiac disease risk has been reported widely in epidemiological studies, this effect has not been found in all studies (103,160,161,165,166). Decker et al. have reported that prolonged breastfeeding was in fact associated with an increased risk for celiac disease (166). Moreover, the actual mechanism of the possible protective effect of breastfeeding remains unsolved. One hypothesis is that continued breastfeeding could reduce the amount of gluten introduced to the infant's diet (165). It has also been suggested that human milk IgA antibodies could decrease the immune response against gluten in the gut of infants (163).

Since early gluten introduction has also been suggested as a trigger for celiac disease, several studies have examined the optimal age for gluten introduction (158,159). The effect of the timing of gluten introduction was suspected in Sweden between 1984 and 1996, when there was a strong increase in the incidence of celiac disease, which was related to new national nutritional recommendations that suggested postponing gluten introduction from 4 to 6 months of age (167,168). The incidence was also reduced when these recommendations were changed in 1996. The new national recommendations of infant feeding in Sweden suggest introducing gluten in small amounts between the age of 4 and 6 months while the child is still breastfed (167,169). Norris et al. have also shown an association between the age at which gluten is introduced and the risk of celiac disease (161). Based on several studies, gluten introduction before the age of 4 months or after 6 months without concurrent breastfeeding has been suggested to increase the risk for development of celiac disease (103,160,161,167,168,170). However, there has been a debate whether later gluten introduction only delays the onset of celiac disease in predisposed individuals instead of actually reducing the risk of the disease. In a recent Norwegian nationwide prospective study it was observed that a higher risk of celiac disease was associated with delayed gluten introduction after 6 months of age, as well as with prolonged breastfeeding over 12 months (171).

Most importantly, recently published large prospective studies have not provided support for the role of breastfeeding or age of gluten introduction for celiac disease risk. In these studies, neither the duration of breastfeeding, age at gluten introduction, nor simultaneous ongoing breastfeeding during gluten introduction have affected the probability of celiac disease (106,172,173).

2.3.2.2 Other environmental factors

Several non-dietary environmental factors have been suggested to be risk factors for celiac disease. These factors include both intrauterine and perinatal conditions (174). Sandberg-Bennich et al. have reported that both neonatal infections and small size for gestational age increase the risk of celiac disease (175). Multiple studies have shown that maternal smoking associates with a lower risk of celiac disease (175-177), and that the season of birth associates with an increased risk of celiac disease (12,13,178). Two large studies have suggested that children born in spring or summer have an increased risk for celiac disease (13,179). This has been explained by the timing of dietary gluten introduction during the wintertime when these children are simultaneously affected by seasonal infectious diseases.

The etiological role of viral infections is one the major area of research in all autoimmune diseases. Ever since Kagnoff and co-workers suggested a possible role for adenovirus in the pathogenesis of celiac disease, several studies have tried to evaluate whether infectious diseases associate with celiac disease (180). So far, only a few of the studies have managed to find any evidence for an association. Similar findings of a potential role of virus infections in T1D has raised the interest of studying this aspect in celiac disease as well, since T1D also confers an increased risk for celiac disease (181-184). Rotavirus infections have been shown to associate with an increased risk for celiac disease in a US population (12). In a German study, it was found that children with celiac disease had more often earlier gastrointestinal infections prior to celiac disease diagnosis (166). In contrast to these reports, one prospective study by Welander et al. reported that infection or gastroenteritis at the age of gluten introduction did not associate with the risk of future celiac disease (185).

Several new environmental hypotheses on the etiology of celiac disease have also recently been presented. The finding of an increased risk of celiac disease associated with caesarean section has been explained by the protection provided by the vaginal bacterial flora colonizing the infant during normal delivery (166), although this has not been demonstrated in all studies (176,186,187). Gut microbiota is an interesting novel point of view in explaining the pathogenesis of all autoimmune diseases. In recent years, the role of the gut microbiota has been studied also in celiac disease. Several studies have shown that the different composition of intestinal bacterial populations, such as increased gram-negative bacteria and decreased bifidobacteria, is associated with celiac disease but the specific role of microbes in the pathogenesis of celiac disease is still unresolved (188-190).

2.3.3 Immunopathogenesis of celiac disease

2.3.3.1 T cell development and function

It is still open for debate, why dietary gluten causes an inflammatory process in the small intestine and leads to the destruction of epithelia in some individuals. Celiac disease has been classified as a T-cell mediated autoimmune type disease. T cells, or T lymphocytes, are characterized by their expression of T cell receptors (TCR) composed of α- and β-chains ($\alpha\beta$-TCR) and are further categorized into helper T cells (CD4+ T cells) and cy-

totoxic T cells (CD8+ T cells) by their phenotypic and functional characteristics (191,192). T cells originate from bone marrow stem cells and their maturation process occurs in the thymus. The progenitor T cells that enter the perimedullary cortex of the thymus are CD4/CD8 double-negative (CD4-CD8-) and as they migrate to the outer region of the thymic cortex, the expression of both CD4 and CD8 molecules on the cell surface is induced (193). Further, as CD4+CD8+ T cells migrate back to the medulla, they undergo positive selection, in which those T cells that do not recognize self-peptides in the context of self HLA-molecules die by apoptosis (194). Only a minority of the CD4+CD8+ T cells, approximately 10%, survives this process. If the T cell recognizes a self-peptide in the context of an HLA class I molecule, CD4 expression is downregulated and the cell becomes single-positive for CD8 expression. Analogously, if the self-peptide is recognized in the context of an HLA class II molecule, CD8 expression is downregulated and the cell becomes single-positive for CD4 expression (193). Subsequently, these single positive CD4+ and CD8+ T cells undergo negative selection in which T cells that recognize a self-peptide too strongly are induced to undergo apoptosis in order to purge the mature T cell repertoire of autoreactive T cells (194). Finally, mature CD4+ and CD8+ T cells are released into the circulation.

Several other subsets of T cells have also been described. The other form of the TCR is composed of γ/δ chains and T cells expressing these types of TCR are called γ/δ T cells. Some of γ/δ T cells express the CD8 molecule but most of them are negative for both CD4 and CD8 molecules (195). The γ/δ T cells are present mostly in the gut epithelium. Another central group of T cells is the natural killer (NK) T cells, which may express both αβ-TCR and receptors that are characteristic for NK cells (196).

Despite effective central tolerance, a minor population of autoreactive T cells can avoid it and enter the circulation. The possible activation of these autoreactive T cells in the periphery is suppressed by several mechanisms of peripheral tolerance, including ignorance, deletion, anergy and active regulation (197,198). Of these mechanisms, active regulation by so-called regulatory T cells (Treg) appears to be the most important one (197). The two main subtypes of Tregs identified are T cells expressing the forkhead box P3 transcription factor (FOXP3+ Tregs) and the T regulatory type 1 (Tr1) cells (197). Tregs are able to suppress the activation and proliferation of effector T cells using several mechanisms, one of which is the inhibition of T cell co-stimulation via cytotoxic T lymphocyte associated protein 4 (CTLA-4), which is expressed on the cell surface of Tregs (199,200). In patients with active celiac disease, effector T cells appear to be resistant to suppression by Tregs, which may contribute to the loss of tolerance to gluten and also to self-antigens (201).

After encountering their cognate antigen naïve CD4+ T cells (see 2.3.3.2) become activated and they begin to proliferate and differentiate into effector T cells. After the exposure to the antigen has ended most of the effector T cells die by apoptosis and only some of the effector T cells return to a resting state and survive as long-living memory T cells (202,203). Naïve and memory T cells in humans have been characterized by their mutually exclusive expression of CD45RA and CD45RO molecules, respectively. In vitro studies demonstrate that upon T-cell activation and proliferation naïve T cells first acquire the expression of CD45RO and subsequently lose the expression of CD45RA (204). CD4+ effector T cells have been classified into further subsets based on their cytokine profile. The first two subsets, Th1 and Th2 cells were first described in the 1980s (205). Th1 cells produce mainly interleukin (IL)-2, interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-13 (206). Although Th cells have their dominant cytokine profile individual T cells may produce heterogeneous patterns of cytokines, such as IL-10 and TGF-β, which have not been shown to be characteristics for either Th1 or Th2 subset. Such T cells producing various mixtures of cytokines have been called Th0 cells (206). The characteristic functions of the two subsets of T cells

(Th1 and Th2) are executed via their dominant cytokines. The main functions of Th1 cells are the elimination of intracellular pathogens and the activation of inflammation and tissue injury (206). Th2 cells, in turn, have a central role in mediating allergy and chronic inflammation and in the protection against helminthic and other parasite infections (206). A subset of T cells, called Th17 cells, has been characterized more recently (207). These Th17 cells produce IL-17 and are involved in the protection against extracellular microbes and fungi. Additional T cell subgroups, such as Th9 and Th22, have also been described (208,209).

2.3.3.2 Innate and adaptive immune response in the pathogenesis of celiac disease

Celiac disease has been classified as a T-cell mediated autoimmune disease in which dietary gliadin causes the activation of both the innate and adaptive immune systems, which gives rise to disease pathogenesis (126,210). The gliadin protein is rich in proline amino acid residues and resistant to degradation by intestinal protease enzymes. Therefore, only

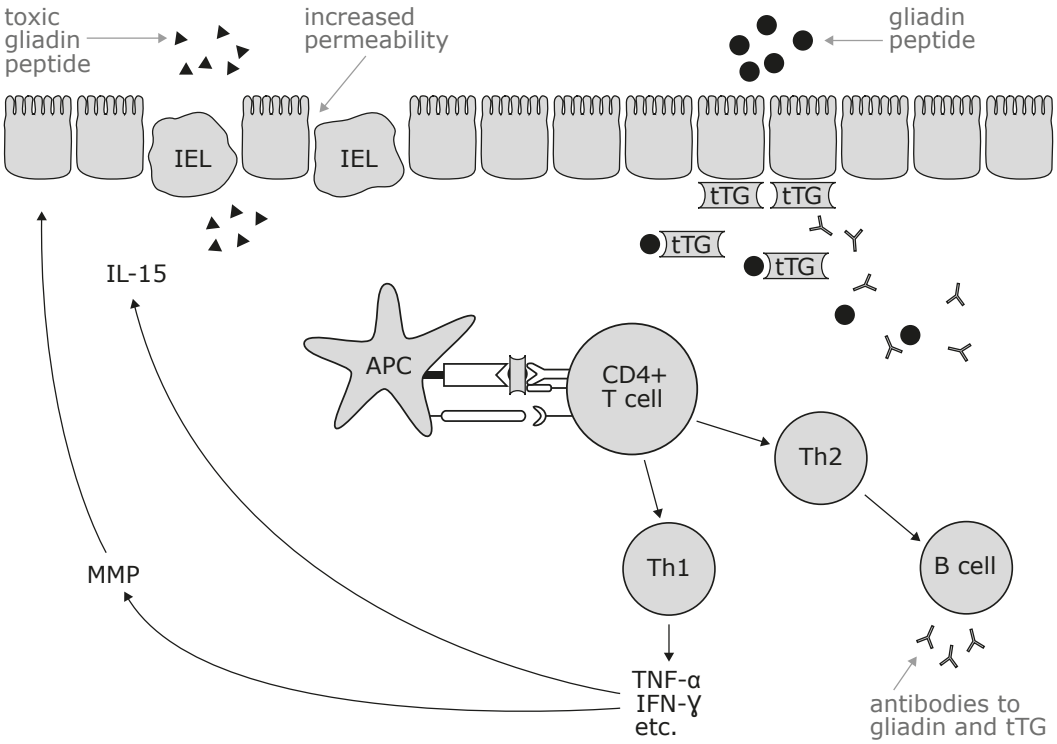

Figure 3. Innate and adaptive immune response mechanisms involved in the pathogenesis of celiac disease. Incompletely digested gluten peptides pass the intestinal epithelium and are deamidated by tissue transglutaminase (tTG). The deamidated peptides are presented to CD4+ T cells by APCs in the context of the HLA-DQ2 or DQ8 molecule in the lamina propria. The activated CD4+ T cells differentiate into Th1 cells that produce proinflammatory cytokines such as IFN-γ and TNF-α. These cytokines, in turn, activate fibroblasts and inflammatory cells of the intestinal epithelium to produce the matrix metalloproteinase (MMP) responsible for matrix degradation, leading to the destruction of the small intestinal mucosa. Differentiated Th2 cells activate B cells to produce antibodies against gliadin and tissue transglutaminase. The toxic gliadin peptide (p31-43) can directly activate intraepithelial lymphocytes (IELs) of the innate immune system via IL-15.

a part of the dietary gliadin is degraded before it enters the intestinal mucosa (18,131). The harmful link between gluten exposure and developing the destruction of intestinal mucosa, as a sign of celiac disease, is the ability of gluten peptides to increase mucosal permeability to macromolecules. This increased intestinal permeability upon gluten exposure occurs in all individuals. However, the mechanism that leads to mucosal damage only in some individuals is still under investigation (211). Zonulin is a eukaryotic protein that modulates tight junctions between cells (212,213). It has been demonstrated that gluten peptides increase zonulin release from enterocytes, which in turn increases the permeability of intestinal mucosa and this phenomenon is enhanced in active celiac disease (213). Since all individuals release zonulin after gluten-exposure, other factors, such as the cytokine profile of intestinal lymphocytes may explain the enhanced permeability in patients with active celiac disease. This question, however still remains unresolved (211). Increased zonulin upregulation leads to reorganization of the cytoskeleton, tight junction opening and increased paracellular passage of antigenic macromolecules into the lamina propria (212,214). Finally, gliadin peptides pass the epithelium, possibly with the help of dendritic cells, by using a transcellular pathway (215). The process of innate and adaptive immune response is described in Figure 3.

After gliadin peptides have passed the epithelium and entered into the mucosal layer they are able to activate an adaptive immune response by CD4+ T cells. Gluten protein itself contains only few negatively charged residues critical for efficient binding to HLA class II molecules. However, the deamidation by tissue transglutaminase present just below the small intestinal epithelium, converts selected glutamine residues to negatively charged glutamic acids and thereby increases the binding affinity of the gluten peptides to certain class II molecules, namely HLA-DQ2 and -DQ8 (216-218). After deamidation, multiple peptides derived from several gluten proteins, α-, γ- and ω- gliadins, are bound strongly by HLA-DQ2 or -DQ8 molecules and expressed on the cell surface of APCs, such as dendritic cells, macrophages and B cells. These deamidated gluten peptide-HLA-DQ2/DQ8 complexes finally activate the antigen-specific CD4+ T cells (218). The activation of CD4+ T cells requires two signals: a TCR-mediated signal and a co-stimulatory signal. TCR-mediated activation occurs when the TCR recognizes the peptide in the context of an HLA-DQ2 or-DQ8 molecule. The co-stimulatory signal is mediated by the binding of the CD28 receptor expressed on the naïve T cells to the B7 co-stimulatory molecules, CD80 or CD86, expressed on activated APCs (219,220). The antigen-specific CD4+ T-cell activation caused by deamidated gliadin peptides, described in Figure 4, is the key reaction in the

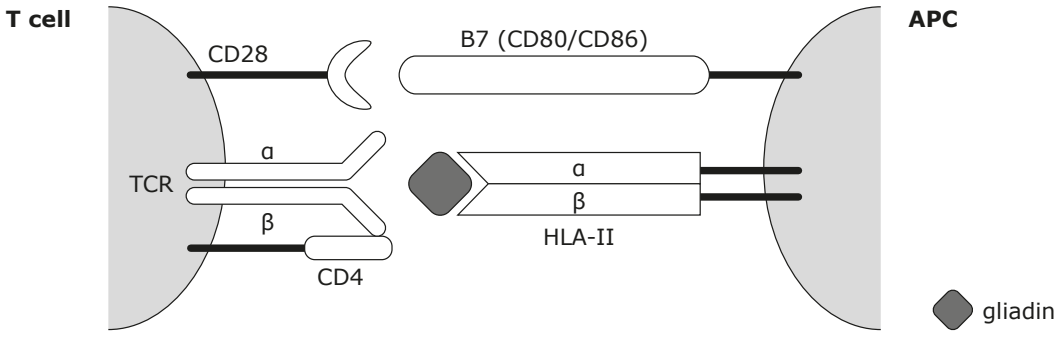

Figure 4. The TCR, expressed on the CD4+ T cell, recognizes the deamidated gliadin peptide in the context of HLA-DQ2 or -DQ8 molecule, expressed on the surface of an APC. The co-stimulatory signal is mediated by the binding of the CD28 receptor, present on the T cells, to the B7 co-stimulatory molecules, CD80 or CD86, expressed on activated APCs.

pathogenic mechanism of celiac disease, since the majority of the gliadin-specific intestinal T cells from celiac disease patients recognize gliadin proteins only after they have undergone deamidation (10,11,216).

Activation of CD4+ T cells leads to their differentiation along both Th1 and Th2 polarized pathways. Th1-polarized cells begin to produce IFN-γ and other proinflammatory cytokines, which leads to the destruction of epithelial cells (see 2.3.3.3) (126,221). Th2-polarized cells, in turn, promote the differentation of B cells into plasma cells capable of producing antibodies not only to gliadin but also to tTG.

T cells specific to tissue transglutaminase have only been demonstrated in the peripheral blood of celiac disease patients but not in the intestinal mucosa whereas B cells specific to tTG are readily detectable there (222-224). It has been suggested that when gliadin and tTG are physically linked during the deamidation reaction, tTG-specific autoreactive B cells can endocytose and process both of these antigens and present peptides derived from gliadin on their HLA class II molecules. Gliadin-specific CD4+ helper T cells that exist in the intestinal mucosa then provide help for these tTG-specific B cells leading to the production of antibodies to tTG but how this happens is still under investigation (222). When gliadin is withdrawn from the diet, T-cell help for anti-tTG specific B cells ceases and consequently the titers of antibodies to tTG decline in patients with celiac disease (222). Recent studies have demonstrated that the antibody response to tTG in celiac disease is dynamically regulated in response to dietary gluten with low-degree maintenance of both autoreactive plasma cells and memory B cells in patients in remission (225,226).

Although the antibody response and the detection of antibodies to tissue transglutaminase is a hallmark of disease development, it is still unclear whether tTGA themselves have a role in the pathogenesis that leads to the destruction of the intestinal mucosa. tTG is expressed at the epithelial border and extracellularly in the subepithelial region and it has been demonstrated that the level of its expression is increased in active celiac disease (216,227). In a study by Kiraly et al. it was shown that tTGA may have an inhibitory effect on tTG whereas Myrsky et al. have demonstrated that these antibodies increase the activity of tTG (228,229). Interestingly, deposits of IgA class tTGA can be found in the intestinal mucosa even before villous atrophy and tTGA seropositivity occur (230,231). This phenomenon could potentially be used for earlier detection of celiac disease.

2.3.3.3 Progression to a destructive immune response

As described above, the activation of Th1-type CD4+ T cells in response to gluten peptides leads to the production of proinflammatory cytokines, mainly IFN-γ and TNF-α, which promotes severe inflammation (232,233). In line with this, IFN-γ has been shown to be the dominant proinflammatory cytokine observed in the gut mucosa of gliadin-challenged celiac disease patients (232,234). In addition to CD4+ T cells, IFN-γ is also produced by CD8+ T cells in both the epithelium and the lamina propria (235,236). Overall, Th1-type cytokines activate tissue resident stromal and immune cells in the intestinal epithelium to produce matrix metalloproteases (MMP), which leads to mucosal damage (237). Gliadin-specific T cells in the gut mucosa also produce high levels of IL-21, which amplifies the Th1-polarized response and enhances the secretion of extracellular matrix-degrading proteases by stromal cells and chemoattractants by epithelial cells (238-242). More recently, it has been suggested that Th17 cells, which produce IL-17A, may also be involved in the pathogenesis of celiac disease of the intestinal mucosa, but the results supporting this hypothesis are still conflicting (233,239,243).

CD8+ T cells also have a central role in the pathogenesis of celiac disease and their appearance in the epithelia is one of the diagnostic markers of celiac disease. CD8+ T cells can recognize gliadin peptides that are 8–12 amino acids long, such as p123-132 derived

from α-gliadin, in the context of HLA-I molecules expressed on the epithelial cells (244-247). Studies have also demonstrated that gliadin activates mucosal CD8+ T cells and support the hypothesis that IELs can present gliadin-derived peptides to both CD4+ and CD8+ T cells.

The destruction of the intestinal mucosa is also mediated by the innate immune response. In addition to deamidated peptides, toxic gliadin peptides, mainly α-gliadin-derived p31-43, can activate the innate immune response to produce IL-15, independently of the adaptive immune response (246-248). IL-15 is a proinflammatory cytokine that is mainly produced by monocytes, macrophages, dendritic cells and epithelial cells. The toxic p31-43 peptide has been shown to induce the production of IL-15 in both the epithelium and lamina propria in patients with active celiac disease and to further increase the migration of IELs and their activation to killer cells (245,249). Furthermore, this toxic peptide has been shown to induce increased expression of the MICA molecule in enterocytes and NKG2D receptor in NK cells, α/β and γ/δ cells and cytotoxic CD8+ T cells (245,249). Activated IELs drive the cells expressing MICA into apoptosis causing further damage to the intestinal mucosa (246). Taken together, IL-15 production by innate immune cells is a hallmark of celiac disease and the most crucial effector mechanism of the innate immune response is the MICA/NKG2D-mediated cytolysis of epithelial cells (246,250).

2.3.3.4 Gliadin epitopes recognized by CD4+ T cells

Gliadin-specific CD4+ T cells were first identified through the analyses of intestinal T cells. Lundin et al. demonstrated that intestinal CD4+ T cells from patients with celiac disease patients recognize gliadin peptides from gluten but not from proteins of other cereals, after in vitro stimulation (10). Since the examination of intestinal gliadin-specific T cells requires isolation of the cells from active celiac disease lesions obtained by intestinal biopsy, this approach is somewhat laborious to perform. Therefore, many of the more recent studies have been performed with peripheral blood, where gliadin-specific T cells are also expected to be present after their initial antigen-driven expansion in the intestine and subsequent recirculation via systemic circulation. Several studies have confirmed that CD4+ T cells specific to deamidated gliadin epitopes can be detected in the peripheral blood in the majority of patients with active celiac disease. However, the frequency of these gliadin-specific CD4+ T cells in the circulation is relatively low and thus in most of the studies patients have been challenged to oral gluten to enhance the number of antigen-specific T cells (14-16,251). A Norwegian study group has recently shown that gliadin-specific CD4+ T cells are detectable in the circulation also without oral gluten challenge by using a sensitive HLA class tetramer method to visualize these cells (252).

Deamidation of gliadin by tTG leads to the conversion of glutamine residues to negatively charged glutamic acid residues. This in turn facilitates the binding of gliadin peptides to the disease-associated HLA-DQ2 and -DQ8 molecules that prefer negatively charged amino acids in their binding pockets (126,216,218). Several studies have demonstrated that CD4+ T cell responses to deamidated gliadin are readily detected in peripheral blood after gluten challenge whereas no responses are seen in celiac disease patients on a gluten-free diet (14-16). It has also been established that CD4+ T cell reactivity to gluten increases after deamidation in patients with active celiac disease but not in healthy controls (15,16,253). Ben-Horin et al. reported that CD4+ memory T cell responses to deamidated gliadin were detected using a CFSE-based in vitro proliferation assay in approximately half of the studied adult celiac disease patients on a gluten-free diet. Interestingly, however, about half of the patients did not show any reactivity to deamidated gliadin (254).

Two deamidated immunodominant epitopes of α-gliadin have been identified, which are predominantly recognized by both intestinal and peripheral blood gliadin-specific

CD4+ T cells from nearly all adult patients with celiac disease (14,16,17). Gliadin peptides QLQPFPQPELPY (Q12Y) and PQPELPYPQPELPY (P14Y) have been demonstrated to contain the immunodominant epitopes α-I (amino acids 57–68) and α-II (62–75), respectively (17). However, Vader et al. have investigated the epitope-specificity of gliadin-specific CD4+ T cells isolated from the small intestine of children with celiac disease and demonstrated that T-cell responses in children appear to be more variable than in adults. T-cell responses in children are directed against multiple gliadin and gluten peptides and also toward native gluten peptides instead of the earlier described immunodominant epitopes of α-gliadin (18). Moreover, Camarca et al. have demonstrated that intestinal T cells from adult patients with celiac disease recognized a heterogeneous population of gluten peptides and only 50% of Italian patients with celiac disease recognized the 33-mer polypeptide (57-89) containing both the α-I and α-II epitopes (255). In addition to these studies on intestinal T cells, Tye-Din et al. have demonstrated that peripheral blood T cells from adult patients recognize several other gluten peptides than those containing the previously reported immunodominant epitopes (256).

Although oral gluten challenge has been proven to be a non-invasive approach to investigate the peripheral blood immune response to gluten in adult patients with celiac disease it is not favourable to perform it in children (257). Circulating gliadin specific T-cell responses in children with celiac disease have not been studied widely. One recent study demonstrated that circulating T cells specific for various deamidated gliadin peptides can also be detected in a high percentage of children with newly diagnosed celiac disease (258).

2.4 DIAGNOSIS OF CELIAC DISEASE

2.4.1 Celiac disease antibodies

2.4.1.1 Reticulin and endomysial antibodies

Antireticulin antibodies (ARA) were the first autoantibodies to be associated with celiac disease (22,23). The ARA test was introduced as a first serological diagnostic assay for celiac disease in 1977 and is routinely performed as an immunofluorescence assay on rat tissue (24). The assay measures both IgA and IgG class antibodies that are directed against reticular fibers of endomysium, a tissue structure of smooth muscle fibers. Nowadays, the ARA assay is no longer recommended as more sensitive and specific tests have been developed (21,25,26).

Endomysial antibodies (EmA), in turn, were discovered in 1983 (27). EmA is still considered the gold standard of celiac disease serological tests, although it measures the same antigen as the later developed tTGA assay (see 2.4.1.2) (27,259). The specificity of the EmA method in the diagnosis of celiac disease in adults is close to 100% both in treated and untreated patients but its sensitivity is lower. Several studies have shown that up to 20% of patients with celiac disease are negative for EmA (260-264). The main drawbacks of the EmA method are that it is laborious, time-consuming and its interpretation can be somewhat subjective.

2.4.1.2 Tissue transglutaminase antibodies (tTGA)

The serological screening of celiac disease has been mainly been based on measuring IgA class tTGA ever since tTG was discovered as a major autoantigen of celiac disease in 1997 (28). tTG is a calcium dependent enzyme of the protein-glutamine γ-glutamyltransferas-

es family and can be found expressed in several tissues, such as heart, liver, skin and the small intestine (265,266). The small intestinal form of tTG, important in celiac disease, has also been named TG2, whereas the epidermal form of tTG, important in Dermatitis herpetiformis, has been named TG3 (267,268). tTG crosslinks proteins through glutamine residues thereby creating inter- and intramolecular bonds that are highly resistant to proteolysis (269,270). Moreover, tTG catalyzes the deamidation of glutamine residues to glutamic acid, a mechanism that is central for the pathogenesis of celiac disease (see 2.3.3.2) (216,271).

tTGA tests have been shown to have both a high sensitivity and a specificity in the diagnosis of celiac disease (21,272). However, the diagnostic performance of the tTGA assay is highly dependent on the methodology used. It has been reported that the solid phase method, used in the radiobinding immunoassays and the liquid phase method, used in the ELISA assays may differ in their sensitivity and specificity, especially in the measurement of IgG class tTGA (273,274). Thus the IgG class tTGA may perform with a lower sensitivity than IgA class tTGA, and it is only recommended to be used in the diagnosis of patients with selective IgA deficiency (21). Several studies have also demonstrated that tTGA levels correlate well with the histological changes observed in the intestinal mucosa, with higher levels observed in subjects with more severe pathology (275-277). Diagnosis of celiac disease in children is also mainly based on measuring tTGA (21,278,279). In young children, specifically in those under 2 years of age, tTGA may have a lower sensitivity (279-282). Moreover, it has been shown that tTGA seropositivity in children may be transient and some of the tTGA positive children may turn seronegative despite continuous gluten exposure (283).

2.4.1.3 Anti-gliadin antibodies and deamidated gliadin peptide antibodies (anti-DGP)

Antibodies to gliadin were described already in 1958 (20). After the enzyme-linked immunosorbent assay (ELISA) method for measuring gliadin antibodies (AGA) was described in the early 1980s, both IgA and IgG class AGAs have been widely used in the diagnosis of celiac disease, in both adults and children (19,284-286). Later studies have demonstrated that gliadin antibodies also appear in many other disorders than celiac disease leading to poor specificity of the assay. Therefore, AGA is nowadays not recommended for celiac disease diagnostics (21,278,287-290). However, although AGA has been shown to perform with low specificity, it may still be useful in the diagnosis of celiac disease in some cases in children under 2 years of age (291-293).

The T-cell response to deamidated gliadin peptides was described at the same time as tTG was demonstrated to be an autoantigen of celiac disease (28,216). Antibodies against these deamidated peptides (anti-DGP), as well as the older AGA are produced against food-derived antigens, which is in contrast to ARA, EmA and tTGA that all are produced against self-tissue structures. Assays for detecting IgA and IgG class antibodies to deamidated gliadin peptides have been developed most recently and have largely replaced the conventional AGA tests (29,294,295). The IgG anti-DGP assay has been shown to be at least as useful or even superior as IgG tTGA in the diagnosis of celiac disease in both adult and pediatric patients with selective IgA deficienc (296,297). Moreover, the IgG anti-DGP may be the only positive serological marker for celiac disease in older patients (298).

Several studies have demonstrated that parallel testing for both IgG anti-DGP antibodies and IgA tTGA has the best specificity in screening for celiac disease in all age groups (272,299-301). Specifically, the IgG anti-DGP assay has shown high specificity in both adults and children with celiac disease (299,300,302,303). Moreover, IgG anti-DGP has been reported to be at least as useful as tTGA for detecting celiac disease in very young children (297,302).

Anti-DGP antibodies may also be the first serological markers of the onset of celiac disease (30,295). It has been demonstrated that anti-DGP antibodies may appear up to a year earlier than tTGA seropositivity in some children (30). Moreover, anti-DGP antibodies have been shown to turn negative faster than tTGA after the start of a gluten-free diet, and they can thus be useful for monitoring diet compliance and as an indirect indicator of the mucosal recovery (300,302,304-306). It has been demonstrated that IgG anti-DGP antibodies together with the tTGA assay have a high negative predictive value in children with celiac disease on a gluten-free diet. Therefore, the use of these two serological tests in follow-up would omit the need for repeat duodenal biopsy in the majority of patients (307).

2.4.2 Small-intestinal biopsy

The European Society of Paediatric Gastroenterology and Nutrition (ESPGHAN) has published diagnostic criteria of celiac disease for pediatric patients since 1969 (308). The first criteria adopted required a sequence of three small intestinal biopsies, first during ongoing gluten containing diet, second during the gluten-free diet and third after gluten rechallenge. These diagnostic criteria have since been revised and new ESPGHAN guidelines published in 2012 indicate that symptomatic children that show tTGA levels at least 10 times above normal, are positive for EmA as well as HLA-DQ2 or -DQ8 no longer need to undergo duodenal biopsies to confirm the diagnosis of celiac disease (21). No unified criteria in adult patients have been published, and thus the diagnosis of celiac disease in adults usually still requires a duodenal biopsy after serological screening. The classification of pathological changes in the duodenal mucosa is based on the Marsh criteria (31). The diagnostic criteria include typical changes of small intestine, crypt hyperplasia and villous atrophy, as well as an increased number of intraepithelial lymphocytes. In addition, the early stage with positive serology and increased IELs but not hyperplasia and villous atrophy in the biopsies has been categorized as potential celiac disease (309). In children with a family history of celiac disease potential celiac disease has been shown to precede the later onset of the disease whereas in adult patients it performs often independently from celiac disease and the histological changes of the small intestinal mucosa do not develop (310,311). Another method, which uses the Watson capsule, also enables obtaining a single biopsy sample of the small intestinal wall. It is mainly used in children in place of upper GI endoscopy. In capsule endoscopy, a video capsule is swallowed to get photographs of the small intestine. This serves as an alternative method for the evaluation of celiac disease. Even though macroscopic pathologic changes of the intestinal mucosa characteristic of celiac disease can be identified by capsule endoscopy, its main limitation is the lack of ability to perform a biopsy. Therefore, capsule endoscopy in diagnosis of celiac disease is limited to patients to whom upper endoscopy is not suitable. Repeated monitoring of the decrease in celiac disease-associated antibodies during the first year is recommended for the assessment of response to the treatment and adherence to gluten-free diet in all patients (21,135). The recommendation of follow-up biopsies varies depending on the country, or even the clinician in charge. However, repeted duodenal biopsy should be considered at least in patients with celiac disease who are symptomatic on a gluten-free diet or have an increased risk of other complications (21,135).

2.4.3 Genetic screening

Genetic analyses are not routinely performed in all individuals with a clinical suspicion of celiac disease. HLA-genotyping for the high-risk HLA-DQ2 and HLA-DQ8 alleles has a high negative predictive value and it has been mainly used to rule out celiac disease

and as a further diagnostic tool in cases where the diagnosis is equivocal. HLA-DQ2 and -DQ8 genotyping is recommended for individuals with suspected celiac disease but who fail to respond to a gluten-free diet as well as for self-treated patients on a gluten-free diet who have never been appropriately tested for celiac disease before commencing the diet (135). HLA-genotyping is also recommended to rule out celiac disease in individuals with a high risk because of first-degree relatives with the disease. HLA-DQ2 and -DQ8 genotyping is recommended specifically for those pediatric patients with a strong clinical suspicion of celiac disease who have high celiac disease-specific antibody levels and for whom small intestinal biopsies are not going to be performed (21). As a result of the new recommendations by ESPGHAN stated above, the significance of HLA-DQ2 and -DQ8 genotyping is likely to increase in the future and the HLA-screening could be used before tTGA test as suggested by Björck et al. (312,313).

2.4.4 New diagnostic tools

Serological tests are often used in the primary diagnosis and clinical follow-up of celiac disease patients, but in some cases their sensitivity is not sufficient. A recent study demonstrated that 2–3% of celiac disease patients are seronegative for all celiac disease antibodies tested and have remarkably low antibody levels or fluctuating seropositivity. Therefore tests, other than those based on serological assays are needed to improve diagnostics of celiac disease (36). Overall, there is a trend for emphasizing non-invasive methods in both the diagnosis and follow-up of celiac disease. Moreover, there is a need for the development of new methods to detect patients at an early stage of the disease when histological changes are not yet detectable. Sollid and co-workers have studied the potential of identifying circulating and intestinal gliadin-specific T cells by an elegant tetramer staining method as a novel diagnostic approach (16,251,314). They recently demonstrated that the HLA-DQ2-gliadin tetramer method from peripheral blood is a sensitive approach for detecting possible celiac disease in most adult patients (314). They also showed that the frequency of intestinal gliadin-specific T cells, as detected by tetramer staining, correlates with the degree of histological damage in the gut mucosa as well as with serum IgA tTGA levels, and therefore this could provide a supplementary diagnostic aspect (251).

2.5 TREATMENT OF CELIAC DISEASE

2.5.1 Gluten-free diet

The only current treatment for all forms of celiac disease is a life-long gluten-free diet, where wheat and related cereals, barley and rye, are avoided. In Dermatitis herpetiformis the drug dapson is often also needed for a clinical response (315). Several studies have shown that extraintestinal manifestations of celiac disease may also disappear during gluten-free diet (316-318). The efficacy of the gluten-free diet is critically dependent on the motivation of the patient. It is known that nonresponsive or refractory celiac disease is often caused by inadequate adherence to the diet. In a majority of the patients, pathological changes in the small intestinal mucosa are healed and the levels of celiac disease-related antibodies significantly decreased after one year of treatment. However, some individuals do not adequately respond to the gluten-free diet, a condition referred to as refractory celiac disease (319).

Since the prolamin structure of avenin in oat differs from that of gliadin in wheat and gliadin-like proteins in barley and rye, several studies have shown that oat may be used

in the diet of celiac disease patients. The safety of oat has been confirmed both in adult and pediatric patients (320-323). However, there is also evidence that not all patients tolerate oat in the diet and the consumption of oat may even lead to villous atrophy (324).

2.5.2 Non-dietary treatments

It is often challenging and expensive to strictly follow a life-long gluten-free diet. Research on non-dietary treatments has been active in recent years and some of the investigated approaches have shown promising results (325). One option to avoiding dietary gluten is the neutralization of gluten. This was shown in a study where a synthetic copolymer neutralized bound gluten and the gluten-induced effects both in mice and human duodenal tissue ex vivo (326). Oral protease enzymes, so-called glutenases, have been shown to increase the breakdown of proteolytically resistant gluten protein (327). Oral proteases are a promising option for non-dietary treatment of celiac disease, and some of them have been examined already in a phase II clinical trial (328,329). Another alternative way to avoid the detrimental reaction of dietary gluten is the inhibition of deamidation by using specific antagonists (330). A recently published study demonstrated that tTG inhibitors reduced gliadin-induced toxicity both in in vitro as well as in ex vivo assays employing biopsy cultures (331). Vaccines targeting the pathogenic T-cell response in celiac disease have also been studied recently. Peptide-specific immunotherapy to induce tolerance towards gluten has been demonstrated in mice (332). Peptide-specific immunotherapy has been shown to be safe in humans but the major limitation of this approach in celiac disease is that the immunodominant T-cell epitopes responsible for pathogenesis are not yet fully defined and the tools to monitor the vaccine-associated immune response are still lacking (332). Importantly, all of the options currently considered for non-dietary treatment are more or less supplementary to the gluten-free diet. For example, it has been estimated that the oral gliadin specific proteases may eliminate only up to two grams of daily gluten, not the entire gluten intake (329).

3 Aims of the study

I to clarify the potential of detection of gliadin-specific T cells in the peripheral blood for the diagnosis of celiac disease in children (I),

II to evaluate the TR-IFMA assay for detecting antibodies to deamidated gliadin peptide in the diagnosis of celiac disease in children, and to analyze the timing of the development of anti-DGP positivity as compared to tTGA seropositivity (II),

III to prospectively analyze the development of gliadin-specific immune responses by following the emergence of tTGA and anti-DGP antibodies and peripheral gliadin-specific T-cell responses in children at a high genetic risk for celiac disease (III).

4 Experimental procedures

4.1 STUDY SUBJECTS

4.1.1 Children with confirmed celiac disease (I-II)

Altogether 92 children with biopsy-confirmed celiac disease from two different cohorts were analyzed in this study. Forty-four of the 92 children with celiac disease (23 girls and 21 boys) were examined because of a clinical suspicion of celiac disease at the Department of Pediatrics of the Kuopio University Hospital (clinical cohort). Blood samples were taken during the clinical visit, at the time of the biopsy, before starting gluten-free diet. Forty-three of these 44 children tested positive for tTGA, one of the children was not tested for tTGA but was highly positive for EmA. The diagnosis of celiac disease was confirmed by small intestinal biopsy at a median age of 6.3 years (range 1.2-15.0) (see 4.2.3). The results of the histological changes in the small intestine biopsy samples in the clinical cohort are shown in Table 1. Thirty-four (82.9%) of the children genotyped were positive for the disease-associated HLA-DQ2 molecule (carried DQA1*05 and DQB1*02 alleles), four (9.8%) were HLA-DQ8 positive (DQB1*03:02) and two (4.9%) were positive for both HLA-DQ2 and -DQ8. HLA-typing was not performed in three of the children.

The second cohort of children with celiac disease (DIPP cohort) consisted of 48 subjects (17 girls and 31 boys) who were participating in the Finnish Type 1 Diabetes Prediction and Prevention study (DIPP) at the Turku and Tampere University Hospitals. Children in the DIPP follow-up study were screened for the HLA-associated genetic risk for T1D at birth and subsequently prospectively monitored for the development of T1D-associated autoantibodies. A subset of children positive for HLA-DQ2 (DQA1*05 and DQB1*02 alleles) or HLA-DQ8 (DQB1*03:02) was also monitored for the development of tTGA positivity, and if the child was tTGA seropositive in two consecutive samples they were biopsied to confirm the diagnosis of celiac disease. The results of the histological changes in small intestine biopsy samples in the DIPP cohort are shown in Table 2. Blood samples from the children in the DIPP cohort were collected every 3 to 6 months from birth until 2 years of age and every 6 to 12 months thereafter. Seventeen of the 48 (35.4%) DIPP children that developed celiac disease were positive for HLA-DQ2 (DQA1*05 and DQB1*02), 13 (27.1%) were positive for both HLA-DQ2 and -DQ8 and 19 of them (39.6%) were positive for HLA-DQ8 (DQB1*03:02). The median age at the time of diagnosis was 5.0 years (range 1.6-11.9 years). The serum sample used for the analysis of anti-DGP assay sensitivity and specificity was either taken at the time of biopsy (n=60) or at the last available follow-up visit before the biopsy (n=32, median 31 days, range 1-252 days).

4.1.2 Healthy controls (I-II)

The control group consisted of 82 healthy children (33 girls and 49 boys) carrying the celiac disease-associated HLA alleles (HLA-DQ2 and/or HLA-DQ8). The control children participated in the Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes (FINDIA) follow-up study (333). Twenty-seven of the controls (32.9%) were positive for HLA-DQ2, 8 (9.8%) were positive for both HLA-DQ2 and -DQ8 and 47 of the children (57.3%) were positive for HLA-DQ8. All of the control children were tested negative for tTGA. Sixty-four of the children were used as controls in study I and their median age at the time of sampling was 5.1 years (range 4.0–6.1). All of the 82 control children were analyzed in study II at a median age of 6.0 years (range 3.0–6.8).

Table 1. Histological results of the duodenal biopsies in the clinical cohort.

Clinical cohort (n=44)	Study	Histology stage of villous atrophy	Clinical cohort (n=44)	Study	Histology stage of villous atrophy
1	II	total	23	II	subtotal
2	II	total	24	II	subtotal
3	II	total	25	II	subtotal
4	II	total	26	I,II	subtotal
5	II	total	27	I,II	subtotal
6	II	total	28	II	partial
7	II	total	29	II	partial
8	II	total	30	II	partial
9	II	total	31	II	partial
10	II	total	32	II	partial
11	II	total	33	II	partial
12	II	total	34	II	partial
13	I, II	total	35	I,II	partial
14	I, II	total	36	I,II	partial
15	I,II	total	37	I,II	partial
16	I,II	total	38	I,II	partial
17	I,II	total	39	I,II	partial
18	I,II	total	40	I,II	partial
19	I,II	total	41	I,II	partial
20	I,II	total	42	II	stage not determined
21	II	total	43	II	stage not determined
22	I,II	total	44	I,II	stage not determined

4.1.3 Children with a high genetic risk for celiac disease (III)

Children with a high genetic risk for celiac disease were recruited from the Kuopio University Hospital and the Kätilöopisto Maternity Hospital in Helsinki for prospective follow-up. Parents of newborns were first met at the maternity hospital and they were briefly informed about the study protocol. Altogether 2013 newborn children were screened for the presence of HLA-DQB1*02 and -DQA*05 alleles, which are associated with the highest risk for celiac disease. The families were informed of the results of the HLA-screening by a phone call when the child was 1 to 2 weeks old. A total of 339 of the children had the appropriate alleles (16.8%) and 291 of these children were followed prospectively. Newborn children were randomized to a dietary intervention group and a control group. The intervention group was given specific nutritional counseling with the aim to time gluten introduction to diet optimally, as described in chapter 4.3. Blood samples were collected at the ages of 9, 12, 18, 24 and 36 months from all children and also at the age of 48

Table 2. Histological results of duodenal biopsies in the DIPP cohort.

DIPP cohort (n=48)	Study	Histology stage of villous atrophy	DIPP cohort (n=48)	Study	Histology stage of villous atrophy
1	II	partial	25	II	subtotal
2	II	partial	26	II	subtotal
3	II	partial	27	II	subtotal
4	II	partial	28	II	subtotal
5	II	partial	29	II	subtotal
6	II	partial	30	II	subtotal
7	II	partial	31	II	subtotal
8	II	partial	32	II	total
9	II	partial	33	II	total
10	II	partial	34	II	total
11	II	partial	35	II	total
12	II	partial	36	II	total
13	II	partial	37	II	total
14	II	partial	38	II	total
15	II	partial	39	II	total
16	II	subtotal	40	II	total
17	II	subtotal	41	II	total
18	II	subtotal	42	II	total
19	II	subtotal	43	II	total
20	II	subtotal	44	II	stage not determined
21	II	subtotal	45	II	stage not determined
22	II	subtotal	46	II	stage not determined
23	II	subtotal	47	II	stage not determined
24	II	subtotal	48	II	stage not determined

months from 85 children at the Kuopio University Hospital. All of the 291 children were followed for tTGA and anti-DGP antibodies. The results of the tTGA test were reported to families, and if the child was tTGA seropositive the families were offered an option for small intestinal biopsy. Although no clinical cut-off value for tTGA was determined, in most of the cases the small intestinal biopsy was performed if the child had high (over 50 IU/ml) tTGA titer whereas children with low tTGA titer were followed with serological analyses. Children with anti-DGP seropositivity only were not biopsied, according to the original study plan. The 85 children followed until 48 months of age were also analyzed for peripheral gliadin specific T-cell responses at the same sampling points as the serological analyses. Due to the small volume of blood obtained in some samples, the serological analyses were given priority and thus T-cell assays were not performed in every sampling point in all of the children studied.

4.2 METHODS

4.2.1 HLA-genotyping (I-III)

The presence of HLA-DQA1 and -DQB1 alleles in all patients and controls were analyzed using sequence-specific oligonucleotide hybridization reactions, as previously described (334-336).

4.2.2 Celiac disease-associated antibodies (I-III)

All serum samples were kept frozen at -80°C until analyzed for celiac disease-associated antibodies. The IgA and IgG class antibodies to a synthetic deamidated gliadin peptide (anti-DGP) (29) were measured by a solid-phase lanthanide-based, time-resolved immunofluorometric assay (TR-IFMA) using Europium-labeled anti-human IgA and Samarium-labeled anti-human IgG, as described earlier (295) (II-III). Commercial ELISA (enzyme-linked immunosorbent assay) -based recombinant human Celikey kit was used to measure IgA class tTGA in children with celiac disease and in controls (Phadia, Freiburg, Germany) (I-III) according to manufacturer's protocol. Values > 8 U/ml were considered positive, as suggested by the manufacturer.

4.2.3 Small intestinal biopsies (I-III)

The diagnosis of celiac disease in all patients was confirmed by small intestinal biopsy. Duodenal biopsy was taken with the Watson capsule method in all of the 44 children examined for clinical suspicion of celiac disease at the Kuopio University Hospital (I-II). Only one biopsy sample from duodenum, after bulbus but before the ligament of Treitz, was taken from each subject with this method. Endoscopic biopsy was done after general anesthesia and 3 to 4 biopsy samples were taken from each subject in the DIPP cohort, as well as from children biopsied in the follow-up study at the Kuopio University Hospital or at the Kätilöopisto Maternity Hospital in Helsinki (III). One of the samples was taken from the bulbus and two or three samples from duodenum. The diagnosis of celiac disease was based on the International Academy of Pathology classification in which the representativeness of the biopsy sample, the presence of villous atrophy and its severity and crypt hyperplasia in the intestinal mucosa biopsies are assessed. Although the Marsh classification (31) was not routinely used for the categorization of biopsy results, namely the number of IELs was not routinely recorded in the pathology report, nearly all of the children with biopsy-confirmed celiac disease belong to the Marsh class III. Thus, the stage of villous atrophy was categorized as partial to total (Table 1 and 2). Staining for CD3+ T cells and gamma delta T cells was also not performed for all of the cases.

4.2.4 T-cell proliferation assays using the CFSE dilution method (I, III)

The development of a gliadin-specific immune response was also analyzed by determining peripheral T-cell responses to deamidated and native gliadin. Antigen-specific T-cell responses were analyzed in vitro from freshly isolated peripheral blood mononuclear cells (PBMC) in children with newly-diagnosed celiac disease and in healthy controls (I), and in children with high genetic risk for celiac disease (III). PBMCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Molecular Probes, USA) and suspended in culture medium (RPMI 1640 supplemented with 5% inactivated human AB serum (Sigma Aldrich), 2 mM L-glutamine, 20 μM 2-mercaptoethanol, 1 mM natrium pyruvate, nonessential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomy-

cin and 10 mM HEPES (all from Lonza, Verviers, Belgium) at 106/ml and stimulated with different antigens in a volume of 200 µl in a 96-well round-bottomed plate (Costar, Corning Incorporate USA). Cells were maintained at 37°C and 5% CO2 in 6 to 8 equal wells per antigen for 10 days, with the exception of PHA in 4 equal wells.

4.2.5 Antigens used in the T-cell proliferation assays (I, III)

The T-cell proliferation assay was performed similarly in studies I and III. Purified tetanus toxoid (TT) was used as an independent control antigen at a final concentration of 1 µg/ml (National Institute of Health and Welfare, Helsinki, Finland). Purified phytohaemagglutinin (PHA) was used as a mitogen control of cell functionality at a final concentration of 2 µg/ml (Remel, UK). Deamidated gliadin antigen was prepared as follows. First, native gliadin from wheat powder (Sigma Aldrich, St. Louis USA) was dissolved in dimethyl sulfoxide (DMSO) and diluted with 4 mM CaCl2 solution (CaCl2 dissolved in PBS) to a concentration of 4 mg/ml. tTG (from guinea pig liver, Sigma Aldrich, St. Louis USA) was dissolved in PBS to a concentration of 0.8 mg/ml. Deamidation of gliadin with tTG was accomplished by incubation of gliadin and tTG in a final volume of 100 µl (25 µl gliadin-dilution, 25 µl tTG-dilution and 50 µl PBS) for 2 h at 37°C. Finally 20 µl of this mixture per 1 ml culture medium was used to stimulate cells, corresponding to a concentration of 20 µg/ml of deamidated gliadin. Native gliadin alone was used at a final concentration of 10 µg/ml and the tTG alone at 2 µg/ml. In study I, PBMCs were stimulated also with 10 µg/ml of synthetic deamidated gliadin peptides QLQPFPQPELPY (Q12Y) and PQPELPYPQPELPY (P14Y) (purity >95%, GL Biochem, Shanghai, China), which contain the earlier reported immunodominant gliadin epitopes α-I and α-II, respectively (17).

4.2.6 Flow cytometry analyses (I, III)

After 10 days of culture, replicate wells for each antigen were pooled, washed with ice-cold wash buffer (PBS, 2% FCS and 0.1% NaN3) and stained on ice for 20 min with the following antibodies: anti-CD4-PerCP-Cy5.5, anti-CD45RO-PE-Cy7, anti-CD45RA-APC and integrin β7-PE (BD Pharmingen, San Jose, USA). Antigen-specific proliferation was analyzed by flow cytometry (FACS Canto II, Becton Dickinson, Mountain View, USA) using the FACSDiva software (BD). The percentage of proliferated CD4+ T cells was determined by gating the CD4+CFSElow subset and calculating its proportion of the total CD4+ cells. The cell division index (CDI) for different antigens was calculated as follows: the percentage of CD4+ CFSElow cells in stimulated cultures divided by the percentage of CD4+CFSElow cells in unstimulated cultures (337). Individual responses to an antigen were considered positive when the CDI was ≥ 2.0 and the difference in the percentage of CD4+CFSElow cells between stimulated and unstimulated cultures was at least 0.5%.

4.2.7 Statistical analyses (I-III)

All statistical analyses were performed by GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). The Mann-Whitney U-test (I-III) and Fisher's exact test (I-III) were used to compare differences between groups when appropriate. Wilcoxon matched-paired signed rank test was used to analyze the changes within a group (II). The Spearman rank correlation test was used to assess the correlation between two parameters (I, II). The cut-off values of the TR-IFMA anti-DGP assay for the highest sensitivity and specificity were determined by receiver operating characteristics (ROC) curves (II). The

Chi-Square test was used to compare whether the optimal gluten introduction between 4 and 6 months was performed more often in the intervention or in the control group in the follow-up study (III). Two-tailed P-values of less than 0.05 were considered significant in all statistical analyses.

4.3 DIETARY INTERVENTION IN CHILDREN WITH A HIGH GENETIC RISK FOR CELIAC DISEASE (III)

Newborn children carrying the HLA-DQA1*05 and -DQB*02 alleles were randomized to an intervention and a control group by using a random number generator. The intervention group was given specific nutritional counseling for the purpose of timing the introduction of gluten to an optimal window between 4 and 6 months of age and while breastfeeding was ongoing, as suggested by previous investigations (103,160,161,165,171,338). Families were advised to start the introduction of gluten-containing cereals with one teaspoon on the first day, two teaspoons on the second day and so on, gradually increasing the amount of gluten up to a maximum of one deciliter during the first week. The families were briefly informed by a phone call about gluten introduction when their child was 1.5 months old, and given a more detailed counseling in person on starting solid foods when the child reached 3.5 months of age. The control group received normal nutritional counseling given to all Finnish families at the child health clinics. Nutritional information was collected similarly in both groups using dietary questionnaires and phone calls (at the age of 1–2 week, 1.5 months, 3.5 months, 6 months and 9 months).

4.4 ETHICS (I-III)

The ethical committees of the participating university hospitals in all substudies approved the study protocol: Kuopio University Hospital (I-III), Helsinki University Hospital (I-II) and Turku University Hospital (II). Written informed consent was obtained from parents/guardians (I-III) and from children over 7 years of age (I-II).

5 Results

5.1 DEVELOPMENT OF tTGA SEROPOSITIVITY AND CELIAC DISEASE IN CHILDREN WITH A HIGH GENETIC RISK (III)

Children with a high genetic risk for celiac disease (all positive for HLA-DQ2) were followed prospectively for the development of tTGA until the age of 3 or 4 years. The cumulative tTGA seropositivity was 6.5% (19 of 291 children) during the follow-up. The median age at tTGA seroconversion was 24 months (range 18–48 months) and there were three children who converted to tTGA seropositivity at the age of 18 months (Original article III, Figure 1). Celiac disease was diagnosed in altogether 9 of the 291 children at the median age of 3.5 years (range 2.6–4.2). The cumulative incidence of celiac disease by the age of 3 to 4 years was 3.1% (Original article III, Figure 3). A total of 2.4% of the children had transient tTGA seropositivity as they converted back to tTGA seronegative during the follow-up. The median level of tTGA at the time of seroconversion was significantly higher in children with confirmed celiac disease than in those with transient tTGA seropositivity ($p=0.008$, median 9.5 IU and 100.0 IU, respectively, Mann-Whitney U-test). Eighteen of all 19 tTGA seroconverted children as well as all of the 9 children diagnosed with celiac disease were girls.

5.2 EVALUATION OF THE TR-IFMA ANTI-DGP ASSAY (II)

In order to evaluate the performance of the in-house TR-IFMA anti-DGP assay in pediatric patients, serum samples were analyzed from children with celiac disease and from healthy controls. All of the children with celiac disease were positive for tTGA and all of the healthy controls tested negative for tTGA. Serum samples were first analyzed at the time of diagnosis of celiac disease or at the latest available sample before the diagnosis. As expected, the median levels of both IgA and IgG class anti-DGP antibodies were significantly higher in celiac disease patients than in healthy controls ($p<0.0001$, Mann-Whitney U-test) (Original article II, Figure 1A). The sensitivity and specificity of the assays were calculated and the optimal cut-off values for anti-DGP seropositivity were determined from ROC-curves (Figure 5A and 5B). Based on the ROC curves the optimal cut-off value for IgA anti-DGP positivity was 153 AU with a sensitivity of 92.4% and specificity of 97.6%. The optimal cut-off value for IgG anti-DGP positivity was 119 AU, with a sensitivity of 98.9% and specificity of 97.6%.

5.3 ANTI-DGP ANTIBODY POSITIVITY AT THE DIAGNOSIS OF CELIAC DISEASE (II, III)

The frequency of seropositivity to a synthetic deamidated gliadin peptide (anti-DGP assay) at the diagnosis of celiac disease was analyzed retrospectively in the DIPP cohort (II) and prospectively in the follow-up study of children with a high genetic risk for celiac disease (III). Overall, the anti-DGP assay performed with a high sensitivity, as all except one of the children with celiac disease in study II were either IgA or IgG anti-DGP positive at the time of diagnosis or at the latest sampling before the diagnosis of celiac disease: 85 of the 92 children (92.4%) were IgA anti-DGP positive, 91 of 92 (98.9%) were IgG anti-DGP positive and 84 of 92 (91.3%) were both IgA and IgG anti-DGP positive at the time of diagnosis. Only two of the 82 tested healthy controls were positive for IgA anti-DGP

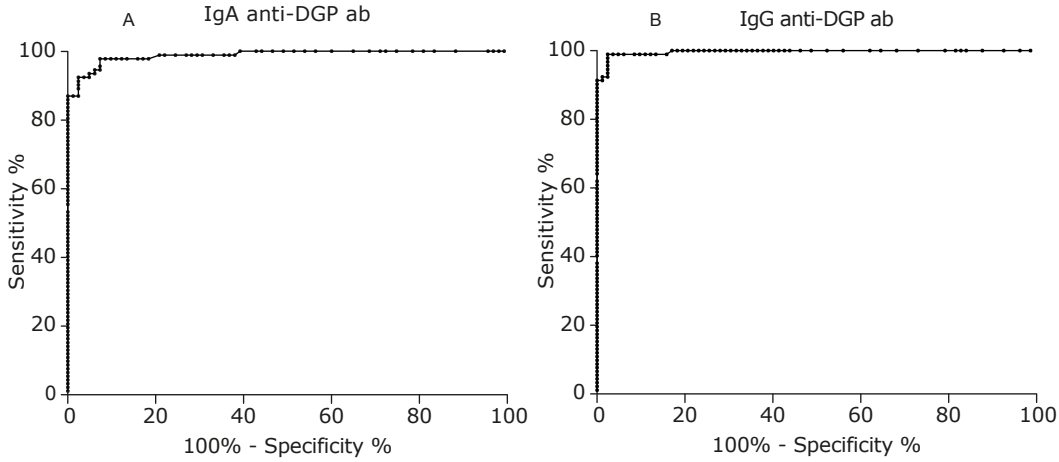

Figure 5. (A) Receiver operating characteristics (ROC) of IgA anti-DGP in all children at or just before diagnosis of celiac disease: area under the curve = 0.99 (95% CI 0.98 – 1.00). (B) Receiver operating characteristics (ROC) of IgG anti-DGP in all children at or just before diagnosis of celiac disease: area under the curve = 0.99 (95% CI 0.99-1.00)

and two other children positive for IgG anti-DGP, which demonstrates that both the IgA and IgG class anti-DGP assays have also a high specificity.

In study III, all except one of the children (18 of 19, 91.7%) who converted tTGA seropositive were also positive for anti-DGP antibodies at least in one sampling point during the follow-up. All of the tTGA seropositive children with confirmed celiac disease were both IgA and IgG anti-DGP positive at the time of tTGA seroconversion, whereas 5 of the 8 children (62.5%) with transient tTGA seropositivity were anti-DGP IgG but not IgA positive at the time of tTGA seroconversion (Original article III, Table II). Among children who remained tTGA seronegative during the follow-up some developed anti-DGP antibodies. The percentage of IgA and IgG anti-DGP positivity in tTGA seronegative children during the follow-up varied between 0.4–1.4% and 4.1–10.0%, at various age points, corresponding to a specificity of 98.6-99.6% and 90.0-95.9%, for IgA and IgG anti-DGP, respectively (Original article III, Figure 4A and B). Most of the tTGA seronegative children who developed anti-DGP antibodies were positive only at one sampling point and turned seronegative in the subsequent sample (36 of 272, 13.2%) but 20 of 272 children (7.4%) were repeatedly IgA or IgG anti-DGP positive (at least in two sampling points).

5.4 IgG-CLASS ANTI-DGP ANTIBODIES PRECEDE tTGA SEROCONVERSION (II, III)

In the retrospective analysis of the DIPP cohort (II), anti-DGP positivity preceded tTGA seropositivity in 72.9% (35 of 48) of children who were later diagnosed with celiac disease. In almost half (21 of 48, 43.8%) of the children with celiac disease only IgG anti-DGP was positive before tTGA seropositivity, 27.1% of the children (13 of 48) were both IgA and IgG anti-DGP positive and only one of the children was positive for IgA but negative for anti-DGP before the appearance of tTGA. Anti-DGP antibodies were detected a median of

1.0 year (range 0.2-7.6) before tTGA seroconversion. Sixteen of these 35 children (45.7%) had IgA or IgG anti-DGP in at least two samples taken before tTGA seroconversion. In 19 of the children with celiac disease (54.3%) anti-DGP antibodies were positive once before tTGA seroconversion. The remaining 13 of 48 (27.1%) of the children converted anti-DGP positive at the time of tTGA seroconversion. The levels of both IgA and IgG anti-DGP antibodies were significantly higher in the last positive serum sample before the diagnosis of celiac disease than in the first positive serum sample (p=0.0013 and p=0.0004, respectively, Wilcoxon matched-paired signed rank test) (Original article II, Figure 3A and B).

In the prospective study, IgG anti-DGP positivity preceded tTGA seroconversion in more than half of the children (5 of 9, 55.5%) that developed tTGA seropositivity and were later diagnosed with celiac disease, a median of 1.0 year earlier (range 0.5-1.5) (Original article III, Table II). In one of the children with confirmed celiac disease IgA class anti-DGP antibodies also appeared 1.0 year earlier than tTGA seropositivity. In addition, in the group of children with transient tTGA seropositivity, over half of the cases (5 of 8, 62.5%) developed IgG anti-DGP positivity a median of 1.2 year earlier (range 0.5-2.9) than tTGA (Original article III, Table II). Two of the children with transient tTGA seropositivity were IgG anti-DGP positive at two sampling points before tTGA seroconversion.

5.5 PRESENCE OF GLIADIN-SPECIFIC CD4+ T CELLS IN THE PERIPHERAL BLOOD (I, III)

Gliadin-specific T-cell responses in peripheral blood were analyzed in children with celiac disease at the time of diagnosis and also followed prospectively in children with high genetic risk for celiac disease (I, III). The frequencies of observed peripheral blood T-cell responses to all antigens tested in studies I and III are shown in Table 3. Circulating CD4+ T cells specific to deamidated gliadin (gTG) were detectable in more than half of the children with active celiac disease as 11 of 20 (55.5%) of the patients had a positive response to gTG compared to 15 of 67 (22.4%) of the healthy controls (p=0.007, Fisher's exact test). The criteria for a positive antigen-specific proliferative T-cell response is described in chapter 4.2.6. The intensity of the proliferative responses to gTG was also significantly stronger in patients than in controls (p=0.02, Mann-Whitney U-test, Original article I, Figure 1) whereas there was no difference in the frequency of T-cell responses to native gliadin between patients and healthy controls (10.5% and 19.7%, respectively). The intensity of proliferative responses to native gliadin also did not differ between patients and healthy controls (Original article I, Figure 1). None of the children with celiac disease, and only 3.6 % of the controls, had T-cell responses to tTG (Table 3).

Altogether 85 children were followed prospectively for peripheral gliadin-specific T-cell responses in study III at the ages of 9, 12, 18, 24, 36 and 48 months. Altogether 306 samples were analyzed during the follow-up. In total 40.5% of the children (17 of 42) had a positive T-cell response to gTG at the age of 9 months whereas only 20.0% of the children (8 of 40) tested had a positive T-cell response to native gliadin at that age (Table 3). The percentage of positive gTG responses decreased during the follow-up to the level of 22.2% at the age of 48 months, which is similar to the frequency detected in healthy controls in study I (Table 3). T-cell responses to native gliadin decreased even more during the follow-up, down to the level of 7 to 9% (Table 3).

In study I, the peripheral blood T-cell responses were also tested to the previously described immunodominant T-cell epitopes α-I (Q12Y) and α-II (P14Y) of gliadin (17). None of the children with celiac disease showed a positive proliferative response to either of the gliadin peptides, and only 7.7% and 5.8% of the healthy controls had a positive response to the Q12Y and P14Y peptides, respectively (Table 3).

Table 3. Frequencies of positive proliferative responses to different antigens in children with celiac disease (CD), in healthy controls, and in children with a genetic risk for celiac disease followed prospectively.

Antigen	Study I		Study III					
	CD children (n=20)	Controls (n=64)	Children positive for HLA-DQA1*05 and DQB1*02 (n=85)					
			9 months	12 months	18 months	24 months	36 months	48 months
	Positive/Analyzed							
gTG	11/20 (55.0%)	15/64 (23.4%)	17/42 (40.5%)	18/56 (32.1%)	16/52 (30.8%)	15/55 (27.3%)	18/59 (30.5%)	10/45 (22.2%)
Gliadin	2/19 (10.5%)	13/64 (20.3%)	8/40 (20.0%)	9/55 (16.4%)	10/52 (19.2%)	4/55 (7.3%)	4/59 (6.8%)	4/45 (8.9%)
tTG	0/16 (0%)	2/55 (3.6%)	2/30 (6.7%)	0/39 (0%)	1/40 (2.5%)	0/42 (0%)	0/48 (0%)	3/38 (7.9%)
TT	18/20 (90.0%)	53/64 (82.8%)	26/32 (81.3%)	29/39 (74.4%)	23/41 (56.1%)	28/43 (65.1%)	41/48 (85.4%)	23/38 (60.5%)
Q12Y	0/15 (0%)	4/50 (8.0%)						
P14Y	0/15 (0%)	3/50 (6.0%)						

5.6 EXPRESSION OF CD45RA-CD45RO+ MEMORY PHENOTYPE IN PERIPHERAL BLOOD GLIADIN-SPECIFIC CD4+ T CELLS (I, III)

Since gliadin-specific T-cell responses to deamidated and native gliadin were seen both in children with active celiac disease and in healthy controls, the cell surface phenotype of these cells was further analyzed (I). The expression of CD45RA and CD45RO was analyzed in vitro to determine the frequency of memory (CD45RA-CD45RO+) CD4+ T cells within the proliferating cells. In children with confirmed celiac disease, the percentage of CD45RA-CD45RO+ cells among proliferating CD4+ T cells was significantly higher in stimulations with gTG than in those with native gliadin (p=0.024, Mann-Whitney U-test, Original article I, Figure 3B), whereas in healthy controls the percentage of CD45RA-CD45RO+ cells in stimulations with both gTG and native gliadin were similar (p=0.35, Mann-Whitney U-test, Original article I, Figure 3B). The expression of memory markers on gliadin-stimulated CD4+ T cells was also analyzed in the follow-up study of genetically predisposed children (III). No differences in the percentages of CD45RA-CD45RO+ cells between stimulations with deamidated and native gliadin were observed in children with a high genetic risk for celiac disease during the follow-up (III).

5.7 EXPRESSION OF B7-INTEGRIN ON PERIPHERAL BLOOD GLIADIN-SPECIFIC CD4+ T CELLS WITH MEMORY PHENOTYPE (I, III)

The expression of β7-integrin, a gut-homing receptor, was analyzed in CD4+ T cells stimulated with different antigens in vitro. The frequency of β7high cells was higher among proliferating CD4+ memory T cells stimulated with gTG (83.0%) than among CD4+ memory T cells stimulated with native gliadin (45.8%) in children with active celiac disease

(p=0.021, Mann Whitney U-test, Original article I, Figure 4). The frequency of β7high CD4+ memory T cells was similar among gTG (median 60.2%) stimulations and native gliadin (median 52.9%) in healthy control children (p=0.37 Mann-Whitney U-test). There was no difference in β7-integrin expression on proliferating CD4+ T cells stimulated with TT, used as an independent control, between patients and controls. The frequency of β7high cells was higher among proliferating CD4+ memory T cells stimulated with both deamidated (median 45.3%) and native gliadin (46.3%) than among CD4+ memory T cells stimulated with TT (29.7%) in children with a genetic risk for celiac disease (p<0.0001 and p=0.0002, respectively, Mann Whitney U-test) (Original article III, Figure 2C).

5.8 DIETARY INTERVENTION (III)

The median age at gluten introduction in all 291 children followed prospectively in study III was 6.0 months (range 3.6-9.7 months). Gluten was introduced slightly earlier in children who were given special nutritional counseling (see 4.3) than in control children (median 5.6 and 6.3 months, p<0.0001, Mann Whitney U-test). Gluten was introduced between the optimal window of 4-6 months in 147 of the 291 children (50.5%). This was performed significantly more often in the intervention group than in controls (71.5% and 43.1%, respectively, p<0.0001, Chi-Square test). In 108 of the children (37.1%), gluten introduction was performed after 6 months of age and only in 3 children (1.0%), was gluten introduced before 4 months of age. In 14 children (4.8%) the age at which gluten was introduced was not reported. Further analyses of the effect of gluten introduction were performed only in those children who had gluten introduction between 4 and 6 months or after 6 months of age, regardless of whether they were included in the original intervention or control group. Altogether 51.2% of the children were breastfed during gluten introduction (149 of 291 children) and in 25.4% (74 of 291 children) breastfeeding was not continued during gluten introduction. The remaining 23.4% were not categorized because of inadequate information (68 of 291 children). The amount of gluten introduced was reported inadequately in several questionnaires, and thus no further analysis was conducted on this.

5.9 THE EFFECT OF GLUTEN INTRODUCTION ON THE DEVELOPMENT OF GLIADIN-SPECIFIC IMMUNE RESPONSES (III)

The timing of gluten introduction did not have a significant effect on the development of celiac disease. The cumulative incidences of tTGA seropositivity during the follow-up in children who had gluten introduction between 4 and 6 months and in children who had gluten introduction after 6 months of age were 6.4% and 6.9%, respectively (p=0.525, Fisher's exact test). Cumulative anti-DGP positivity at the age of 48 months in children who had gluten introduction between 4 and 6 months and in children who had gluten introduction after 6 months of age was also equal (21.8% and 21.3%, respectively, p=0.383, Fisher's exact test).

Among the 85 children followed prospectively for peripheral blood gliadin-specific T-cell responses, gluten was introduced at a median age of 5.8 months (range 3.6-9.7). In 46 children (54.1%) gluten was introduced between 4 and 6 months of age and in 32 children after 6 months of age (37.6%). In the remaining 5 children the age of gluten introduction was not reported. Gluten introduction did not have a significant influence on the development of gliadin-specific T-cell responses. The percentages of positive T-cell responses to gTG in children who had gluten introduction between 4 and 6 months and in children who had it after 6 months of age were 56.5% and 68.8%, respectively (p=0.197, Fisher's exact test).

6 Discussion

The diagnosis of celiac disease is nowadays based on serological screening and subsequent confirmation by the detection of typical histological changes in duodenal biopsies. The duodenal biopsy is taken by upper GI endoscopy, which requires a general anesthesia in pediatric patients and is somewhat an uncomfortable procedure. An alternative method for duodenal biopsy in children is a Watson capsule endoscopy, which in turn does not require anesthesia but allows taking only a single biopsy sample compared to upper GI endoscopy by with 3 to 4 samples can be collected. New criteria recently proposed by The European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) emphasize the significance of serological testing in the diagnosis of celiac disease in children and suggest that the diagnosis can be based on high levels of celiac disease-specific antibodies, reducing the need for duodenal biopsy (21). Although serological screening has a central role in the diagnosis of celiac disease, it is known that T-cell mediated inflammatory processes in the intestinal mucosa are already ongoing when celiac disease-associated antibodies appear. The aim of this thesis was to determine whether gliadin-specific antibody and T-cell responses can be detected before the onset of the clinical disease by using immunological tests. For this purpose, celiac disease-associated antibodies and gliadin-specific T-cell responses were analyzed in peripheral blood samples from children with active celiac disease at the time of diagnosis, and also prospectively in children with a high genetic risk for the disease.

6.1 THE DEVELOPMENT OF CELIAC DISEASE-ASSOCIATED ANTIBODIES

Serological screening has largely been based on the measurement of serum IgA tTGA, which has both high sensitivity and specificity in the diagnosis of celiac disease (272,302,303,339). tTGA may, nevertheless, perform with a lower sensitivity especially in young children under two years of age (297,302). It is also known that young children may have transient tTGA seropositivity and not all of the tTGA seropositive individuals will eventually develop clinical disease (340,341). In a recently published study it was shown that even up to 28% of children with T1D who developed tTGA seropositivity later became seronegative despite ongoing gluten consumption (342). EmA have been considered as the golden standard for the diagnosis of celiac disease as they perform with extremely high specificity. Although the method for detecting EmA is time-consuming, and the results depend on the quality and experience of the laboratory, the new criteria by ESPGHAN recommend the EmA test as a celiac disease-specific antibody test and tTGA for primary screening of celiac disease (21). Moreover, the ESPGHAN guidelines have emphasized the importance of antibody levels. Based on these recommendations, the duodenal biopsy may be avoided in children positive for tTGA at levels over 10 times the upper limit of normal and when a false-positive result has been excluded by positive EmA and positivity for HLA-DQ2 or HLA-DQ8 (21).

Assays for detecting IgA- and IgG-class antibodies to deamidated gliadin peptides (anti-DGP) have been developed most recently and are widely used because of their good sensitivity and specificity, especially in children. The sensitivity of IgA anti-DGP has been reported to range between 80.7% and 95.1% and that of IgG anti-DGP between 80.1% and 98.6% (272). In several studies, the specificity of the IgG anti-DGP assay has been demonstrated to be extremely high, both in adults and in children (299,300,302,303). The specificity has been reported to be up to 98.9% for IgG anti-DGP in adults and even 100% in children under 2 years of age (302,303). However, the reported sensitivity and specificity of anti-DGP antibodies has a wide range presumably due to the wide variety of methods

and commercial kits that have been used in these studies (272). Recent studies have indicated that parallel testing for both tTGA and IgG anti-DGP antibodies may have the best specificity in the screening for celiac disease, both in adults and children (30,296).

The performance of a TR-IFMA anti-DGP assay in the diagnosis of celiac disease in children was analyzed in study II. Serum samples from children taken at the time of diagnosis of celiac disease or the latest sample available before the diagnosis were analyzed in the DIPP cohort and in healthy, age- and HLA-matched controls. A ROC curve analysis demonstrated that the optimal cut-off value for IgA anti-DGP was 153 AU and for IgG anti-DGP 119 AU. All of the 92 children with celiac disease were either IgA or IgG anti-DGP positive. The cut-off values determined with pediatric samples in this study are very similar to the earlier reported cut-offs of 150 AU for both IgA and IgG anti-DGP, which are based on the analyses of adult serum samples (295) Only one of the children with celiac disease was not IgG anti-DGP positive and seven children were IgA anti-DGP negative, indicating sensitivities of 98.9% and 92.4%, respectively. The sensitivity of the TR-IFMA assay was remarkably high compared to findings observed recently in children using commercial ELISA tests, as outlined above.

In study II, only two of the 82 control children were positive for IgA anti-DGP and IgG anti-DGP, indicating a specificity as high as 97.6% for both IgA and IgG anti-DGP assays. None of the controls were positive for both IgA and IgG anti-DGP. The control children were not followed and although these children were tested negative for tTGA, it is possible that some of these healthy but genetically predisposed children will develop celiac disease later on. However, the four positive samples in the control group had relatively low levels of IgA or IgG anti-DGP antibodies. We also demonstrated that both the IgA and IgG anti-DGP antibody levels correlated with the severity of the villous atrophy in the intestinal biopsies (II) emphasizing the importance of high levels of antibodies in the diagnosis of celiac disease.

In study III, altogether 291 children positive for HLA-DQA1*05 and -DQB1*02 alleles were followed prospectively for the development of celiac disease-associated antibodies. The cumulative incidence of tTGA seropositivity by the age of 3 to 4 years in this cohort was 6.5%, which is in accordance with earlier studies, in which the frequency of tTGA seropositivity in genetically predisposed children was reported to vary between 2.6% and 6.0% at the age of 5 to 7 years (87,161,340,343). The cumulative incidence of confirmed celiac disease in our study was 3.1%. Importantly, the majority of the children with biopsy-confirmed celiac disease were diagnosed after tTGA seropositivity without clinical suspicion, which support the notion that presently many of the children with celiac disease are asymptomatic (344). The median age at the time of seroconversion was 24 months and three of the children seroconverted tTGA positive at the age of 18 months. Eight of all 291 children (2.7%) had transient tTGA seropositivity and they turned tTGA seronegative during the follow-up. Interestingly, the children with transient tTGA seropositivity including one child with a negative biopsy, had significantly lower tTGA titers at the time of seroconversion than the nine children who were later confirmed with celiac disease by duodenal biopsy, supporting the significance of antibody levels in the diagnosis of celiac disease (21).

In study III, all the children with a high genetic risk for celiac disease who converted tTGA seropositive and were confirmed with celiac disease were both IgA and IgG anti-DGP positive at the time of tTGA seroconversion. Over half of the children with transient tTGA seropositivity were also IgG anti-DGP positive at the time of tTGA seroconversion but none of them were IgA anti-DGP positive, confirming the high specificity of IgA class anti-DGP assay for celiac disease as already shown in study II. Not only tTGA seroconverted children but also some of the children who remained tTGA seronegative developed anti-DGP antibodies. Altogether 76 of 1151 serum samples analyzed

from children who remained tTGA seronegative during the follow-up were positive for IgG but only 17 of the children were positive for IgA anti-DGP, with the corresponding specificities of 93.4% and 98.5% for IgG and IgA anti-DGP, respectively. The specificities of the IgA and IgG anti-DGP assays were more or less similar to those reported in study II. Importantly, in the majority of tTGA seronegative children, that were positive for anti-DGP antibodies, only one sample taken during the follow-up was positive, suggesting that transient anti-DGP-positivity is relatively common. However, in children with transient positivity to anti-DGP antibodies, the levels were lower than in children with confirmed celiac disease. Most of the tTGA seronegative children, repeatedly positive for IgG anti-DGP, were positive also at the last sampling point. Since the follow-up time was limited, it is possible that some of these repeatedly anti-DGP seropositive children will develop celiac disease later on. These findings may be associated with the slightly lower specificity of the IgG anti-DGP assay in study III compared to the results of study II. Altogether, both of these studies emphasize the significance of the levels of anti-DGP antibodies in the diagnosis of celiac disease in pediatric patients.

Since several studies have suggested that the best sensitivity and specificity for diagnosis of celiac disease, especially in young children, can be achieved by combining assays detecting IgA tTGA and IgG anti-DGP antibodies, we further analyzed whether anti-DGP antibodies appeared before tTGA positivity. The earlier appearance of anti-DGP antibodies was demonstrated both retrospectively in the DIPP cohort (II) and prospectively in the cohort of children with a high genetic risk for celiac disease (III). In the DIPP cohort, anti-DGP antibody positivity preceded tTGA seroconversion in up to 72.9% (35 of 48) of children with confirmed celiac disease. In 13 of the children with confirmed celiac disease both IgA and IgG anti-DGP antibodies preceded tTGA seroconversion. Only one of the children had IgA but not IgG anti-DGP antibodies preceding tTGA positivity. In the DIPP cohort the first IgA or IgG anti-DGP seropositivity was detected a median 0.9 or 1.0 year earlier than tTGA seropositivity, respectively. In the prospective follow-up study, IgG class anti-DGP antibodies appeared a median 1.0 year earlier than tTGA seroconversion in over half of the children with confirmed celiac disease. In contrast, IgA class anti-DGP positivity preceded tTGA seroconversion only in one child. The results obtained in these studies are in line with the only prospective study published so far (30). However, in that study, anti-DGP positivity preceded the appearance of tTGA in only 19% of the subjects studied, which is a much lower frequency than what was observed in the studies described here. Importantly, in the prospective follow-up study, anti-DGP positivity was observed to preceed tTGA positivity also in children with transient tTGA seropositivity. Therefore, it appears that IgG anti-DGP antibodies perform with a good sensitivity to predict tTGA seropositivity but they do not clearly discriminate between celiac disease-associated and transient tTGA seropositivity. This may complicate the clinical usefulness of the phenomenon. As transient tTGA seropositivity has been reported in young children, it is similarly possible that anti-DGP positivity may also be a transient phenomenon especially in young that does not necessarily predict the clinical onset of celiac disease (340).

6.2 PERIPHERAL BLOOD GLIADIN-SPECIFIC T-CELL RESPONSES

Since it is known that the T-cell mediated inflammation process in the intestinal mucosa has already begun when celiac disease-associated antibodies appear, the detection of gliadin-specific T-cell responses in peripheral blood could provide an interesting possibility for monitoring the celiac disease-associated immune response. Moreover, it is possible that the emergence of these peripheral blood T cells might even precede the antibody response to gliadin peptides. Gliadin epitopes recognized by CD4+ T cells were first de-

scribed through an analysis of intestinal T cells (10). Later, it was shown that CD4+ T cells specific to deamidated gliadin epitopes can also be detected in the peripheral blood of a majority of patients with active celiac disease. The frequency of gliadin-specific CD4+ T cells in the circulation is relatively low and thus in most of the studies patients have been challenged to oral gluten to enhance the gliadin-specific immune response in the gut, which also leads to an increase of gliadin-specific T cells in the circulation (14-16,251). All of the studies mentioned above have analyzed gliadin-specific T-cell responses in adult patients. Only one recently published study demonstrated that these gliadin-specific T cells are detectable also in children with celiac disease, even without gluten challenge (87). The Norwegian study group has recently shown that gliadin-specific CD4+ T cells are detectable in the circulation of adult patients also without oral gluten challenge by using a sensitive HLA class II tetramer technique (252).

In study I, we demonstrated, for the first time, that CD4+ T cells specific to gTG are also detectable in the peripheral blood of children with newly diagnosed celiac disease, and that their frequency is higher than that observed in healthy control children. T-cell responses to gTG were likewise detected in HLA-DQ2-positive children followed prospectively from the age of 9 months in study III. Peripheral T-cell responses were detected more often to gTG than native gliadin in children with active celiac disease. These results are in accordance with the model that the deamidation of gliadin epitopes enhances their T-cell reactivity in the context of celiac disease-associated HLA molecules (271,345,346). The deamidation of gliadin by tTG leads to the conversion of glutamine residues to negatively charged glutamic acid residues, enhancing the capacity of gliadin peptides to bind to HLA-DQ2 and -DQ8. Although peripheral blood T-cell responses were detected more often to gTG in children with celiac disease, T-cell responses to native gliadin were detected at a similar frequency in children with celiac disease and healthy children with a genetic risk for celiac disease (I, III). This is in line with one of the early studies, in which peripheral T-cell responses to native gliadin were observed to be common in healthy control subjects (347). Moreover, studies by Anderson et al. have shown that some of the healthy controls also had specific T-cell responses to native gliadin in the peripheral blood after an oral gluten challenge, whereas responses to deamidated gliadin increased only in patients with confirmed celiac disease (15). Ráki et al. reported similar results, detecting gliadin-specific peripheral blood T cells by HLA tetramers in celiac disease patients but not in controls after a short-term gluten challenge (16). Finally, in the study by Ben-Horin et al., CD4+ memory T-cell responses to deamidated gliadin were detected in about half of adult patients on a gluten-free diet (254). Importantly, almost half of the patients did not show any reactivity to deamidated gliadin. Also according to our studies, T-cell responses to native gliadin seems to be relatively common in healthy control children (I and III). In our study, CD4+ T-cell responses were detected significantly more often to gTG as compared to native gliadin in children with active celiac disease, whereas healthy controls recognized native gliadin as often as gTG (I). In healthy controls, T-cell responses to gTG were as common in DQ8-positive as they were in DQ2-positive healthy controls (I). Thus, healthy individuals carrying the DQ2 and DQ8 alleles do not have enhanced T-cell responses to deamidated gliadin epitopes. Taken together, the increased T-cell recognition of deamidated gliadin in children with active celiac disease may result from an increased amount of gTG-specific memory T cells in the peripheral bllod. Nevertheless, the frequency of gTG-specific memory T-cells appears to be low without oral gluten challenge. In study I, 11 of the 20 children with confirmed celiac disease responded to gTG (I), which is in accordance with the study by Ben-Horin et al., in which half of the patients with celiac disease did not show reactivity to deamidated gliadin using a CFSE dilution method (254).

Peripheral T-cell responses in children with celiac disease and in healthy controls were

also tested to the peptides QLQPFPQPELPY (Q12Y) and PQPELPYPQPELPY (P14Y), which contain the previously identified immunodominant epitopes of α-gliadin: α-I and α-II, respectively (17). Interestingly, none of the patients and only a few of the control children recognized these peptides, indicating that these epitopes do not explain the peripheral blood T-cell reactivity to deamidated gliadin in children. These results are in accordance with an earlier study that demonstrated that intestinal T-cell responses in children with celiac disease are more variable than in adults, and are directed against multiple deamidated gliadin and gluten peptides as well as toward native gluten peptides, instead of the earlier described immunodominant epitopes of α-gliadin (18). Other epitopes recognized by intestinal T cells from adult patients have also been characterized and these studies demostrate that intestinal T cells in adult patients with celiac disease also recognize several epitopes of gliadin and not only the so-called immunodominant epitopes (255,256,349). Differences in the T cell epitopes recognized may also be explained by the first cereals that are introduced into the diet of Finnish children, usually rye and barley together with wheat. Therefore, T-cell responses to wheat gluten may be relatively less important in Finnish children and this may explain the absence of T-cell responses to the immunodominant epitopes of wheat α-gliadin in our study.

T-cell reactivity to the self-antigen tTG was extremely rare in this study. In study I, none of the 16 children with celiac disease, and only two of 55 control children (3.6%), showed T-cell responses to tTG, and in study III the T-cell responses to tTG varied between 0% and 7.9% at different sampling points. Peripheral blood T-cell reactivity to tTG was remarkably lower than that reported in earlier studies (223,224). Comerford et al. demonstrated T-cell responses to tTG in up to 80% of untreated and in 40% of treated adults patients with celiac disease but also in 38% of healthy controls (224). They also demonstrated that T cells specific to tTG produced the proinflammatory cytokines IL-17A and IL-21, which may have relevance for the pathogenesis of celiac disease.

Both in study I and III, we demonstrated that peripheral blood T cells specific to native and deamidated gliadin had mainly a memory phenotype and they expressed high levels of the gut-homing molecule β7-integrin. The frequency of the memory phenotype (CD45RA-CD45RO+) on gTG-specific T cells was higher than on T cells specific for native gliadin, but only in patients with celiac disease. This finding suggests that in healthy children, some of the observed T-cell responses to gliadin and gTG may in fact be derived from in vitro-activated naïve T cells, whereas gTG-specific responses in patients with celiac disease are more clearly of memory origin. Similar findings were detected in the healthy, genetically predisposed children in the prospective follow-up study (III) as no difference was observed in the percentages of memory CD4+ T cells between stimulations with deamidated and native gliadin. It is known that upon activation and proliferation in vitro naïve T cells first acquire the expression of CD45RO and subsequently lose the expression of CD45RA, making the timing of the analysis of CD45 molecule expression in vitro critically important (204). Regardless of this, it can be assumed that the percentage of proliferating CD4+ T cells with the CD45RA-CD45RO+ phenotype more or less reflects the frequency of memory cell-derived antigen-specific precursor T cells. Our results are in accordance with the study by Ráki et al., which used an elegant HLA-tetramer method to visualize CD4+ T cells specific to deamidated gliadin directly ex vivo (16). In most of the patients with celiac disease the tetramer-positive cells analyzed were of the CD45RA-CD45RO+ memory phenotype T cells (16). In our studies, we also observed that gliadin- and gTG-specific T cells expressed high levels of the β7-integrin molecule in both children with active celiac disease and in children with a genetic risk for celiac disease. This finding supports the hypothesis that peripheral blood CD4+ T cells specific to both native and deamidated gliadin are originally activated in the gut and are capable of trafficking back to the intestine.

6.3 GLUTEN INTRODUCTION

Since the most important celiac disease risk allele combination of HLA-DQA1*05 and -DQB1*02 is relatively common in the general population, it has been suggested that environmental factors must also have a pivotal role in the development of celiac disease. Several studies have suggested that the timing of gluten introduction to infants influences the risk of celiac disease in genetically susceptible children (103,160,161,167,168,170). Specifically, these studies suggest that an optimal window of gluten introduction for prevention of celiac disease exists between 4 and 6 months of age while breastfeeding is ongoing. Based on these observations, the secondary aim of this thesis was to evaluate whether the timing of gluten introduction has a significant effect on the development celiac disease-associated immune responses. For this purpose a dietary intervention was performed in order to analyze whether gluten introduction has an effect on the development of celiac disease-associated antibodies and the development of clinical disease. The dietary intervention group was given special nutrition counseling with the aim of targeting gluten introduction to between 4 and 6 months of age while breastfeeding was ongoing, and starting cereal consumption in small but gradually increasing doses (103,160,161,165,338). The control group received the normal advice given to all Finnish families at the child health clinics. Approximately half of the children (54%) in the study had gluten introduced to their diet within the optimal window 4 and 6 months of age, and this was successful more often in the intervention than in the control group. The median age of gluten introduction in the study was 6.0 months. Although the power of this study is limited due to the relatively low number of children studied, neither the timing of gluten introduction nor breastfeeding appeared to affect the development of celiac disease or associated autoantibodies. Importantly, our findings are in accordance with the results of recently published larger prospective studies (106,172,173). Contrary to the earlier hypothesis, these studies did not support the importance of either the age at gluten introduction or concurrent breastfeeding in the development of celiac disease (106,172,173).

6.4 LIMITATIONS OF THE STUDY AND FURTHER CHALLENGES

When looking at the results of this study, certain limitations, which may have had an impact to the conclusions of the study, need to be considered.

Peripheral blood T-cell responses to deamidated gliadin were not seen in all children with active celiac disease, which may, at least partly, be due to the low number of antigen-specific T cells in the circulation. Other methods than the CFSE dilution assay utilized here could be more sensitive in detecting gliadin-specific T cells in the peripheral blood, and should be considered in future studies. As described earlier, the gliadin epitopes are presented to the CD4+ T cells in the context of HLA-DQ2 or -DQ8 molecules. In our studies, we did not directly test whether the peripheral blood gliadin-specific T-cell responses observed were HLA-DR or -DQ restricted, which could have allowed a more specific analysis of the pattern of T-cell responses in patients and controls. We neither tested whether the peripheral blood gliadin-specific T-cell responses differed from their cytokine production profiles which could allow for more specific analysis as well.

Due to the original screening for genetic risk for T1D in the DIPP study, the proportion of HLA-DQ8-positive children with celiac disease in study II was higher than observed in earlier clinical studies. The control group consisted of healthy age-matched children positive for HLA-DQ2 or -DQ8 risk alleles, which may be criticized since healthy children may have lower levels of anti-DGP-antibodies than symptomatic subjects with other GI or autoimmune diseases than celiac disease, leading to the overestimation of the speci-

ficity of the assay. Further analysis of the anti-DGP assay with a control group of symptomatic children with normal histological findings in duodenal biopsies would allow the calculation of positive and negative predictive values, which would be more useful in the clinical evaluation of the performance of the test.

To analyze the development of gliadin-specific immune responses in study III, blood samples were taken from children at the ages of 9, 12, 18 months and every 12 months thereafter. The percentage of children with anti-DGP seropositivity, as well as the percentage of T-cell responses to deamidated gliadin was relatively high at the age of 9 months. As no earlier samples were taken, it remains unclear whether the gliadin-specific immune responses could be detectable even earlier and whether they are induced by the introduction of gluten. As the primary aim of the study was to analyze whether the gliadin-specific immune response could be detected by immunological tests before the damage of the intestinal mucosa and the clinical onset of the disease, the duodenal biopsy should had been performed at the time of anti-DGP seropositivity or at least after repeated anti-DGP positivity. Peripheral blood T-cell responses were analyzed only in 85 of the children and thus the analysis of the correlation between the development of T-cell responses to gliadin, the appearance of tTGA and anti-DGP antibodies and the clinical disease was not feasible. In the future, a larger study population and possibly more frequent sampling would be needed for a full evaluation of the significance of peripheral blood T-cell responses to deamidated gliadin in pediatric patients.

7 Conclusions

In conclusion, our studies demonstrate that the TR-IFMA assay for detecting antibodies to a synthetic deamidated gliadin peptide performs with a high sensitivity and specificity in the diagnosis of celiac disease in pediatric patients. Moreover, we demonstrated, first retrospectively that specifically IgG anti-DGP antibodies appeared a median 1.0 year earlier than IgA tTGA in the majority of the children with celiac disease. In the follow-up study of children with a high genetic risk for celiac disease, altogether 6.5% of children converted tTGA seropositive by the age of 4 years and the final incidence of celiac disease was 3.1%. Anti-DGP antibodies also preceded tTGA seropositivity a median 1.0 year earlier in this study. This was observed both in the children with confirmed celiac disease as well as those with transient tTGA seropositivity. Collectively, the results from our two studies indicate that an earlier detection of celiac disease may be possible by monitoring tTGA and anti-DGP antibodies frequently in genetically susceptible children.

In these studies, we also demonstrate for the first time that CD4+ T cells specific to deamidated gliadin are detectable in the peripheral blood of children with newly diagnosed celiac disease and also in the blood of children with a high genetic risk for celiac disease already in early infancy. Although responses to gTG were observed both in children with celiac disease and in healthy but genetically predisposed children, they were significantly less common in the latter group. Deamidation of gliadin increased the frequency of T-cell responses in children with active celiac disease, whereas the responses to deamidated and native gliadin did not differ in healthy controls. In the follow-up study, T-cell responses to gTG were relatively common already at an early age of 12 months in HLA-DQ2-positive children but the frequency of responses decreased during the follow-up to a similar level that was observed in the healthy control children in study I. In both children with celiac disease as well as in healthy children with a genetic risk for celiac disease, CD4+ T cells specific to gTG appeared to have a memory phenotype and they expressed the gut-homing marker, β7-integrin. In all, our results support the widely accepted model for the importance of T-cell responses to deamidated gliadin epitopes in the pathogenesis of celiac disease also in pediatric patients. Based on our results, it seems that responses to gTG are relatively common at an early age in genetically predisposed children, but they appear to gradually decrease in children who remain healthy whereas they increase in those who develop celiac disease.

Although the power of the follow-up study was limited, our results support the notion that neither the timing of gluten introduction nor breastfeeding affects the development of celiac disease-associated autoimmunity. Importantly, these results are in accordance with other large prospective studies that have been published recently (106,172,173) .

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Anne Lammi

Gliadin-specific immune responses in the development and prediction of celiac disease in children

The aim of this thesis was to analyze, by using immunological methods, whether gliadin-specific immune responses can be detected prior to the development of the clinical phase of celiac disease. Our results indicate that earlier detection of celiac disease in genetically susceptible children may be possible by monitoring tTGA and anti-DGP antibodies whereas monitoring peripheral blood gliadin-specific T-cell responses did not enhance early diagnosis. Anti-DGP antibodies may be the first marker of celiac disease in a majority of pediatric patients.

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