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NATURAL HISTORY OF CELIAC DISEASE-ASSOCIATED ANTIBODIES AND PROGRESSION TO OVERT DISEASE IN CHILDREN AT INCREASED GENETIC RISK

by

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To Ville and Konsta

ABSTRACT

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Natural history of celiac disease-associated antibodies and progression to overt disease in children at increased genetic risk. Department of Pediatrics, University of Turku, Turku, Finland

Background: Celiac disease is a lifelong, gluten-sensitive, autoimmune-mediated chronic enteropathy, tightly associated with risk alleles at the HLA class II genes.

Aims: This study was carried out as a part of the population-based Type 1 Diabetes Prediction and Prevention (DIPP) Project. The first aim was to study the natural history of celiac disease-associated antibodies before the diagnosis of celiac disease was made. The second aim was to describe when and in which order celiac disease-associated and type 1 diabetes-associated antibodies appeared in children with genetic risk for both diseases.

Subjects and Methods: Antibodies against tissue transglutaminase (TGA) and other celiac disease-associated antibodies were measured in serum samples collected at 3- to 12-month intervals of children at genetic risk for celiac disease who participated in the DIPP project. Celiac disease was confirmed by duodenal biopsy. Type 1 diabetes-associated antibodies were measured in all samples that had been collected. Overt disease was diagnosed according to World Health Organization criteria. Follow-up continued until a diagnosis of type 1 diabetes or until the end of a defined follow-up period.

Results: TGA appeared in children at genetic risk for celiac disease only after the first year of life, but anti-gliadin antibodies often emerged significantly earlier, at age 6 months. The data show that spontaneous disappearance of celiac disease-associated antibodies, transient or persisting, is a common phenomenon, at least in prepubertal children. In children with genetic susceptibility to type 1 diabetes and celiac disease, celiac disease-associated antibodies usually develop earlier than the type 1 diabetes-associated antibodies.

Conclusions: The transient nature of celiac disease-associated antibodies emphasizes the significance of establishing seropositivity repeatedly in screening detected celiac disease before gastroscopy and duodenal biopsy are considered and emphasized the importance of duodenal biopsy for diagnosing celiac disease.

Keywords: antibodies, celiac disease, children, transglutaminase, type 1 diabetes, follow-up study

TIIVISTELMÄ

Satu Simell

Keliakiaan liittyvien vasta-aineiden ja keliakian kehittyminen geneettisesti alttiilla lapsilla. Lastenklinikka, Turun yliopisto, Turku

Tausta Keliakia on elinikäinen sairaus, jossa viljan sisältämä gluteeni aiheuttaa autoimmuunityyppisen suolistotulehduksen perimältään sille alttiilla ihmisillä.

Tavoitteet Väitöskirjatyön tavoitteena oli tutkia keliakiaan liittyvien vasta-aineiden ilmaantumista ja luonnollista kulkua suurentuneessa geneettisessä riskissä olevilla lapsilla ennen keliakian diagnosoimista. Toisena tavoitteena oli kuvata keliakiaan sekä tyypin 1 diabetekseen liittyvien vasta-aineiden ilmaantumista lapsilla, joilla on molemmille taudeille altistavat HLA-geenialueen perintötekijät.

Menetelmät Väitöskirjatutkimukseni perustuu pitkittäiseen tyypin 1 ennustaminen ja ehkäisy -seurantatutkimukseen (DIPP). Kudostransglutaminaasivasta-aineita (TGA) sekä muita keliakiaan liittyviä vasta-aineita mitattiin 3 – 12 kuukauden välein otetuista seeruminäytteistä. Keliakia diagnosoitiin ohutsuolikoepaloista. Tyypin 1 diabetekseen liittyviä vasta-aineita mitattiin jokaisesta otetusta verinäytteestä ja diabetes diagnosoitiin WHO:n kriteerein. Lapsia seurattiin tyypin 1 diabeteksen kehittymiseen asti tai kunnes seuranta-aika päättyi.

Tulokset Kudostransglutaminaasivasta-aineita (TGA) ilmaantui lapsille ensimmäisen ikävuoden jälkeen, mutta gliadiinivasta-aineita voitiin todeta jo 6 kuukauden iässä. Keliakiaan liittyvät vasta-aineet katosivat tilapäisesti tai pysyvästi jopa 50 %:lla geneettisesti alttiilta lapsilta myös ilman gluteenitonta dieettiä. Keliakiaan liittyvät vasta-aineet ilmaantuivat aiemmin kuin diabetekseen liittyvät vasta-aineet.

Johtopäätökset Keliakiaan liittyvien vasta-aineiden spontaani katoaminen korostaa seulonnalla todettujen toistettujen positiivisten näytteiden merkitystä ennen ohutsuolikoepalojen ottoa. Keliakiadiagnoosin perustuminen ohutsuolen limakalvon löydöksiin on entistä tärkeämpää.

Avainsanat: keliakia, kudostransglutaminaasi, lapset, seurantatutkimus, tyypin 1 diabetes, vasta-aineet

TABLE OF CONTENTS

ABBREVIATIONS

AGA	antigliadin antibodies
APC	antigen presenting cell
ARA	antireticulin antibodies
CD	celiac disease
DAISY	Diabetes Autoimmunity Study in the Young
DGP-ab	deamidated gliadin peptide antibodies
DIPP	Type 1 Diabetes Prediction and Prevention study
ELISA	enzyme-linked immunosorbent assay
EMA	endomysium antibodies
ESPGAN	European Society of Paediatric Gastroenterology and Nutrition
GADA	autoantibody against the 65 kD glutamic acid decarboxylase
HLA	human leukocyte antigen
IAA	insulin autoantibody
IA-2A	autoantibody against the protein tyrosine phosphatase-related IA-2 protein
ICA	islet cell autoantibody
INF-γ	interferon γ
IEL	intraepithelial lymphocyte
IgA and IgG	immunoglobulin A and G
IL	interleukin
JDFU	Juvenile Diabetes Foundation Unit
MICA	major histocompatibility complex class I chain-related gene A
MMP	matrix metalloproteinase
NKG2D	natural-killer cell receptor
PCR	polymerase chain reaction
TCR	T cell receptor
T1D	type 1 diabetes
TG	tissue transglutaminase
TGA	autoantibody against tissue transglutaminase
TNF-α	tumor necrosis factor α
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by Roman numerals I – III. The original communications have been reproduced with permission from copyright holders.

I Simell S, Kupila A, Hoppu S, Hekkala A, Simell T, Ståhlberg M-R, Viander M, Hurme T, Knip M, Ilonen J, Hyöty H, Simell O. Natural history of transglutaminase autoantibodies and mucosal changes in children carrying HLA-conferred celiac disease susceptibility. *Scand J Gastroenterol* 2005; 40:1182-1191

II Simell S, Hoppu S, Hekkala A, Simell T, Ståhlberg M-R, Viander M, Yrjänäinen H, Grönlund J, Markula P, Simell V, Knip M, Ilonen J, Hyöty H, Simell O. Fate of five celiac disease-associated antibodies during normal diet in genetically at-risk children observed from birth in a natural history study. *Am J Gastroenterol* 2007; 102:2026-2035

III Simell S, Hoppu S, Simell T, Ståhlberg MR, Viander M, Routi T, Simell V, Veijola R, Ilonen J, Hyöty H, Knip M, Simell O. Age at development of type 1 diabetes and celiac disease-associated antibodies and clinical disease in genetically susceptible children observed from birth. *Diabetes Care* 2010; 33:774-779

1. INTRODUCTION

Celiac disease is a lifelong autoimmune-mediated disorder previously thought to affect only the mucosa of the small intestine. However, recent data have convincingly shown that the disease may influence the whole organ system and lead to highly variable clinical symptoms and findings. There is a genetic susceptibility to celiac disease, which is strongly associated with the human leukocyte antigen (HLA) DQ2, which occurs in nearly 90% of all patients with celiac disease. Most of the patients with celiac disease who are HLA DQ2 negative are HLA DQ8 positive (Sollid et al. 1989, Polvi et al. 1996). However, genetic susceptibility alone is not a sufficient condition for the development of celiac disease, because 20 – 30% of the general population is DQ2 positive (Polvi et al. 1996).

In addition to genes, environmental triggers are needed to activate the autoimmune process in celiac disease. So far, the only established environmental contributor is continuous exposure to dietary gluten. Once the inflammation in the intestinal mucosa has begun, disease-specific antibodies appear in the circulation. The discovery of these antibodies has made population-based screening of celiac disease possible.

Celiac disease has long been known to associate with type 1 diabetes (Walker-Smith and Grigor 1969, Visakorpi 1969). Often, type 1 diabetes is diagnosed first and celiac disease develops later (Saukkonen et al. 1996, Barera et al. 2002). The reason for this comorbidity is usually explained by the same susceptible HLA alleles for both diseases (Sollid et al. 1989, Smyth et al. 2008).

The purpose of this study was to elucidate the natural history of celiac disease-associated antibodies and progression to overt celiac disease in a cohort of genetically susceptible children. The children were followed closely from birth. Another purpose was to describe the coexistence of antibodies related to celiac disease and to type 1 diabetes in children at increased genetic risk for both diseases.

2. REVIEW OF THE LITERATURE

2.1 History of celiac disease

The first description of what has been called celiac disease dates from the first and second centuries A.D. by Aretaeus the Cappadocian (Adams 1856). The history of modern celiac disease was described by Samuel Gee in 1888 (Gee 1888). He described severe steatorrhoea and cachexia in people of any age but mainly in children aged 1 to 5 years. Gee realized the importance of diet, "Errors in the diet may perhaps be a cause, but what error?"(Gee 1888). It took over 60 years until the link between celiac disease and wheat was recognized by the Dutch pediatrician Willem Dicke and colleagues (Dicke 1950). A few years later alcohol-soluble gliadin was identified as the toxic component in wheat gluten (van de Kamer et al. 1953). Very soon after that, the duodenal mucosal damage with short villi, crypt hyperplasia and inflammation typical of celiac disease was described by Paulley (Paulley 1954). A few years later the instruments for peroral intestinal biopsies were developed and this enabled a specific diagnosis of celiac disease (Royer et al. 1955, Shiner 1957).

The first serological markers, antigliadin antibodies (AGA), were described in 1958 (Berger 1958), but they were introduced to clinical practice only several years later (Ferguson and Carswell 1972). At the same time also reticulin antibodies (ARA), the first antibodies against the connective tissue components in celiac disease were found (Seah et al. 1971a). The endomysium antibodies (EMA) were described in 1983 (Chorzelski et al. 1983) and almost 15 years later tissue transglutaminase was identified as the celiac disease-associated endomysial autoantigen. Finally, tissue transglutaminase antibodies (TGA) were identified in 1997 (Dieterich et al. 1997).

2.2 Epidemiology of celiac disease

One of the oldest epidemiological studies of celiac disease was published in 1950. At that time, it was reported that the incidence was 1/8000 in England and Wales and 1/4000 in Scotland (Davidson and Fountain 1950). After that, the published figures on the prevalence of celiac disease have varied considerably, depending on the country and the diagnostic criteria. If only classic celiac disease is taken into account, the prevalence in the Netherlands was 1:6000 in 2004 (Schweizer et al. 2004). However, this prevalence figure strongly underdiagnoses celiac disease which presents with non-classical symptoms. In Finland, active case-finding revealed the prevalence of 1:370 (Collin et al. 1997) whereas a population-based screening study reported a prevalence of 1:99 (Mäki et al. 2003). Lohi and colleagues showed that the total prevalence of celiac disease among adults in Finland increased from 1:92 in 1978–1980 to 1:52 in 2000–2001 (Lohi et al. 2007). Recently Myléus and associates reported a high prevalence of 29:1000 (3%) among 12-year-old children in Sweden (Myléus et al. 2009). A global prevalence

of approximately 1% of the population has been reported (Carlsson et al. 2001, Fasano et al. 2003a, Bingley et al. 2004, Tommasini et al. 2004). However, in the Far East celiac disease is rare (Fasano and Catassi 2001).

2.3 Clinical picture

2.3.1 Classic celiac disease

In the 1970s the classic symptoms reported and observed by children with disease were diarrhea, steatorrhea, failure to thrive, malabsorption syndrome and abdominal distension. The affected children were typically young, usually under two years of age (Visakorpi et al. 1970, Young and Pringle 1971). It was reported that the incidence of celiac disease decreased in the 1980s (Challacombe and Bayliss 1980, Stevens et al. 1987), but rather than disappearing (Logan et al. 1983, Mäki et al. 1988, Kelly et al. 1989, Mäki and Holm 1990), the symptoms of celiac disease became milder and celiac disease was often diagnosed among older children and adults. A recent retrospective study from Finland examined the symptoms of 197 children at the time of diagnosis of celiac disease. Children younger than 3 years of age most often had chronic diarrhea (67%), whereas the main symptom of older children was abdominal pain (38%). Growth had not been affected in most children, because less than 7% had severe growth failure. However, anemia and iron deficiency were common (25% and 43%, respectively) (Savilahti et al. 2010). The number of children with no symptoms has increased markedly as targeted population screening has been carried out (Mäki et al. 1988, Ludvigsson et al. 2004). In Spain, on the other hand, children are more often diagnosed on the basis of classical symptoms than adults and the symptoms are most pronounced in children younger than 2 years old (Vivas et al. 2008).

2.3.2 Silent celiac disease and celiac disease detected by screening

Silent celiac disease is characterized by celiac disease associated-antibodies and small bowel mucosal lesions, which are cured when the subjects adhere to a gluten free diet: these patients have no overt symptoms related to the disease (Ferguson et al. 1993). However, many of the patients with silent celiac disease are not truly asymptomatic, because often when gluten free diet has been introduced, they recognize marked improvement in their general well-being (Mustalahti et al. 2002, Hoffenberg et al. 2004, Viljamaa et al. 2005a, McGowan et al. 2009). Modern serological testing identifies silent celiac disease among people at risk of celiac disease, i.e. first-degree relatives to patients with established celiac disease and people with conditions that are frequently associated with celiac disease.

Cross-sectional studies have shown that celiac disease is a common disease already among young children. In a Swedish population-based screening study, the prevalence of celiac disease was at least 1% in children aged 2.5 years (Carlsson et al. 2001). In

Finland, the prevalence of celiac disease is 1% in school-aged children (Mäki et al. 2003).

The natural course of celiac disease in childhood has been studied in some recent prospective studies. In those studies children at increased risk for celiac disease, defined by either genetic risk or being a relative for a patient with type 1 diabetes, have been followed up and TGA analyzed at regular intervals. These studies have shown that TGA appears only after the age of 2 years (Hummel et al. 2000, Hoffenberg et al. 2003, Liu et al. 2003, Castano et al. 2004, Hummel et al. 2007). Although the TGA levels have fluctuated during follow-up, they have remained consistently positive in most of the affected children (Liu et al. 2003).

2.3.3 Extraintestinal symptoms

Celiac disease is not restricted to the gastrointestinal tract. Probably the best known extraintestinal manifestation of celiac disease is dermatitis herpetiformis, a blistering skin disease affecting primarily the elbows, knees, buttocks and scalp. It was first described in 1884 but it was only in the 1960s when the connection to celiac disease was detected (Duhring 1884, Marks et al. 1966). The diagnosis of dermatitis herpetiformis is based on pathognomonic IgA deposits in the dermal papillae of uninvolved skin (van der Meer 1969). Almost all patients with dermatitis herpetiformis have some degree of enteropathy, but only 10 – 30% have gastrointestinal symptoms (Reunala et al. 1984).

Untreated celiac disease poses a risk for decreased bone mineral density regardless of the patient's age and the clinical picture of the disease (Mora et al. 1998, Mustalahti et al. 1999). Consequently, the risk of fractures increases (Olmos et al. 2008). Bone mineral density improves when a gluten free diet is introduced (Kemppainen et al. 1999, Mora et al. 2001). Celiac disease may also cause non-specific arthritis or arthralgia (Collin et al. 1992, Lubrano et al. 1996).

The mineralization of permanent teeth is markedly affected by untreated celiac disease. Dental enamel defects occur in 10 – 96% of celiac disease patients (Aine et al. 1990, Pastore et al. 2008, Cheng et al. 2010). Recurrent aphthous stomatitis occurs in over 40% of celiac disease patients, and about 5% of patients with aphthous ulcers have celiac disease (Pastore et al. 2008, Cheng et al. 2010).

Between 10 and 30% of the patients with untreated celiac disease may have various neurological manifestations, including ataxia, peripheral neuropathy, dementia, and cerebral calcifications and epilepsy (Luostarinen et al. 1999, Luostarinen et al. 2001, Bushara 2005, Hadjivassiliou et al. 2010). Psychiatric disorders, especially depression and anxiety, also associate with celiac disease (Pynnönen et al. 2004, Ludvigsson et al. 2007b, Addolorato et al. 2008).

About half of all untreated patients with celiac disease have hypertransaminasemia, which disappears after a few months of gluten free diet. Celiac disease occurs in 3 – 7%

of patients with autoimmune hepatitis, primary biliary cirrhosis, or primary sclerosing cholangitis. A gluten free diet has no effect or only a minor effect on the course of these autoimmune disorders (Volta et al. 1998, Ludvigsson et al. 2007a, Caprai et al. 2008, Volta 2009).

Untreated celiac disease affects reproduction through unknown mechanisms, and approximately 4% of women with unexplained infertility have nonsymptomatic celiac disease (Collin et al. 1996). Untreated celiac disease shortens the reproductive span of females and celiac disease of the mother is associated with an increased frequency of spontaneous abortion, and poor intrauterine growth and low birth weight of the neonate (Özgör and Selimoglu 2010).

Refractory sprue or refractory celiac disease is a rare complication of celiac disease, characterized by persistent symptoms and marked histological changes despite strict adherence to gluten free diet (Rubio-Tapia and Murray 2010). In ulcerative jejunoileitis mucosal ulcerations develop mainly in the jejunum, and a gluten free diet has no effect on the progression of the disease (Biagi et al. 2000). The prognosis of both of these complications of celiac disease is, regrettably, rather poor.

Malignancy of the alimentary tract is a complication associated with long-lasting, untreated celiac disease. The affected individuals are at increased risk of adenocarcinoma of the small intestine, pharynx and esophagus. T-cell non-Hodgkin's lymphoma and especially enteropathy-associated T-cell lymphoma have a strong association with celiac disease (Catassi et al. 2002, Green et al. 2003, Catassi et al. 2005).

The overall mortality rate among patients with celiac disease is increased (West et al. 2004, Viljamaa et al. 2006). In a recent study from Sweden the hazard ratio for mortality was 1.39 in celiac disease patients, 1.72 in patients with small bowel inflammation and 1.35 in latent celiac disease. Malignancy and cardiovascular disease were the main causes of death (Ludvigsson et al. 2009).

2.4 Diagnosis of celiac disease

The diagnostic criteria for celiac disease were first defined in 1969 by the European Society of Paediatric Gastroenterology. These so-called Interlaken criteria defined celiac disease as a permanent condition of gluten intolerance. The diagnosis required three small bowel biopsies – the first during the gluten containing diet, which had to show "flat" mucosa; the second, during gluten free diet showing improvement in villous structure, and the third, at gluten challenge 2 years later which had to show histological relapse (Meeuwisse 1970). In 1977 the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) performed a survey to evaluate how accurately the Interlaken criteria were implemented for diagnosing celiac disease in practice. The requirement of biopsy-documented flat mucosa was occasionally regarded too demanding and the degree of mucosal changes before and after gluten reintroduction also needed clarification

(McNeish et al. 1979). In 1990 the ESPGAN criteria were revised (Walker-Smith et al. 1990). Gluten challenge was no longer required in obvious cases, as long as the typical villous atrophy was confirmed during a gluten containing diet and when full clinical and, in some cases, also histological recovery could be confirmed. In most cases antibodies during gluten containing diet and the disappearance of antibodies during a gluten free diet was considered sufficient to confirm the diagnosis. However, gluten challenge was still recommended in some specific cases, i.e., if there was any doubt about the initial diagnosis or if the clinical response to gluten free diet was inadequate (Walker-Smith et al. 1990). The diagnostic criteria for celiac disease have not been modified after 1990, in spite of the fact that the clinical picture of celiac disease has changed and there is mounting evidence that novel serological and histological tests are fairly well associated with the conventional diagnostic criteria.

2.4.1 Serological tests

Serological tests are important for selecting individuals who require small bowel biopsy examination for confirmation of the diagnosis of celiac disease. Their role is currently even more important than earlier, because the classical picture of celiac disease is nowadays only rarely encountered and symptoms vary. Serological tests alone or in combination with genetic analysis have also made screening studies possible and increased our knowledge of the prevalence and clinical features of celiac disease. Serological tests also help to screen individuals, who are at a particular risk of celiac disease, e.g., patients with autoimmune diseases or relatives of patients with celiac disease. As a non-invasive procedure, antibody assays are a good first-step test before making a decision to recommend the patient to undergo small bowel biopsy. Serological tests are also widely used for evaluation of compliance of the patients with a gluten free diet: disappearance of celiac disease-associated antibodies during a gluten free diet is commonly regarded as an affirmation of the diagnosis.

Currently, the presence of celiac disease-associated antibodies by serological testing is frequently the reason to suspect celiac disease and to perform a small bowel biopsy. However, such biopsies should also be taken when there is a strong clinical suspicion of celiac disease, irrespective of whether serological tests are positive or negative (Green and Cellier 2007). Because most serological tests detect specific IgA antibodies, subjects with IgA deficiency and celiac disease may go undiscovered. This association is even more significant among patients who do have celiac disease, since almost 8% of subjects with IgA deficiency have celiac disease (Meini et al. 1996) and, vice versa, approximately 2% of patients with celiac disease are IgA deficient (Cataldo et al. 1998).

2.4.1.1 Gliadin antibodies

Gliadin, the alcohol-soluble component of gluten, is the major storage protein of wheat. It is the toxic component in celiac disease and causes immunological reactions in the gut. Both IgA- and IgG-class antibodies against gliadin (AGA-IgA and AGA-IgG) are

Table 1. Sensitivity and specificity of serum AGA-IgA and AGA-IgG in children with untreated celiac disease.

Reference	Study population				AGA-IgA		AGA-IgG	
	Number of patients	Mean/ *median age (years)	Number of controls	Mean/ *median age (years)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Ståhlberg et al. 1986	31	5.5	278	5.9	90	86	94	67
Not et al. 1993	22	ND	185	ND	100	100	98	91
Lerner et al. 1994	28	9.6	41	8.4	52	94	88	92
Carroccio et al. 1996	36	*1.4	72	*1.2	72	90	89	72
Sacchetti et al. 1996	32	*5.0	42	*2.0	84	93	87	74
Bottaro et al. 1997	50	*2.5	25	*3.0	92	68	100	36
Sulkanen et al. 1998	136	*10.7[1]	207	*10.0[1, 2]	85	82	69	73
Vitoria et al. 1999	27	5.0	34	5.9	96	97	ND	ND
Wolters et al. 2002	52	4.0	49	5.1	83	86	83	80
Baudon et al. 2004	30	*2.0	116	ND	60	99	93	90
Lagerqvist et al. 2008	428	*1.3	87[nb]/129	*2.4[nb]/*3.1	96	95	ND	ND

[1] Study population includes also a group of adults

[2] Three control groups; 154 controls with clinically suspected celiac disease, median age 10.0 years; 32 controls with inflammatory bowel disease, median age 14.3 years; 21 controls with type 1 diabetes, median age 11.9 years

nb = no biopsy

ND = no data

often present in the serum of untreated patients with celiac disease (Stern et al. 1979, Unsworth et al. 1981, Savilahti et al. 1983, Bürgin-Wolff et al. 1989). However, gliadin antibodies are not disease-specific, since they are found in patients with food allergies, gastrointestinal diseases (Kumar et al. 1984, Lindberg et al. 1985, Kull et al. 1999) and also in healthy individuals (Bonamico et al. 1997, Kaukinen et al. 2000).

Several methods have been used to analyze AGA, but currently ELISA is the most used method (Volta et al. 1985, Wolters et al. 2002). Both AGA-IgA and AGA-IgG have been used widely in the search for celiac disease. The sensitivity of AGA-IgA varies between 52% and 100% and of AGA-IgG between 69% and 100%. The specificities vary between 68% and 100% and 36% and 92%, respectively (Table 1). The sensitivity of both AGA-IgA and AGA-IgG is higher in children under 2 years than in older children and in adults (Savilahti et al. 1983, Bürgin-Wolff et al. 1991, Lagerqvist et al. 2008, Maglio et al. 2009). The use of AGA testing alone is not recommended for screening of celiac disease because of the great variability and low precision of the test (Hill 2005a).

Deamidated gliadin peptide antibodies (DGP-ab) have recently been described as contributory to the pathogenesis of celiac disease (Aleanzi et al. 2001) and a commercial ELISA method has been developed for analysis of DGP-ab (Prince 2006). Although DGP-ab is more sensitive (88%) and more specific (94%) than native gliadin antibodies, its accuracy is still inferior to that of TGA (Lewis and Scott 2010).

2.4.1.2 Endomysial and reticulin antibodies

In 1971 Seah et al. discovered antibodies against connective tissue reticulin fibers (antireticulin antibodies, ARA) in the serum of patients with celiac disease and with dermatitis herpetiformis (Seah et al. 1971a, Seah et al. 1971b). ARA is best detected by an indirect immunofluorescent method using unfixed cryostat sections of rat liver and kidney as antigens. The sensitivity of the IgA-class ARA in untreated celiac disease patients varies from 65% to 97% and the specificity from 92% to 100% (Table 2).

Another type of connective tissue antibody, endomysium antibodies (EMA), was identified by Chorzelski et al in 1983. They detected a new IgA-class tissue antibody which reacted with the endomysium of smooth muscle, particularly of monkey esophagus (Chorzelski et al. 1983). EMA was strongly associated ARA (Hällström 1989). Later human umbilical cord replaced monkey esophagus as a substrate, as it was less expensive and an ethically more acceptable alternative (Ladinser et al. 1994). The EMA test has a sensitivity from 89 to 100% and a specificity from 90 to 100% (Table 2). EMA is somewhat age-dependent, and thus the sensitivity among children below two years of age is lower than that in older children (Bürgin-Wolff et al. 1991, Maglio et al. 2009). The major disadvantages of EMA as a screening tool are that EMA is expensive, requires much work and the reading of the results is subjective (Mäki 1995).

Table 2. Sensitivity and specificity of serum ARA and EMA in children with untreated celiac disease.

Reference	Study population				ARA		EMA	
	Number of patients	Mean/ *median age (years)	Number of controls	Mean/ *median age (years)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Mäki et al. 1984b	29	8.3	245	5.7	97	98	ND	ND
Lerner et al. 1994	28	9.6	41	8.4	65	100	97 (ME)	98 (ME)
Sacchetti et al. 1996	32	*5.0	42	*2.0	94	100	93 (UC) 97 (ME)	100 (UC) 100 (ME)
Kolho and Savilahti 1997	53	*6.5	114	ND	96	92	94 (UC)	100 (UC)
Bottaro et al. 1997	50	*2.5	25	*3.0	74	100	94 (UC) 96 (ME)	100 (UC) 96 (ME)
Sulkanen et al. 1998	136	*10.7[1]	207	*10.0[1, 2]	92	96	100 (UC)	100 (UC)
Vitoria et al. 1999	27	5.0	34	5.9	ND	ND	100 (ME)	100 (ME)
Wolters et al. 2002	52	4.0	49	5.1	ND	ND	92 (ME)	90 (ME)
Baudon et al. 2004	30	*2.0	116	ND	ND	ND	90 (ME)	100 (ME)
Lagerqvist et al. 2008	428	*1.3	87[nb]/129	*2.4[nb]/*3.1	ND	ND	89 (ME)	96 (ME)

[1] Study population includes also a group of adults

[2] Three control groups; 154 controls with clinically suspected celiac disease, median age 10.0 years; 32 controls with inflammatory bowel disease, median age 14.3 years; 21 controls with type 1 diabetes, median age 11.9 years

nb = no biopsy

ND = no data

UC = umbilical cord (human)

ME = monkey esophagus

2.4.1.3 Transglutaminase antibodies

In 1997 Dieterich et al. showed that tissue transglutaminase (TG) is the main if not the sole endomysial autoantigen recognized by the EMA. They demonstrated that the characteristic immunofluorescence staining of endomysial structures was lost if the serum of a patient with celiac disease was preincubated with TG (Dieterich et al. 1997). TG is a widely distributed calcium-dependent intracellular enzyme that catalyses the covalent and irreversible cross-linking of proteins and deamidates glutamyl donors. TG is released into the extracellular space because of mechanical or inflammatory stress, infection, or apoptosis (Schuppan 2000, Koning et al. 2005).

The first ELISA method for TGA analysis was developed using guinea pig liver tissue transglutaminase as the antigen (Sulkanen et al. 1998). However, the sensitivity and especially the specificity were lower than those in the EMA assay. A new test which used recombinant human tissue transglutaminase as antigen was soon developed and that test showed improved sensitivity and specificity which are comparable with those of EMA tests (Table 3). Later, Korponay-Szapó and colleagues showed that EMA, ARA and TGA were virtually identical (Korponay-Szabó et al. 2000, Korponay-Szabó et al. 2003)

Using TGA rather than EMA as a serological marker of celiac disease resolved some of the problems mentioned above concerning the EMA assay. An ELISA method for TGA measurement is relatively inexpensive, objective, and requires much less work than the indirect immunofluorescence method used for EMA.

Table 3. Sensitivity and specificity of serum TGA in children with untreated celiac disease.

References	Study population				TGA	
	Number of Patients	Mean/ *median age (years)	Number of Controls	Mean/ *median age (years)	Sensitivity (%)	Specificity (%)
Sulkanen et al. 1998	136	*10.7[1]	207	*10.0[1, 2]	95 (GP)	94 (GP)
Vitoria et al. 1999	27	5.0	34	5.9	100 (GP)	94 (GP)
Troncone et al. 1999	48	5.7	63	4.2	92 (GP)	98 (GP)
Vitoria et al. 2001	42	4.9	28	6.1	95 (RH)	100 (RH)
Wolters et al. 2002	52	4.0	49	5.1	96 (GP) 96 (RH)	92 (GP) 100 (RH)
Hansson et al. 2002	57	*4.0	24	*5.0	100 (RH)	96 (RH)
Baudon et al. 2004	30	*2.0	116	ND	93 (RH)	97 (RH)
Leach et al. 2008	32	8.5	44	9.0	93 (RH)	91 (RH)
Lagerqvist et al. 2008	428	*1.3	87[nb]/129	*2.4[nb]/*3.1	90 (RH)	98 (RH)

[1] Study population includes also a group of adults

[2] Three control groups; 154 controls with clinically suspected celiac disease, median age 10.0 years; 32 controls with inflammatory bowel disease, median age 14.3 years; 21 controls with type 1 diabetes, median age 11.9 years

nb = no biopsy
ND = no data
GP = guinea pig
RH = recombinant human

2.4.2 Small bowel biopsy

The diagnosis of celiac disease is based on histological evidence of small-intestinal villous atrophy with crypt hyperplasia and inflammation in the epithelium and lamina propria (Paulley 1954). In 1992, Marsh and colleagues described four stages of gradually developing mucosal changes characterizing the progression of changes related to the small intestine of patients with celiac disease (Marsh 1992). First, lymphocytic infiltrative lesion develops, in which the number of intraepithelial lymphocytes (IEL) is increased in otherwise normal mucosa (Marsh stage 1). Next, a hyperplastic lesion with normal villous architecture is seen, but in addition to an increased number of IELs, also the crypts show hyperplasia (Marsh stage 2). Then, a destructive lesion develops: villi are short and the crypts enlarged (Marsh stage 3). Later, the Marsh stage 3 lesions were subdivided into three categories: 3a for mild villous atrophy, 3b for marked villous atrophy and 3c for total villous atrophy (Oberhuber et al. 1999). Marsh stage 4 is very rare and may be found in some cases of complicated celiac disease. It consists of a hypoplastic lesion characterized by a flat mucosa but normal crypt height and normal IEL count (Marsh 1992).

Because the mucosal changes in celiac disease may be patchy (Bonamico et al. 2004), it is important to take several biopsies at different loci; the number of biopsies may be up to six (Green and Cellier 2007). Mucosal changes may occasionally be evident only in the bulb of the duodenum, and this emphasizes the importance of taking a biopsy also from the bulb (Bonamico et al. 2008, Rashid and MacDonald 2009).

2.5 Genetics of celiac disease

The genetic component of celiac disease is evident, since the prevalence among first degree relatives is almost 10% (Mäki et al. 1991, Högberg et al. 2003a), and the concordance rate between monozygotic twins exceeds 80%, while the concordance rate among dizygotic twins is as for siblings, 11% (Greco et al. 2002, Nisticò et al. 2006).

Celiac disease is strongly associated with major histocompatibility complex (MHC) genes located in the short arm of chromosome 6 (6p21), especially with alleles of the human leukocyte antigen (HLA) class II genes encoding the DQ2 and DQ8 molecules (Sollid et al. 1989). These molecules are expressed on antigen presenting cells, such as monocytes, dendritic cells, macrophages and B cells. The association is strongest with the HLA DQ2 heterodimer which is encoded by alleles DQA1*05 and DQB1*02. Over 90% of celiac disease patients carry these alleles compared to 20 – 30% found in the general population (Tosi et al. 1983, Sollid et al. 1989, Polvi et al. 1996). DQ2 can be formed either when both DQA1 and DQB1 alleles are located in one chromosome (DR3-DQ2- or DQA1*05-DQB1*02-positive subjects, *cis*), or when one allele is inherited from each of the parents (DR5-DQ7/DR7-DQ2 or DQA1*05-DQB1*0301/DQA1*0201-DQB1*02 heterozygous subjects, *trans*) (Sollid et al. 1989). Individuals who are homozygous for DQ2 have a higher risk for developing celiac disease than those who are heterozygous for DQ2 (Ploski et al. 1993). Most DQ2-negative celiac disease patients carry HLA DQ8 (DR4-DQ8) encoded by DQA1*03 and DQB1*0302 (Sollid et al. 1989). A few patients carry only half of the DQ2 heterodimer encoded either by DQA1*05 or DQB1*02 (Karell et al. 2003).

The genetic effect attributable to the HLA-alleles (locus named COELIAC1) seems to explain 30 – 53% of the genetic risk (Sollid and Lie 2005, Hunt and van Heel 2009), implying that there are other predisposing genes to celiac disease. So far, at least 12 other loci have been reported to associate with the development of celiac disease (Schuppan et al. 2009). These loci contain genes which contribute to the encoding of immunologically important proteins affecting the function of antigen-presenting cells or T cells (Jabri and Sollid 2009). However, the collective effect of these non-HLA genes seems to contribute only from 3 – 4% of the celiac disease heritability (Hunt and van Heel 2009).

2.6 Pathogenesis of celiac disease

Genes are necessary but not sufficient factors in the development of celiac disease. Some environmental triggers are needed, but so far the only proven environmental factor contributing to celiac disease is gluten. Gluten consists of storage proteins, prolamines and glutenins, which provide grain with the baking properties. The prolamine of wheat is gliadin, of barley hordein, of rye secalin and of oat avenin. These prolamines have high contents of glutamine and proline. The high content of proline makes these peptides resistant to degradation by intestinal proteases. In oat the amount of proline is approximately half of that in wheat, barley or rye, thus decreasing markedly its toxicity to

patients with celiac disease (Shewry and Tatham 1990, Vader et al. 2003, Wieser 2007). In celiac disease patients gluten-derived glutamine- and proline-rich peptides penetrate the intestinal epithelium (Shan et al. 2002). These peptides cause intestinal inflammation by synergism between innate and adaptive immunity (Figure 1).

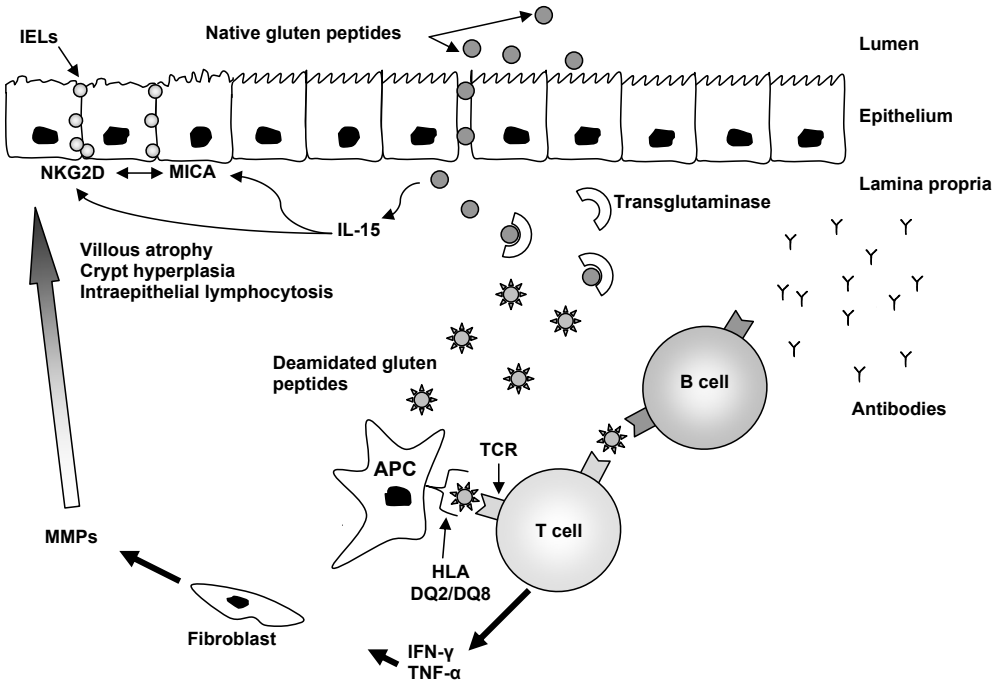

Figure 1. Simplified sketch of the mechanisms of pathogenesis in celiac disease. IEL = intraepithelial lymphocyte; IL-15 = interleukin 15; NKG2D = natural-killer cell receptor; MICA = major histocompatibility complex class I chain-related gene A; TCR = T cell receptor; APC = antigen presenting cell; INF-γ = interferon γ; TNF-α = tumor necrosis factor α; MMP = matrix metalloproteinase.

Various hypotheses have been presented to explain the mechanisms of how peptides reach the lamina propria of the gut. The paracellular pathway is one possibility. The increased permeability of the intestinal epithelium might be caused by the increased amount of zonulin which opens the tight junctions giving the toxic gluten-derived peptides access to the lamina propria (Fasano et al. 2000). It has been shown that an immunodominant 33 amino acid fragment of α-gliadin (p57-89) (Shan et al. 2002) uses a transcellular route by an interferon-γ-dependent transcytosis to travel to the lamina propria (Schumann et al. 2008). Harmful gluten peptides may also reach the lamina propria by retrotranscytosis of secretory IgA through transferrin receptor CD71 (Matysiak-Budnik et al. 2008).

Celiac disease-toxic gluten peptide, the non-immunodominant peptide 31-43 (p31-43), induces mucosal damage via a non-T-cell-dependent pathway (innate immunity). This peptide upregulates interleukin-15 production in the mononuclear and dendritic cells of

the lamina propria (Maiuri et al. 2000). Interleukin-15 (IL-15) is involved in activating and expanding intraepithelial lymphocytes (IELs) (Maiuri et al. 2001), in upregulation of the cell-surface antigen, MICA (Hüe et al. 2004), and in increased expression of the natural-killer cell receptor NKG2D, the receptor of MICA molecules in CD8 $\alpha\beta^+$ and $\gamma\delta^+$ T cells (Roberts et al. 2001). The interaction of MICA and its receptor may be partly responsible for enterocyte apoptosis and celiac disease-specific mucosal lesions (Hüe et al. 2004).

After penetration of the gut epithelium by the gluten peptides, especially the digestion-resistant 33-mer peptide fragment (p57-89), and arrival at the lamina propria the adaptive immune system is activated (Shan et al. 2002). The glutamine residues are deamidated by tissue transglutaminase into glutamic acid. This deamidation makes gliadin peptides negatively charged and even more immunostimulatory and thus more capable to bind to HLA DQ2 or DQ8 on the surface of the antigen presenting cells, such as dendritic cells (Molberg et al. 1998, Van De Wal et al. 1998, Arentz-Hansen et al. 2000). Gluten reactive $CD4^+$ T cells are then activated and proinflammatory cytokines, like interferon γ (IFNγ) (Nilsen et al. 1995) and tumor necrosis factor α (TNF-α), are produced. These proinflammatory cytokines stimulate, in turn, fibroblasts which produce tissue-damaging matrix metalloproteinases (MMPs), which degrade the mucosal matrix – villous atrophy ensues (Schuppan 2000).

B cells are activated and plasma cells begin to produce antibodies against tissue transglutaminase and gluten (Gianfrani et al. 2005). Antigliadin antibodies are produced by peripheral blood lymphocytes (Troncone et al. 1987), and celiac disease-specific TGA are produced locally in the mucosa of the small intestine (Marzari et al. 2001). The role of antibodies in the pathogenesis of celiac disease is controversial, but TGA has been shown to prevent the generation of transforming growth factor beta thus contributing to mucosal damage (Halttunen and Mäki 1999).

2.7 Environmental factors and celiac disease

It is well known that gluten causes the autoimmune-like process of celiac disease. However, evidence of other triggers, mostly related to early infant feeding, have been presented, as well. A longer breastfeeding period seems to reduce the risk of celiac disease (Auricchio et al. 1983, Greco et al. 1988, Fälth-Magnusson et al. 1996, Peters et al. 2001, Ivarsson et al. 2002), but this finding has been contested (Ascher et al. 1997, Norris et al. 2005). A prospective study suggested that early (before 3 months of age) as well as late introduction of gluten (after 7 months of age) increases the occurrence of celiac disease-associated antibodies (Norris et al. 2005). A low amount of dietary gluten intake might also prevent the development of celiac disease (Ascher et al. 1993, Mitt and Uibo 1998, Ivarsson et al. 2002). Many studies show that introduction of gluten to the infant's diet when infant is still in the age of being breastfed has markedly reduced the risk of celiac disease (Fälth-Magnusson et al. 1996, Peters et al. 2001, Ivarsson et al. 2002).

In addition to dietary factors, some infectious agents could increase the risk of celiac disease, e.g. adenovirus and rotavirus infections (Kagnoff et al. 1984, Kagnoff et al. 1987, Stene et al. 2006). On the other hand, a reduced amount of microbial contacts and infections in early childhood might increase the prevalence of celiac disease, which would provide support to the so called hygiene hypothesis (Kondrashova et al. 2008). The hygiene hypothesis claims that reduced exposure to microbes in the industrialized world leads to an imbalance in the immune system resulting in an increasing prevalence of autoimmune and allergic disorders (Bach 2002).

2.8 Treatment of celiac disease

The only known effective treatment for celiac disease is a gluten free diet, which has to be continued throughout life. All gluten containing grains, i.e., wheat, rye and barley, must be strictly avoided. Because oat belongs to a different grass species than the domestic grains of Finland, oat is non-toxic and tolerated by patients with celiac disease (Janatuinen et al. 1995) even in the long term (Kemppainen et al. 2007). Also children with celiac disease tolerate oat in their diet (Högberg et al. 2004, Holm et al. 2006).

Adherence to a strict gluten free diet results in the disappearance of celiac disease-associated antibodies within a few months to a year (Bürgin-Wolff et al. 2002). For monitoring of diet adherence celiac disease-associated antibodies should be assessed, although they are not completely reliable markers of gluten free diet (Kaukinen et al. 2002, Hill et al. 2005b). Small-intestinal biopsy samples usually show histological remission in most children within two years of gluten free diet but in adults recovery may take twice as long (Wahab et al. 2002, Rubio-Tapia et al. 2010).

Several studies have proven that a diet entirely free of gluten is hard to follow and easily leads to non-compliance. Recent studies indicate that compliance ranges from 42% to 91% of the affected subjects (Hall et al. 2009). In an Italian study of 22 screening-detected, asymptomatic adolescents with celiac disease, only 23% followed closely a gluten free diet, whereas 68% of subjects diagnosed because of symptoms followed the diet appropriately (Fabiani et al. 2000). In a Finnish study, compliance was high and did not differ between screening-detected and symptom-detected patients 14 years after diagnosis (96% and 93% were compliant, respectively) (Viljamaa et al. 2005a). Studies concerning the effect of age at diagnosis and compliance to gluten free diet are controversial. Högberg and associates showed that adults complied at a rate of 80%, when the diagnosis was made before the age of 4 years and 36% when made later in life (Högberg et al. 2003b). On the other hand, Ciacci and colleagues reported that patients diagnosed after 20 years of age had better dietary compliance (82%) than those who had been diagnosed at an earlier age (62%) (Ciacci et al. 2003).

Since adhering to a gluten free diet is challenging, there have been efforts to find other treatment options for patients with celiac disease. Our improved understanding of the pathophysiological events of celiac disease has enabled researchers to explore new

therapies. There are attempts to modify the harmful gluten peptides of grains through transgenic technology or selective breeding (Vader et al. 2003, Molberg et al. 2005). However, these modifications still require that the patient modifies his or her diet. Recently, targeted nondietary therapies have been introduced. One trend is to eliminate the immunodominant gliadin peptides by exogenous endoproteases (Gass et al. 2007, Mitea et al. 2008). Another possibility is to introduce interactions with the mechanisms gluten is involved in patients with celiac disease. Examples of such therapies are tissue transglutaminase inhibitors (Choi et al. 2005), HLA DQ2 antagonists (Xia et al. 2007), zonulin receptor antagonist (Paterson et al. 2007), and anti-interferon γ antibody (Costantino et al. 2008). The safety of these new therapies has not been established and need, of course, extensive nonclinical and clinical study before they can be introduced for daily use.

2.9 Celiac disease in combination with other autoimmune diseases

Celiac disease is often associated with other autoimmune diseases. The prevalence of celiac disease in patients with type 1 diabetes is 4 – 10% (Collin et al. 2002), with autoimmune thyroid diseases 2 – 5% (Collin et al. 1994, Berti et al. 2000, Meloni et al. 2001), with Sjögren's syndrome 5 – 15% (Iltanen et al. 1999, Szodoray et al. 2004) and with Addison's disease 3 – 8% (Myhre et al. 2003, Biagi et al. 2006, Betterle et al. 2006). The reason for this comorbidity may lie in a common genetic background or to similar immune mediated disease mechanisms. Ventura and colleagues showed that the duration of gluten exposure was related to the prevalence of autoimmune diseases in celiac disease patients (Ventura et al. 1999). If the diagnosis of celiac disease had been made before age 2 years, the prevalence of autoimmune disorders was 5.1% but if it was made after age 10 the prevalence was 23.6%. However, the effect of the duration of gluten exposure has not been confirmed in other studies, although older age at diagnosis does increase the amount of associated autoimmune diseases (Sategna Guidetti et al. 2001, Viljamaa et al. 2005b).

2.9.1 Celiac disease in combination with type 1 diabetes

Celiac disease has long been known to associate with type 1 diabetes (Walker-Smith and Grigor 1969, Visakorpi 1969). Undiagnosed celiac disease may cause abdominal pain, malabsorption, diarrhea, stunted growth, and unstable diabetes and it may also increase the risk of hypoglycemia in patients with diabetes (Thain et al. 1974, Mohn et al. 2001, Narula et al. 2009). However, under these circumstances celiac disease is most often symptom-free (Mäki et al. 1984a, Savilahti et al. 1986, Barera et al. 2002). Usually type 1 diabetes is diagnosed first and celiac disease develops typically 5 – 6 years later (Saukkonen et al. 1996, Barera et al. 2002, Cerutti et al. 2004, Larsson et al. 2008). However, in some cases celiac disease is diagnosed first, as individuals with celiac disease have a two- to threefold risk of developing type 1 diabetes when compared to individuals in the general population (Ludvigsson et al. 2006). When celiac disease is

Table 4. Prevalence of celiac disease in children with type 1 diabetes according to studies published since 2000.

References	Country	Number of Patients	Median Age (years)	Antibodies Screened	Celiac Disease Diagnosed Before Screening	Screening Positivity	Confirmed Celiac Disease by Biopsy	Prevalence of Celiac Disease
Kordonouri et al. 2000	Germany	520	14.2	TGA EMA AGA-IgA	0	23 (4.4%)	9	1.7%
Agardh et al. 2001	Sweden	165	Follow-up	TGA EMA AGA-IgA AGA-IgG	3	9 (5.5%)	6	5.5%
Peretti et al. 2004	France	284	Follow-up	TGA EMA AGA-IgA AGA-IgG	2	10 (3.5%)	9	3.9%
Cerutti et al. 2004	Italy	4322	11.8	EMA AGA-IgA AGA-IgG	34	no data	258	6.8%
Hansen et al. 2006	Denmark	269	10.9	TGA EMA AGA-IgA AGA-IgG	5	33 (12.3%)	28	12.3%
Poulain et al. 2007	France	950	Follow-up	TGA EMA ARA AGA-IgA AGA-IgG	1	14 (1.5%)	14	1.6%
Salardi et al. 2008	Italy	331	Follow-up	EMA	2	29 (8.8%)	22	6.6%
Larsson et al. 2008	Sweden	300	Follow-up	EMA	2	27 (9.0%)	27	9.7%

diagnosed in children with type 1 diabetes, introduction of a gluten free diet improves the diabetes-related metabolic control and growth (Amin et al. 2002, Saadah et al. 2004). This observation has not been universal: some studies have reported a contradictory effect or no effect at all (Lorini et al. 1996, Kaukinen et al. 1999). Frequent screening for celiac disease antibodies is recommended for patients with type 1 diabetes (Saukkonen et al. 1996, Larsson et al. 2008, Fröhlich-Reiterer et al. 2008). The overall prevalence of celiac disease in children with type 1 diabetes ranges from 1.6% to 12.3% (Table 4).

There is a paucity of studies on the prevalence of autoimmunity in subjects before type 1 diabetes and/or celiac disease emerges. Two cross-sectional studies, one from Great Britain and one from Germany, the prevalence of diabetes-associated autoantibodies was reported in a group of non-diabetic first degree relatives to patients with type 1 diabetes. In Great Britain the prevalence was 11.6% and in Germany 12.9%. The prevalence of celiac disease-associated antibodies was 7.0% and 7.3%, respectively, and a total of 0.7% and 0.9% of the individuals were simultaneously positive for type 1 diabetes-associated antibodies and celiac disease associated-antibodies (Williams et al. 2001, Jaeger et al. 2001). In a prospective observational study from USA (the Diabetes Autoimmunity Study in the Young, DAISY), only 0.2% of the children had both diabetes- and celiac disease-associated antibodies (Norris et al. 2005). In a German prospective follow-up study, the BabyDiab study, the cumulative risk of developing TGA by age 8 years was 4.9% and for developing diabetes-associated antibodies 7.8%. Only 0.2% of the children had both diabetes-associated and celiac disease-associated antibodies by age 8 years. The diabetes-associated autoantibodies developed first (Hummel et al. 2007) (Table 5).

The comorbidity of these two chronic diseases is usually explained by shared HLA alleles and other predisposing non-HLA gene regions (Sollid et al. 1989, Smyth et al. 2008). Also, both diseases need some environmental triggers, e.g., gluten, and some triggers may play a role in the development of both diseases (Norris et al. 2003, Ziegler et al. 2003, Norris et al. 2005). However, removal of dietary gluten after type 1 diabetes has developed, does not seem to influence the course of diabetes neither in the short nor in the long term. In a study involving 7 children with type 1 diabetes-associated antibodies, gluten withdrawal for 12 months followed by re-introduction had no effect on the appearance of diabetes-associated autoantibodies or the progression to overt type 1 diabetes (Hummel et al. 2002, Füchtenbusch et al. 2004).

2.9.1.1 Epidemiology of type 1 diabetes

The global incidence of type 1 diabetes is increasing, especially among children under 5 years of age (Onkamo et al. 1999, EURODIAB ACE Study Group 2000), but the incidence varies greatly: the incidence in China and Venezuela is the lowest, in Finland and Sardinia the highest (Onkamo et al. 1999). In 1953 the incidence of type 1 diabetes in Finland was 12/100 000 per year (Somersalo 1954), in 2005 it was 64.2/100 000 (Harjutsalo et al. 2008).

Table 5. Prospective follow-up studies of type 1 diabetes and celiac disease.

Stydy	Country	Recruitment	Inclusion criteria	Follow-up started	Follow-up schedule	T1D-associated antibodies	CD-associated antibodies
DAISY	USA	1993 – 2004	• specific HLA-alleles screened from the general population • siblings of above mentioned group • first-degree relatives of patients with T1D	• from birth • from different ages • from birth to 8 years	9, 15 and 24 months, then annually	IAA, IA-2A, GADA	TGA
BabyDiab	Germany	1989 – 2000	• offspring of a parent with type 1 diabetes	• < 3 months	9 months and 2, 5, 8, 11 and 14 years	IAA, IA-2A, GADA	TGA, EMA, AGA-IgG, AGA-IgA
DIPP	Finland	1994 –	• specific HLA-alleles screened from the general population • siblings of above mentioned group	• from birth • from different ages	3 to 6 months until 2 years, 6 to 12 months thereafter	ICA, IAA, IA-2A, GADA	TGA, EMA, ARA, AGA-IgG, AGA-IgA

T1D = type 1 diabetes
CD = celiac disease

2.9.1.2 Pathogenesis of type 1 diabetes

In the developed countries, type 1 diabetes is the most common autoimmune disease in children and adolescents. The disease progresses by immune-mediated destruction of insulin producing beta cells in the pancreatic islets (Atkinson and Maclaren 1994). There is a strong genetic susceptibility for type 1 diabetes, but regulatory genetic mechanisms are also able to protect from the disease. The most important contributing genes are located in the HLA class II locus in chromosome 6 (6p21) (Todd et al. 1987). Most patients with type 1 diabetes carry the DR4-DQ8 (DQB1*0302/x, x ≠ *02, *0301, *0602, or *0603) or DR3-DQ2 (DQA1*0501-DQB1*02) haplotype and those who are heterozygous for DR4-DQ8/DR3-DQ2 (DQB1*02/*0302) are at particularly high risk (Redondo and Eisenbarth 2002). In the Finnish population, the disease risk by age 15 is approximately 7% among persons with the high risk genotype (DR4-DQ8/DR3-DQ2), and 2 – 3%, with the moderate risk genotype (DR4-DQ8 or DR3-DQ2) is (Ilonen et al. 1996, Hermann et al. 2004). In addition to the HLA-loci, numerous other genes or genetic regions affect the susceptibility to type 1 diabetes (Barrett et al. 2009).

Many different factors either alone or in combination may trigger the autoimmune process leading to type 1 diabetes in genetically susceptible individuals. The most probable environmental culprits involve enteroviruses or other infectious agents, and dietary factors, e.g., cow's milk proteins, gluten, or early introduction of foreign proteins to the infant's feeding (Peng and Hagopian 2006). Before type 1 diabetes becomes overt, there is a preclinical phase that lasts from months to years when diabetes-associated autoantibodies are detectable in the blood.

2.9.1.3 Autoantibodies in type 1 diabetes

The first autoantibody described as being related to type 1 diabetes was the islet cell autoantibody (ICA) (Bottazzo et al. 1974). ICA is directed against cytoplasmic components of the islet cells (Genovese et al. 1992). Since then, autoantibodies against biochemically characterized target antigens have been detected. Insulin autoantibodies (IAA) (Palmer et al. 1983), autoantibodies against glutamic acid decarboxylase (GADA) (Baekkeskov et al. 1990) and autoantibodies against protein tyrosine phosphatase-related IA-2 protein (IA-2A) (Rabin et al. 1994) have been identified and are used for prediction of type 1 diabetes. The most recent autoantibody related to type 1 diabetes is targeted against zinc transporter 8 (ZnT8), an islet β-cell secretory granule membrane protein (Wenzlau et al. 2007, Yang et al. 2010).

Type 1 diabetes-associated autoantibodies are useful markers of preclinical disease and identify individuals at risk of type 1 diabetes (Bingley et al. 1994, Ziegler et al. 1999). Many prospective studies have increased our knowledge about the natural course of preclinical type 1 diabetes. The Finnish Type 1 Diabetes Prediction and Prevention Study (DIPP) is a population based prospective cohort study which screens the genetic risk for type 1 diabetes by sampling cord blood. Individuals at risk are followed up at 3- to 12-month intervals by autoantibody analysis (Kupila et al. 2001). In the American

Diabetes Autoimmunity Study in the Young (DAISY) two different groups of children are investigated: children with first degree relatives with type 1 diabetes and infants who by screening have been identified as being at increased genetic risk for type 1 diabetes (Rewers et al. 1996). In the German BabyDiab study children who have one parent with type 1 diabetes are followed up, but less frequently than in DIPP and DAISY (Table 5).

When present in serum alone, even persistently, most autoantibodies are not associated with an increased risk of type 1 diabetes. However, if the child expresses multiple autoantibodies, i.e., simultaneously positive for two or more diabetes-associated autoantibodies, the risk of overt disease increases markedly (Siljander et al. 2009). In young children, IAA and ICA are usually the first autoantibodies to appear. Recent data suggests that 95% of all IAA, GADA and IA-2A seroconversions occur in a cluster, 12 months before or 8 months after the ICA seroconversion. IA-2A usually tests positive later than the other. However, the rate of seroconversion to antibody positivity is varies highly (Kupila et al. 2002, Siljander et al. 2009).

3. AIMS OF THE STUDY

The aims of the present study were to describe the natural course of celiac disease and celiac disease-associated antibodies before diagnosis and to compare the appearance of celiac disease-associated antibodies and type 1 diabetes-associated autoantibodies. The specific objectives were:

1. to determine at what age tissue transglutaminase autoantibodies emerge in children with increased genetic risk to celiac disease and who were followed-up frequently since birth or early childhood (I)

2. to explore the natural history of antibodies against tissue transglutaminase, endomysium, reticulin and gliadin in children carrying a HLA-conferred risk for celiac disease and observed frequently from birth (II)

3. to compare the ages and sequence in which antibodies associated with celiac disease and type 1 diabetes appear and overt diseases develop in children with HLA-conferred susceptibility to both diseases (III)

4. RESEARCH DESIGN AND METHODS

All three original studies (I-III) of this thesis involved subjects participating in the Finnish Type 1 Diabetes Prediction and Prevention Study (DIPP).

4.1 Study design

The DIPP study is an ongoing population-based prospective follow-up study aiming to explore the natural course of preclinical type 1 diabetes. The DIPP study was launched in November 1994 in Turku, in September 1995 in Oulu and in October 1997 in Tampere. The university hospitals in these three cities cover an annual birth rate of 11 000 representing nearly 20% of all births in Finland. The majority (>90%) of the parents to the newborn babies born were and are willing to participate in cord blood screening to assess HLA-conferred susceptibility to type 1 diabetes. Frequent follow-up is offered to the children with an increased risk for type 1 diabetes who carry high risk alleles (HLA DQB1*02/*0302) or moderate risk alleles (HLA DQB1*0302/x, x ≠ *02, *0301, *0602 and males born in Turku with DQA1*05/y-DQB1*02/z, y ≠ *0201, z ≠ *0301, *0302, *0602, *0603). In Turku, the follow-up visits are at 3-month intervals for the first 2 years of life and at 6-month intervals thereafter, and in Oulu and Tampere at 3, 6, 12, 18 and 24 months and annually thereafter. The follow-up continued until the child progressed to overt type 1 diabetes or until the end of the defined follow-up period was reached (Kupila et al. 2001).

Genetic testing is also offered to the siblings of the at-risk newborns participating in the follow-up (index children). The siblings with high or moderate risk alleles are invited to the follow-up.

A venous blood sample is drawn at every follow-up visit for analysis of four type 1 diabetes-associated autoantibodies. To document when diabetes autoimmunity begin, islet cell autoantibodies (ICA) are first measured from every blood sample drawn. If the sample is ICA positive, autoantibodies against biochemically characterized autoantigens insulin (IAA), glutamic acid decarboxylase (GADA) and protein tyrosine phosphatase-related IA-2 protein (IA-2A) are also analyzed in all samples drawn from that child since birth. All four diabetes-associated autoantibodies are analyzed in all samples drawn from the children born on or after January 1, 2003. If diabetes-associated autoantibodies are detected, follow-up visits are scheduled at 3-month intervals thereafter in every study center.

The study exploring the natural history of celiac disease was launched in 1999 and all children with HLA DQB1*02/DQB1*0302 and boys with HLA DQA1*05/y-DQB1*02/z, (y ≠ *0201, z ≠ *0301, *0302, *0602, *0603) were included. IgA-class tissue transglutaminase autoantibody (TGA) was used as the primary marker of celiac disease autoimmunity. TGA was first measured in the samples drawn during the year

2000 from all children with HLA-conferred diabetes and celiac disease susceptibility. Later, TGA was first analyzed at age 1 year in the children with genetic susceptibility to celiac disease. When a child became TGA positive, antibodies against endomysium (EMA), reticulin (ARA) and gliadin (AGA-IgA and AGA-IgG) were analyzed also in the child's all previous and forthcoming samples. TGA was also measured from the last available samples from the six children who withdrew from the follow-up before the year 2000. The age at seroconversion to antibody positivity was defined as the age when the first positive sample was drawn, irrespective of the slight variation in the intervals between the last autoantibody-negative and the first autoantibody-positive sample.

The diagnosis of type 1 diabetes was based on the WHO criteria (World Health Organization/ Department of Noncommunicable Disease Surveillance 1999). Duodenal biopsies were recommended for all TGA-positive children. If biopsies showed villous atrophy, celiac disease was diagnosed and gluten-free diet was instituted.

4.2 Subjects

Table 6. Characteristics of the study subjects

	Study I	Study II	Study III
Target group	Index children and siblings	Index children	Index children
Subjects born	11/1994 – 12/1999 [i] 09/1980 – 08/1997 [s]	11/1994 –12/2002	11/1994 – 12/2005
Genetic risk analyzed, n	41 127 38 781 [i] 2 346 [s]	64 467	100 846
Genetic risk found, n	6 968 5 854 [i] 1 114 [s]	10 596	15 458
Continued in follow-up, n (date)	4 424 (12/2001) 3 583 [i] 841 [s]	5 616 (12/2003)	7 126 (3/2007)
Antibodies measured, n	1 101 906 [i] 195 [s]	1 320	2 052
Screening	TGA	TGA EMA ARA AGA-IgA AGA-IgG	TGA EMA ARA AGA-IgA AGA-IgG ICA IAA GADA IA-2A
Follow-up period	11/1994 – 12/2002	11/1994 –12/2003	11/1994 – 03/2007
Follow-up of antibody positive children	11/1994 – 06/2003	11/1994 –08/2004	11/1994 – 03/2007
Gender, n			
Male	659 556 [i] 103 [s]	877	1 332
Female	442 350 [i] 92 [s]	443	720

[i] = index children (children followed from birth)
[s] = siblings (older siblings of an index child)

4.3 Methods

4.3.1 Genetic screening

The HLA alleles were analyzed from cord-blood spots dried on filter paper using a semi-automated technique (Nejentsev et al. 1999). DNA sequences extracted from the blood spots were PCR amplified, then hybridized in solution with allele-specific, lanthanide chelate-labelled oligonucleotide probes, and the hybridization products were measured using time-resolved fluorometry. Six predisposing and protective HLA DQB1 alleles (*02,

*0301, *0302, *0602, *0603 and *0604) were analyzed, and children with DQB1*02/DQB1*0302 and DQB1*0302/x (x ≠ DQB1*02, DQB1*0301 or DQB1*0602) were invited for follow-up. In addition, HLA DQA1*0201 and*05 alleles were analyzed in boys with DQB1*02/z (z ≠ DQA1*0201-DQB1*02, DQB1*0301, *0302, *0602 or *0603) in Turku, as the risk of type 1 diabetes is higher in Turku among boys than girls carrying the DR3-DQ2 haplotype. The celiac disease-associated DQA1 alleles were analyzed also from children with the DQB1*02/*0302 genotype.

4.3.2 Type 1 diabetes-associated autoantibodies

Islet cell antibodies (ICA) were quantified by a standard indirect immunofluorescence method on sections of frozen human pancreas from a blood group O donor (Bottazzo et al. 1974). The end-point dilution titers of ICA-positive samples were recorded and the results expressed in Juvenile Diabetes Foundation (JDF) units. The detection limit of the assay was 2.5 JDF units. The assay sensitivity was 100% and specificity 98% in the fourth round of the International Workshop on the Standardization of the ICA Assay (Lernmark et al. 1991).

The antibodies to glutamic acid decarboxylase (GADA) were measured with specific radiobinding assays, as described (Savola et al. 1998a). The antibody results were expressed in relative units (RU) based on a standard curve run on each plate. The cut-off limit for GADA positivity was 5.36 RU, representing the 99th percentile in a group of 373 healthy Finnish children and adolescents. The disease sensitivity of the assay was 82% and the specificity 96%, based on the Autoantibody Standardization Program (DASP) workshop in 2005. All samples with antibody levels between the 97.5th and 99.5th percentiles were retested to confirm the antibody status.

IA-2A were quantified using a modification (Savola et al. 1998b) of a radioligand method (Bonifacio et al. 1995). The cut-off limit for IA-2A positivity was set at 0.43 RU, which represents the 99th percentile of 374 non-diabetic Finnish children and adolescents. The disease sensitivity of this assay was 72% and the specificity 100% in the 2005 DASP workshop.

Insulin autoantibodies (IAA) were analyzed with a radiobinding microassay using 5 µl of serum as described (Ronkainen et al. 2001). The cut-off limit for IAA positivity was 1.56 RU, representing the 99th percentile in 371 Finnish children and adolescents. The disease sensitivity of the assay was 58% and the specificity 98% in the 2005 DASP workshop.

4.3.3 Celiac disease-associated antibodies

Serum samples were stored at -70ºC until analyzed in duplicate in 1:100 dilution. Serum IgA antibodies against tissue transglutaminase were measured using a recombinant human TGA kit (Celikey™; Pharmacia Diagnostics, Freiburg, Germany) with values 5–8 U/ml regarded as equivocal and > 8 U/ml as positive, as suggested by the manufacturer. To analyze TGA-IgG in children with IgA deficiency a TGA-IgG kit from the same manufacturer was used.

Serum EMA-IgA were determined by an indirect immunofluorescence method using human umbilical cord as substrate (Ladinser et al. 1994). Serum ARA-IgA were measured

by an indirect immunofluorescence method using unfixed rat stomach, liver and kidney as substrate (Mäki et al. 1984b). Serum AGA-IgG and AGA-IgA were analyzed by ELISA (Savilahti et al. 1983).

ELISA was used to measure serum IgA concentration and IgG class antigliadin antibodies in children with IgA values < 0.05 g/l (Savilahti et al. 1983).

4.3.4 Small bowel biopsies

Upper gastrointestinal videoendoscopy under anesthesia with duodenal biopsies was recommended for children positive for TGA. If the child reseroconverted to TGA negativity in a follow-up sample taken before endoscopy, the procedure was withheld, but the follow-up and regular analysis of antibodies continued. The biopsy specimens were evaluated for villous morphology, crypt hyperplasia and intraepithelial lymphocytes (IEL) at the Departments of Pathology of the three universities of this study. The specimens were assessed according to a modified Marsh score (Oberhuber 2000) as 0 (no inflammation), 1 (normal villi but increased number of IEL), 2 (normal villi but increased number of IEL and crypt hyperplasia), 3a or 3b (mild or marked villous flattening, respectively) or 3c (flat mucosa). Scores 0 and 1 were classified as normal, and scores 2, 3a, 3b and 3c as diagnostic for celiac disease. If celiac disease was diagnosed the child and the child's parents were provided counseling aiming at exclusion of gluten from the child's diet.

4.3.5 Ethics

The Ethics Committees of the participating three university hospitals approved the study. Written informed consent was obtained of the parents for the analysis of HLA-alleles, for type 1 diabetes-associated and celiac disease-associated antibody analysis and for the small-intestinal biopsies required of children with positive celiac disease-associated antibodies. The child's consent was also requested for the small-intestinal biopsies if the child's age exceeded 7 years.

4.3.6 Statistics

In studies I and II descriptive data are shown as mean values with standard deviation (SD) and ranges. In study III descriptive data are shown as median values and ranges. In study II the ages of children at the time of seroconversions to positivity for the different antibodies were compared with Mann-Whitney-Wilcoxon Signed Rank Test for paired samples. A P value < 0.05 was regarded as implying a significant difference between the groups. In study III Wilcoxon's test was used to compare differences between the median ages at seroconversion and Cox's regression analysis to compare the time to development of diabetes-associated and celiac disease-associated antibodies. Tests were considered as implying significant between the study groups, if the two-sided P value was < 0.05. The statistical analyses were performed using SAS version 8.2 (Studies I and II) and SAS version 9.2 (SAS Institute, Cary, NC) (Study III).

5. RESULTS

The follow-up of the study cohorts, which comprises the main part of the three original papers and this thesis, started in November 1994. In the first study (**I**), 906 index children were followed-up from birth until the end of June 2003, when the median age of the children was 3.6 years. Altogether 29 children seroconverted to TGA positivity during this follow-up period; in addition, two IgA-deficient children seroconverted to AGA-IgG positivity, which also suggested commencement of celiac disease autoimmunity. Consequently, 31 index children (3.4%) showed celiac disease autoimmunity at the end of this follow-up period. Study **I** also provided follow-up data covering 195 older siblings of the index children; 6 of them (3.1%) seroconverted to TGA positivity. Only three of the six siblings with TGA positivity seroconverted during the follow-up, the other three siblings had a positive or equivocal value already in the first sample.

Altogether 1320 index children were included in the second study (**II**), in which the cohort children were followed-up until the end of August 2004. During that study period, 51 children (3.9%) seroconverted to TGA positivity or AGA-IgG positivity, if the child was IgA-deficient (n = 2). The median age of the children at the end of this follow-up (cohort **II)** was 4.0 years.

At the end of March 2007, when the follow-up period of the third study (**III**) ended, the median age of the children was 5.5 years. Altogether 2052 index children were included in this analysis; 88 of them (4.3%) had seroconverted to positivity for TGA or were IgA-deficient and had seroconverted to AGA-IgG positivity (n = 2) (Table 6 and Table 7).

5.1 Appearance of celiac disease-associated antibodies (I, II, III)

All children who seroconverted to TGA positivity did so only after age 1.0 year. This was confirmed in all three studies, as in study **I** the youngest child who seroconverted did so at the age of 1.3 years, and in studies **II** and **III** the seroconversions to TGA positivity occurred at age 1.0 years or later. After the age of 1 year, the annual seroconversion rate was continuously approximately 1% in all three cohorts. The median age of the children in cohort **III** at seroconversion to TGA positivity was 3.0 (range 1.0 – 9.0) years. The older siblings in the families of the index children followed-up from the median age of 7.3 (2.8 – 20.3) years serovonverted to positivity for TGA at a median age of 8.3 (5.8 – 10.3) years (**I**).

The median ages at seroconversion to EMA and ARA positivity were 3.0 (1.0 – 9.0) years and 3.0 (1.0 – 10.5) years, respectively, i.e., similar to the ages at seroconversion to TGA positivity. However, the age at seroconversion to AGA-IgA and in particular to AGA-IgG positivity showed a different pattern: the youngest children seroconverting to AGA-IgG positivity did so already at age 0.5 years. The median age at seroconversion to AGA-IgG positivity was 1.5 (0.5 – 11.5) years, also markedly less than the median ages of seroconversion to TGA, EMA or ARA positivity ($p < 0.001$).

The age at seroconversion to IgA class AGA positivity was similar to that of the children who seroconverted to IgG class AGA positivity, as the youngest children seroconverted to AGA-IgA positivity already at age 0.6 years. However, the median age at AGA-IgA seroconversion was 3.0 (0.6 – 10.0) years, i.e., later than seroconversion to AGA-IgG positivity (median age 1.5 (0.5 – 11.5) years) (Table 7 and Figure 2).

AGA-IgG was the first antibody to appear in 21 of the 49 children with an HLA-conferred genetic celiac disease susceptibility who seroconverted during the follow-up (**II**). An additional 13 children seroconverted to AGA-IgG positivity at the same time as to some other celiac disease-associated antibody (TGA, EMA, ARA or AGA-IgA). Seven children seroconverted to AGA-IgG positivity at a median of 0.5 (0.3 – 2.1) years later than they seroconverted to positivity to the first-appearing antibody. The remaining 8 children seroconverted to positivity against one or more of the other celiac disease-associated antibodies, but were permanently AGA-IgG negative. This result was confirmed in a larger cohort of children in study **III**, where 66 of the 86 TGA positive children seroconverted first to AGA-IgG positivity alone or in combination with another celiac disease-associated antibody. In 40 of the 66 children AGA-IgG first seroconverted alone. The median time from AGA-IgG seroconversion to TGA, EMA, ARA or AGA-IgA positivity was 0.8 (0.2 – 6.1) years.

Table 7. Number of study subjects, proportions of antibody positive children, median ages at seroconversion to positivity for celiac disease-associated antibodies, and proportion and median ages of children with confirmed celiac disease in the three study groups.

	Study I*	**Study II**	**Study III**
n	906	1320	2052
Age at the end of follow-up Years, median (range)	3.6 (1.0 – 8.1)	4.0 (1.0 – 9.5)	5.7 (1.0 – 12.3)
Positive for TGA (or AGA-IgG, if IgA-deficient) (n, %)	31 (3.4%)	51 (3.9%)	88 (4.3%)
Age at seroconversion Years, median (range)			
TGA	3.0 (1.3 – 6.5)	3.0 (1.0 – 7.0)	3.0 (1.0 – 9.0)
EMA	NA	3.0 (1.0 – 7.0)	3.0 (1.0 – 9.0)
ARA	NA	2.6 (1.0 – 7.0)	3.0 (1.0 – 10.5)
AGA-IgA	NA	2.5 (0.6 – 7.0)	3.0 (0.6 – 10.0)
AGA-IgG	NA	1.5 (0.5 – 6.6)	1.5 (0.5 – 11.5)
Biopsy-confirmed celiac disease (n, %)	11 (1.2%)	22 (1.7%)	44 (2.1%)
Age at diagnosis Years, median (range)	3.5 (2.4 – 6.0)	3.6 (2.4 – 7.1)	4.3 (1.6 – 9.8)

* only index children of Study I are included

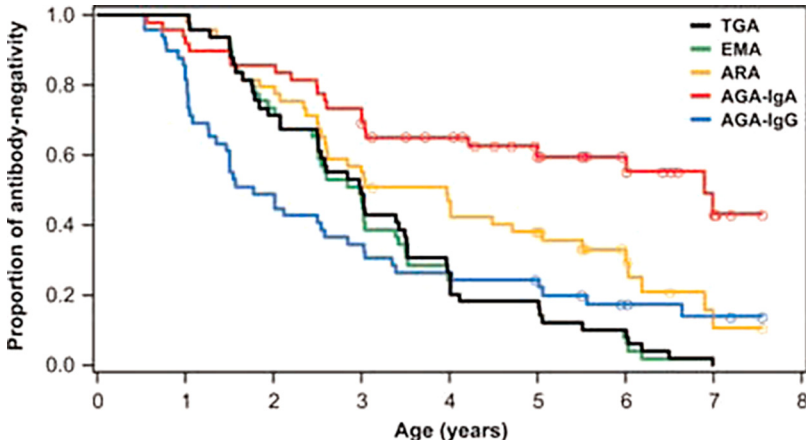

Figure 2. Life-table analysis of the emergence of five celiac disease-associated antibodies.

5.2 Transient and fluctuating antibodies (I, II, III)

A large proportion of children with celiac disease-associated antibodies spontaneously lost the antibodies without any dietary interventions, first shown in a group of 35 TGA positive children (**I**). Of these 35 children 18 (51%) first seroconverted to TGA positivity, but further follow-up documented that the antibodies disappeared spontaneously after one to three positive samples. Three of these 18 children first seroconverted to TGA positivity after which the antibodies disappeared, reappeared and in two children disappeared again. However, children with persistent TGA had higher mean and peak TGA values [103 (± 198; 9–1583) U/ml and 248 (± 364; 38–1583) U/ml] than the children who had transient TGA [24 (± 27; 8–113) U/ml and 28 (± 31; 8–113) U/ml] or fluctuating TGA [27 (± 30; 9–88) U/ml and 39 (± 42; 14–88) U/ml] during the follow-up ($p < 0.001$).

The phenomenon of spontaneously disappearing celiac disease-associated antibodies was not restricted to TGA, as EMA, ARA, AGA-IgG and AGA-IgA were also occasionally transiently positive (**II**). Of the 49 children who tested positive at least once for TGA, 24 (49%) became spontaneously antibody-negative. During follow-up, 15 of these 24 children were positive for two to five types of antibodies, which all later disappeared completely. Transient or fluctuating antibody values were seen in 45% of the EMA positive (22 of 49), 43% of the ARA positive (16 of 37), 41% of the AGA-IgA positive (9 of 22) and 32% of the AGA-IgG positive (13 of 41) subjects. This phenomenon was also observed in 86 children in study **III,** as shown in Figure 3. Although 23 of the 86 TGA positive children were TGA positive in only one sample, all except one showed positivity in the very same sample also for AGA-IgG, AGA-IgA, EMA and/or ARA.

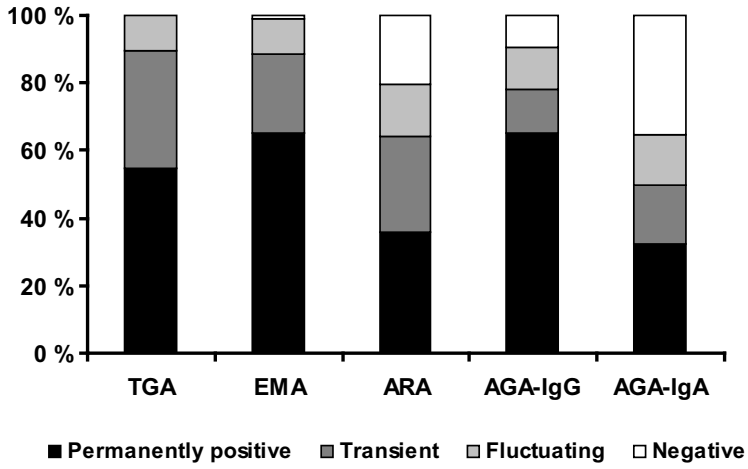

Figure 3. Proportion of children with permanent, transient or fluctuating positivity for TGA, EMA, ARA, AGA-IgG or AGA-IgA among the 86 study children (**III**).

5.3 Small bowel biopsies and confirmed celiac disease (I, II, III)

In study **I**, 31 children were positive for TGA, or positive for AGA-IgG, if they were IgA deficient. Upper gastrointestinal videoendoscopy was performed on 16 of the 31 TGA positive children; 10 had villous atrophy. As one child developed skin biopsy-confirmed dermatitis herpetiformis, altogether 11 index children (1.2%) had biopsy-proven celiac disease; the diagnosis was made at a median age of 3.5 (2.4 – 6.0) years. In addition, duodenal biopsies were taken of 5 of the 6 TGA positive older siblings; celiac disease was found in 3.

Duodenal (or skin) biopsies were taken of 29 of the 51 children who tested positive for TGA (AGA-IgG, if IgA-deficient). Celiac disease or dermatitis herpetiformis was confirmed in 22 children (1.7%); the median age was 3.6 (2.4 – 7.1) years (**II**).

In study **III** altogether 88 children were positive for TGA, or positive for AGA-IgG, if IgA-deficient. The parents of 55 children consented to upper gastrointestinal videoendoscopy and duodenal biopsies. Villous atrophy was confirmed in 43 children and one additional child was diagnosed with dermatitis herpetiformis (2.1%). The median age at diagnosis of celiac disease was 4.3 (1.6 – 9.8) years.

In all three overlapping study cohorts a sizable number of children, who in at least one sample had been positive for at least one celiac disease-associated antibody, did not proceed to small-intestinal biopsy. The main reason for biopsy cancellations (in 76% to 86% of the cases) was that antibodies had disappeared from the serum of the children spontaneously. Only a few of the parents of the antibody positive children refused gastroscopy and biopsy (9% to 24%). Of the 44 children with confirmed

celiac disease, only 1 has not adhered to a gluten free diet. During frequent follow-up through over 5 years this subject has continuously been positive for celiac disease-associated antibodies, but has not had any clinical symptoms and continues to grow normally.

In the study cohorts, the time interval between seroconversion to TGA positivity and small bowel biopsy failed to predict the biopsy findings. The index children with total villous atrophy (Marsh 3c) and the children with partial villous atrophy (Marsh 3a and 3b) had been TGA positive before the biopsy for 0.8 (± 0.6; 0.3–1.5) years and 1.8 (± 1.0; 0.3–3.5) years, respectively, while the index children who had normal villous morphology in their biopsy samples (Marsh 0 or Marsh 1) had been TGA positive before the biopsy for 1.0 (± 0.7; 0.3–2.4) years (**I**).

In study **II** upper gastrointestinal videoendoscopy with duodenal biopsies was performed on 26 TGA positive children. The median antibody concentrations increased, as the Marsh scores of the duodenal biopsy samples increased, although random variation was substantial (Table 8).

5.4 Celiac disease-associated and type 1 diabetes-associated antibodies (III)

Study **III** focused on determining type 1 diabetes-associated and celiac disease-associated antibodies in children who participated in the DIPP study. In this study, 342 children were positive for ICA in at least one sample, while 146 children tested positive for at least one biochemical diabetes-associated autoantibody (IAA, GADA or IA-2A) in at least two consecutive samples. These children included 19 who were continuously ICA negative. Altogether 215 children were positive only for ICA; of them, 17 were ICA positive in only one sample. Altogether 88 children were regarded as positive for celiac disease-associated antibodies, as 86 children were positive for TGA and two children with IgA-deficiency were positive for AGA-IgG.

Seroconversion to positivity for the first-appearing celiac disease-associated antibody occurred at a median age of 1.5 (0.5 – 7.5) years, i.e., at a markedly younger age than seroconversion to positivity for the first diabetes-associated autoantibody (3.0 years, 0.4 – 11.1 years; $P < 0.001$). If only persisting biochemical diabetes-associated autoantibodies were accepted for analysis, the children seroconverted at a median age of 2.5 (0.5 – 10.1) years, also clearly later than the children seroconverted to celiac disease-associated antibody positivity ($P = 0.007$). If only the celiac disease-associated antibody TGA is considered, the children seroconverted at a median age of 3.0 (1.0 – 9.0) years, i.e., at the same age as the children seroconverted to positivity for diabetes-associated autoantibodies, but later than they seroconverted to positivity for the first biochemically defined diabetes-associated autoantibody ($P = 0.011$) (Table 9).

Table 8. Median values (range) of five measured antibodies at the time of duodenal biopsy.

Antibody measured	Cut-off limit for positivity	Marsh score of the biopsy sample			
		Marsh 0 (n = 6)	Marsh 3a (n = 6)	Marsh 3b (n = 8)	Marsh 3c (n = 6)
TGA	8 U/ml[a]	4.6 (0.2 – 11.1)	56.6 (11.5 – 96.7)	88.4 (29.3 – 363.6)	259.4 (41.7 – 1074.2)
AGA-IgA	0.2 EU/ml[b]	0.1 (0.1 – 0.1)	0.1 (0.1 – 0.2)	0.7 (0.2 – 2.5)	1.2 (0.1 – 2.8)
AGA-IgG	5.0 EU/ml[c]	4.4 (1.0 – 16.1)	35.1 (7.1 – 81.3)	39.1 (10.5 – 170.2)	89.7 (13.5 – 205.4)
EMA	5	0.5 (0 – 10)	80 (20 – 160)	160 (20 – 1280)	240 (40 – 1280)
ARA	5	0 (0 – 5)	80 (5 – 320)	90 (0 – 320)	480 (10 – 1280)

[a] 5 – 8 U/ml equivocal values
[b] In children < 2 years of age cut-off value was 0.5 EU/ml
[c] In children < 2 years of age cut-off value was 10.0 EU/ml

Table 9. Median age at seroconversion to positivity for type 1 diabetes-associated and celiac disease-associated antibodies.

First seroconversion to positivity for any antibody in the group	N	Median age (range) (years)
Celiac disease-associated antibodies		
TGA, AGA-IgA, AGA-IgG, EMA or ARA	88	1.5 (0.5 – 7.5)
Celiac disease-associated antibodies (excluding AGA)		
TGA, EMA or ARA	86	2.5 (1.0 – 9.0)
Diabetes-associated autoantibodies		
ICA, IAA, GADA or IA-2A	342	3.0 (0.4 – 11.1)
Biochemical diabetes-associated autoantibodies		
IAA, GADA or IA-2A	146	2.5 (0.5 – 10.0)
First seroconversion to positivity for each antibody separately	**N**	**Median age (range) (years)**
TGA	86	3.0 (1.0 – 9.0)
AGA-IgA	50	3.0 (0.6 – 10.0)
AGA-IgG	78	1.5 (0.5 – 11.5)
ARA	74	3.0 (1.0 – 10.5)
EMA	85	3.0 (1.0 – 9.0)
ICA	342	3.2 (0.4 – 11.1)
IAA	110	2.0 (0.5 – 10.3)
GADA	107	3.0 (0.7 – 10.1)
IA-2A	90	3.0 (0.5 – 9.8)

During the follow-up, 51 of the 342 children positive for diabetes-associated autoantibodies progressed to overt diabetes, and 43 of the 88 children positive for celiac disease-associated antibodies progressed to biopsy-proven celiac disease. One additional child developed skin biopsy-confirmed dermatitis herpetiformis at age 4.3 years. Although celiac disease-associated antibodies developed, on average, at a slightly younger age than the diabetes-associated autoantibodies, overt diabetes and celiac disease were diagnosed at age 4.5 (1.4 – 11.6) years and 4.3 (1.6 – 9.8) years, respectively (P = 0.257) (Figure 4).

Nineteen (5.6%) children developed both diabetes-associated and celiac disease-associated antibodies. Eight children seroconverted first to positivity for diabetes-associated autoantibodies, eight first to celiac disease-associated antibodies, and three simultaneously to positivity for the two types of antibodies. The median age of the 19 children who seroconverted to positivity for the first diabetes-associated autoantibody was 1.6 (0.5 – 6.5) years and the first celiac disease-associated antibody 1.5 (0.8 – 7.2) years. Accordingly, the children who seroconverted to positivity for both diabetes- and celiac disease-associated antibodies seroconverted at a younger age than those who were positive only for diabetes-associated antibodies (1.6 years vs. 3.0 years; P = 0.026).

Of the 19 children with both diabetes-associated autoantibodies and celiac disease-associated antibodies, 10 were positive for one or more of the three biochemical diabetes-associated autoantibodies. Four of them developed first IAA, GADA or IA-2A,

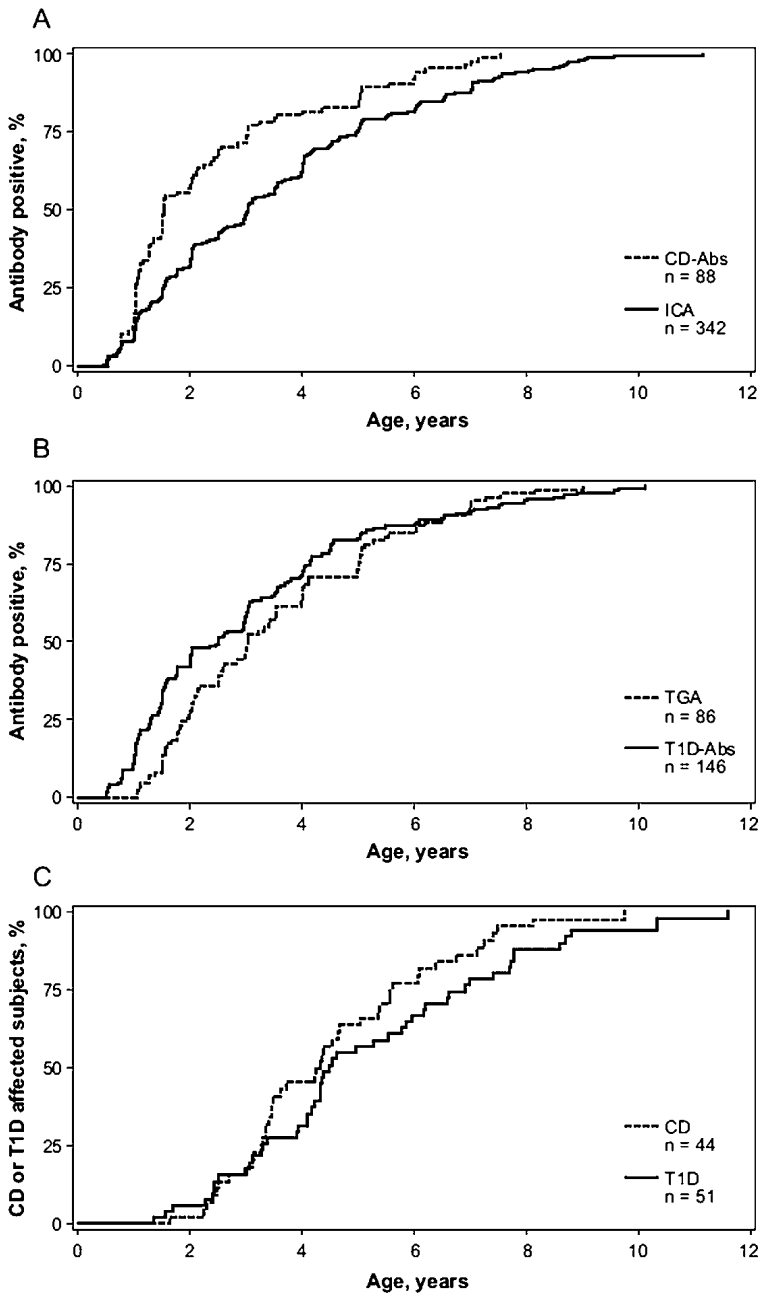

Figure 4. Cumulative seroconversion to positivity for type 1 diabetes- and celiac disease-associated antibodies and progression to overt disease in the DIPP study children with HLA-conferred genetic type 1 diabetes and celiac disease susceptibility. CD-Abs = at least one sample positive for TGA (IgA or IgG) and / or AGA-IgA, AGA-IgG, EMA or ARA; ICA = at least one sample positive for ICA or ICA and IAA, GADA and / or IA-2A; TGA = at least one sample positive for TGA; T1D-Abs = at least two consecutive samples positive for IAA, GADA and/or IA-2A (= persisting biochemical diabetes-associated autoantibodies); CD = celiac disease; T1D = type 1 diabetes.

four celiac disease-associated antibodies, and two both diabetes-associated and celiac disease-associated antibodies at the same age. The children seroconverted to positivity for biochemical autoantibodies and celiac disease-associated antibodies at median ages of 1.4 (0.5 – 8.9) years and 1.4 (0.8 – 2.6) years, respectively.

The three children who progressed to clinical type 1 diabetes and celiac disease during the follow-up developed antibodies, clinical type 1 diabetes and celiac disease in random order (Figure 5).

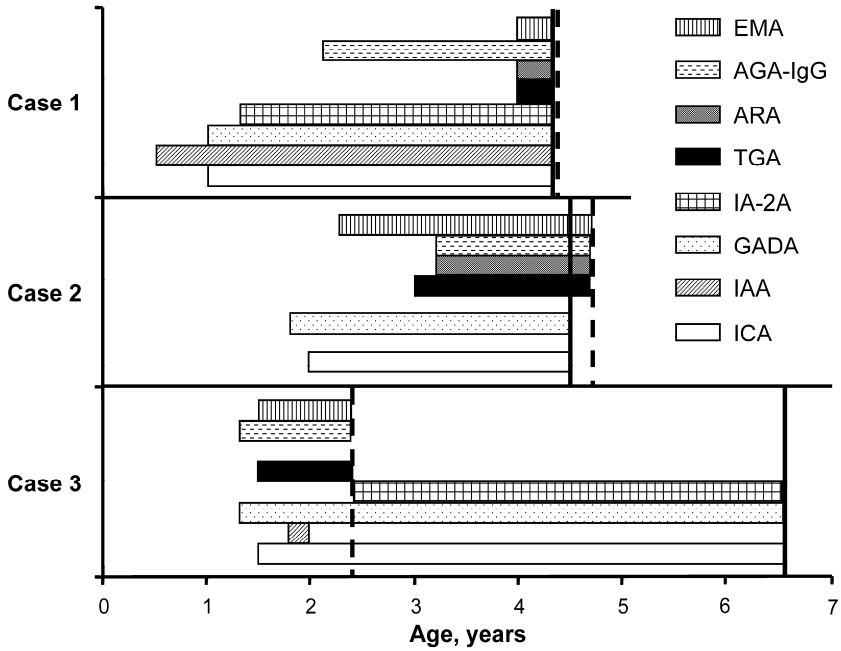

Figure 5. Age at seroconversion to antibody positivity in the three children who progressed both to type 1 diabetes and celiac disease. The age at diagnosis of type 1 diabetes is shown by a vertical solid line and the age at diagnosis of celiac disease by a vertical dashed line. One child seroconverted first to IAA-positivity at age 6 months, and to ICA-, GADA- and IA-2A-positivity at 1.3 years. He developed AGA-IgG at 2.1 years, but only at 4.0 years did he become positive for TGA, EMA and ARA. Diabetes and celiac disease were finally diagnosed simultaneously when he was 4.3 years. Another child seroconverted to GADA positivity at 1.8 years and to ICA positivity 3 months later. He developed EMA at 2.3 years and TGA at 3.0 years. Diabetes was diagnosed when he was 4.5 years, and small bowel biopsies two months later showed marked villous atrophy confirming overt celiac disease. A third boy who was IgA-deficient seroconverted to GADA and AGA-IgG positivity at 1.3 years and to ICA positivity 3 months later. He was TGA-IgG positive for the first time at 1.5 years, and celiac disease was confirmed at 2.4 years of age. He started gluten-free diet but has constantly been TGA-IgG-positive but AGA-IgG negative. He progressed to clinical diabetes when he was 6.6 years.

6. DISCUSSION

This thesis illustrates the natural course of celiac disease-associated antibodies and progression to overt celiac disease in children with increased HLA-conferred genetic risk and monitored closely since birth. Much has been learned about the etiopathogenesis of celiac disease, but less is known about the natural course of celiac disease and antibodies associated with celiac disease autoimmunity, especially before the diagnosis of celiac disease.

6.1 Natural course of the antibodies associated with celiac disease

We have shown in the prospective DIPP study (the Finnish Type 1 Diabetes Prediction and Prevention study) that the earliest age for TGA to become positive is when the child is about 1 year. The seroconversions occurred significantly earlier than has been reported in previous follow-up studies. In the American prospective follow-up study DAISY, no children were reported to be TGA positive before age 2.6 years (Hoffenberg et al. 2003). The results of that study were similar to the German BabyDiab follow-up study, in which TGA was never found in samples drawn before the child was 2 years (Hummel et al. 2007). An explanation for the differences in the first seroconversions to TGA positivity in these three studies may relate to the different schedules of venous blood sampling. In the DIPP study samples are taken every 3 to 6 months for the first 2 years and every 6 to 12 months thereafter, which makes the recognition of early TGA seroconversions possible. In DAISY, samples are taken when the child is 9, 15 and 24 months and annually thereafter, and in the BabyDiab study at 9 months and the at 2, 5, 8, 11 and 14 years. Also, in a Spanish population based screening study, where the children were examined and TGA was measured at the ages of 1.5 years and 2.5 years of age, the first positive TGA value was measured only at the second visit when the children were 2.5 years (Castano et al. 2004). However, in a Swedish case-control study the first emergence of TGA and EMA in the serum of children was at the age of 7.5 months, but these children were symptomatic (Lagerqvist et al. 2008). The study included children who were diagnosed during a period when Sweden experienced an epidemic of celiac disease in young children, which may also have influenced the results (Ivarsson et al. 2000). The average age at seroconversion to TGA positivity was lower in the DIPP study (median age 3.0 years), than in DAISY (mean age 4.7 years) and in BabyDiab (mean age 4.9 years) (Norris et al. 2005, Hummel et al. 2007).

When studying the chronology of celiac disease-associated antibodies we have shown that TGA, EMA and ARA usually develop at the same age. This is understandable, since these antibodies are virtually identical, as shown by Korponay-Szapó and colleagues (Korponay-Szabó et al. 2000, Korponay-Szabó et al. 2003). Usually these antibodies occur simultaneously: in our study for example only one TGA positive child did not seroconvert to EMA positivity whereas 14% of TGA positive children did not have ARA.

The earliest age at which a child developed IgG-class antigliadin antibodies in the present study was 6 months. The median age for AGA-IgG seroconversion was, however, 1.5 years, which was significantly earlier than seroconversion to positivity for the other celiac disease-associated antibodies at the age of 3.0 years. Similar results were reported in the BabyDiab study, although the first seroconversion to AGA-IgG positivity was reported in children aged 1.0 year (Hummel et al. 2000). Interestingly, seroconversion to AGA-IgA positivity in the DIPP study occurred notably later, i.e. at a median age of 3.0 years, although the first children seroconverted already at age 6 months. A previous study reported that during gluten challenge anti-gliadin antibodies usually appear before EMA (Bürgin-Wolff et al. 1991). In a Swedish case-report Lagerqvist and colleagues reported that AGA-IgA is better than TGA and EMA in identifying celiac disease in children younger than 18 months of age. Especially in children who were diagnosed with celiac disease under the age of one year high AGA-IgA occurred in 95% of the patients, whereas TGA and EMA was found in 66% and 62%, respectively. In children older than 18 months, high TGA and EMA levels occurred in 99%. Unfortunately, in that study AGA-IgG was not analyzed (Lagerqvist et al. 2008).

There is no explanation for the early seroconversion of AGA-IgG. Unlike TGA, EMA and ARA, which are directed against self-antigens, gliadin antibodies are directed against food antigens. It has been shown that antibodies directed against cow's milk proteins and other food-related antibodies often rise soon after the specific food item has been introduced for the first time. However, the level of antibody values decreases with time (Tainio et al. 1988, Vaarala et al. 1995). This is also one possible explanation for the early AGA-IgG seroconversion seen in our study population of young children: a nonspecific immune response to gluten containing food when introduced for the first time.

AGA-IgG is present in the sera of the general population much more often than clinical celiac disease (Catassi et al. 2000), and only some of the AGA-IgG positive children progress to overt celiac disease. The incidence of gliadin antibodies also seems to increase with age (Kumar et al. 1984). Regardless of the fact that AGA-IgG is the first antibody to appear in most antibody-positive children of our study, it must be considered poorly suited for primary screening of celiac disease and for deciding whether or not gastrointestinal endoscopy and biopsy should be performed. Although gliadin antibodies are directed against food antigens, they may yet prove to be important for searching for other triggers of celiac disease than gluten. In some individuals AGA-IgG may specify the time when the permeability of the intestine has increased and gluten peptides gain access to the lamina propria, which initiates the cascade terminating in celiac disease.

In our prospective follow-up study, 4.3% of the children with an HLA-conferred risk for celiac disease were positive for TGA at least in one sample, quite similar to in other prospective studies of at-risk children. In the BabyDiab study the proportion was 3.5% and in DAISY 4.1% (Hummel et al. 2000, Hoffenberg et al. 2003). The prevalence of celiac disease-associated antibodies in our study is also similar to the prevalence of 4.2% reported by Fasano and colleagues in an American cross-sectional screening study of at-

risk children (Fasano et al. 2003a). The figures in our study are in agreement with studies looking for celiac disease-associated seropositivity in children with type 1 diabetes (Kordonouri et al. 2000, Agardh et al. 2001, Peretti et al. 2004). However, the prevalence is understandably lower (1.5%) in a population of unselected school-aged children than in a study involving children with an increased genetic risk for celiac disease (Mäki et al. 2003). In the DIPP study celiac disease was confirmed by biopsy in 2.1% of the whole cohort which is comparable with the 1.2% and 1.9% prevalences of celiac disease in BabyDiab and in DAISY, respectively (Hummel et al. 2000, Hoffenberg et al. 2003).

Because upper gastrointestinal endoscopy is an invasive procedure, it would be certainly helpful if celiac disease could be diagnosed without duodenal biopsy. Furthermore, in our study the time interval from seroconversion to TGA positivity and the severity of the small bowel biopsy findings correlated poorly. The celiac disease-associated antibody values increased as the Marsh scores of the duodenal biopsy samples increased, although substantial variation occurred, as has been reported in another prospective study (Liu et al. 2003). Thus, diagnosing celiac disease only on the basis of high titers of antibodies is not appropriate.

6.2 Transient antibodies

DAISY, the Denver, Colorado based prospective follow-up study, reported that celiac disease-associated antibody values are not stable in young children with increased genetic risk for celiac disease. In DAISY, 42 children were observed since birth or early childhood by serial testing (at ages of 9, 15 and 24 months) for TGA. The study found that in children not yet diagnosed with celiac disease, positive TGA values fluctuated 10 – 100-fold over 3 to 12 months. However, most children (39/42) remained consistently TGA positive after seroconversion (Liu et al. 2003). These findings thus differ markedly from our prospective follow-up data, as up to 51% of the children once positive for TGA (18/35) spontaneously reseroconverted to TGA negativity. Our prospective study is the first to report transiently positive TGA values in a large proportion of children. The obvious reason for the discrepancy in the results was due to our more frequent sampling and follow-up.

In many studies in which antibody positivity has been detected in only one sample, the result has often been considered as a false positive (Hill et al. 2000, Chan et al. 2001). In a Finnish study, one-third of TGA or EMA positive children turned negative during 1 to 2 years follow-up (Bister et al. 2005). Our data also strongly suggest that such transiently positive values do exist and that such fluctuating values may, in fact, occur quite commonly. The transient nature of celiac disease-associated antibodies has also lately been reported in a screening study from the Netherlands (Hogen Esch et al. 2010). Disappearance of TGA was also confirmed in our group of 49 children: 24 (49%) became spontaneously TGA negative. Clearly, the positive values were not due to laboratory errors, because 32 – 45% of the other celiac disease-associated antibodies,

EMA, ARA, AGA-IgA and AGA-IgG, also disappeared in parallel with the TGA during the follow-up.

The reason or reasons and mechanisms which make the celiac disease-associated antibodies disappear are not known. During gluten challenge the anti-gliadin antibodies may disappear in spite of continuous gluten intake (Bürgin-Wolff et al. 1991). One possibility which may have caused disappearance of antibodies is that the immune system of these individuals is able to strictly limit autoimmunity-related process.

It is unlikely that the disappearance of the antibodies is the result of dietary changes and gluten withdrawal. When TGA seropositivity was found and the result was told to the family, we emphasized that the family should not make any changes to the child's diet or restrict the child's gluten intake before a small bowel biopsy had been taken and celiac disease had been confirmed or excluded. Unconscious restriction of gluten intake is also very improbable because dietary changes were asked during every visit at the follow-up clinic. Complete removal of gluten from the diet is also very unlikely, at least among children who ate in day-care centers or schools, because these institutions require a physician's certificate before any individual dietary adjustments are made to a child's diet. It is also well documented that even small amounts of gluten in the diet sustain the mucosal changes and autoimmune processes (Laurin et al. 2002, Tommasini et al. 2004). All these circumstances reduce the possibility that the spontaneous disappearance of celiac disease-associated antibodies was due to dietary changes related to the parents' awareness of an increased risk of celiac disease. In Finland, children with biopsy-proven celiac disease receive a state-funded allowance to cover the extra expenses caused by the gluten-free diet, which may also reduce the willingness of the parents to adhere to the diet before biopsy had been taken. Finally, some of the sample series showing transient celiac disease-associated antibody values had been collected to the DIPP biobank many years before permission to study celiac disease development was requested of the ethics committee and parents.

6.3 Celiac disease autoimmunity and type 1 diabetes autoimmunity

According to the present findings celiac disease-associated antibodies usually develop earlier than type 1 diabetes-associated autoantibodies. When all celiac disease-associated antibodies (TGA, EMA, ARA, AGA-IgA and AGA-IgG) were taken to analysis, seroconversion to positivity for celiac disease-associated antibodies appeared at a median age of 1.5 (0.5 – 7.5) years, i.e. earlier than seroconversion to positivity for type 1 diabetes-associated autoantibodies [median age 3.0 (0.4 – 11.1) years]. These results differ clearly from those reported by the BabyDiab study, in which diabetes-associated autoantibodies developed at a median age of 2.3 years, much before celiac disease-associated antibodies emerged (at age 4.9 years) (Hummel et al. 2007). However, in the BabyDiab study IAA, GADA and IA-2A were defined as type 1 diabetes-associated antibodies and celiac disease autoimmunity was only based on TGA. When the results of

the BabyDiab study are compared with our results, it is obvious that our study provides more information on the early phases of type 1 diabetes and celiac disease autoimmunity, because of our more frequent follow-up schedule and inclusion of information delivered also by AGA-IgA, AGA-IgG, EMA and ARA findings measured from all samples drawn from children who at any time point were positive for TGA.

6.4 Strength and limitations of current studies

The strength of our study is a long and frequent follow-up of a large number of children at genetic risk for celiac disease observed since birth. In the DIPP study, the blood samples have usually been taken at 3 to 6 months intervals or at least annually. These short intervals permit description of the appearance and disappearance of celiac disease-associated antibodies and allow illustration of the natural course of the antibodies before overt celiac disease. This tight follow-up also allows documentation of the changes that emerge rapidly and also disappear within a relatively short time period, as such changes are easily missed if the children are examined less frequently. We have now also followed-up some of the children for over 12 years, although the youngest ones have been followed-up for only one year. The massive DIPP biobank with serum samples stored at -70°C provides also excellent possibilities for further analyses in the future.

The present studies have limitations. The DIPP study was launched for the type 1 diabetes prediction and prevention. Thus, the inclusion criteria were based on the children's genetic risk for type 1 diabetes. Consequently, the participation and follow-up has been offered to children with increased genetic risk for type 1 diabetes. Although the risk alleles are largely the same for type 1 diabetes and for celiac disease, only some of the children with the highest risk alleles for celiac disease, i.e. children with HLA DQA1*0501-DQB1*02 (DR3-DQ2), are included in the DIPP study. However, celiac disease screening was offered to all children with a high risk for type 1 diabetes who had HLA DQB1*02/DQB1*0302. In that group, most children have DR3-DQ2/DR4-DQ8 (HLA DQA1*05-DQB1*02/*0302) and the remaining children have DR4-DQ8/y [HLA DQA1*0201-DQB1*02/*0302 (y ≠ DR3-DQ2)]. Participation in a celiac disease study was not offered to children with moderate risk for type 1 diabetes and carrying HLA DQB1*0302/x (x ≠ *02, *0301, *0602) as the risk for celiac disease in these children is very low. Although these studies do not include all children at increased risk for celiac disease, they provide a remarkably good picture of the natural course of celiac disease in symptom-free at-risk children before the diagnosis. However, due to the study design, we are not able to draw any conclusions about the natural history of celiac disease-associated antibodies in an unselected population. In any case, celiac disease rarely develops to subjects who do not carry these HLA risk alleles.

In a follow-up study which lasts for many years some subjects are always lost to follow-up. However, most of the children in our study continued for many years and adhered well to the tight follow-up and regular blood draws. The follow-up schedules of the

children at the three study centers in Finland differed slightly, as the children were seen more frequently in Turku than in Oulu and Tampere. The effect of these differences in the follow-up was analyzed in the study concerning the appearance of celiac disease-associated antibodies and type 1 diabetes-associated autoantibodies. The analysis showed that the longer follow-up intervals postponed the seroconversion ages slightly as concerns celiac disease-associated antibodies in Oulu and Tampere but not for diabetes-associated autoantibodies. The reason for this difference is not known. However, the changes in the seroconversion ages were minor and do not affect the conclusions of our study.

We used TGA as the primary antibody for celiac disease autoimmunity screening for children with an HLA-conferred celiac disease risk. TGA is a highly sensitive and specific marker of celiac disease and in most comparisons it exceeds other celiac disease-associated antibodies regarding sensitivity and specificity (Hill 2005a, Rostom et al. 2005). TGA has been well accepted and applied widely as the sole screening assay for celiac disease in children (Hoffenberg et al. 2000, Liu et al. 2003, Hoffenberg et al. 2003, Tommasini et al. 2004). However, due to our study design, we may have missed occasional children who only developed non-TGA celiac disease-associated antibodies. We also included rather unspecific antigliadin antibodies, AGA-IgG and AGA-IgA, in the group of celiac disease-associated antibodies. However, we consider that analyzing AGA together with other celiac disease-associated antibodies is justified, since seroconversion to AGA positivity may identify the time when the celiac disease process really begins in children who develop also other celiac disease-associated antibodies. Antigliadin antibodies may also be important when other celiac disease triggers than gluten are looked for. We have previously measured antibodies to the deamidated gliadin peptide in part of the currently studied children (Ankelo et al. 2007); the findings showed high concordance with the AGA and TGA results.

In many cases we were not able to arrange upper gastrointestinal endoscopy immediately after TGA-positivity was established. In some cases the seroconversion had occurred already years or months before the first antibody measurement. This was a typical situation particularly in the beginning of the study: the oldest children were 5 years old when TGA was measured for the first time. The first seroconversion was determined in samples collected during follow-up and stored frozen in our biobank. Due to the delays in arranging for the biopsy samples or to the parents' request to postpone the procedure, some children spontaneously lost TGA. When a symptom-free child does not have celiac disease-associated antibodies, there is no indication for duodenal biopy samples. Consequently, we were not able to analyze any changes in the duodenal mucosa in most children with transient seropositivity.

We used ICA as the primary autoantibody for diabetes autoimmunity screening, although we also analyzed the three biochemical autoantibodies in all samples drawn from the ICA-positive children. Consequently, we probably missed a few children who were ICA-negative but had other diabetes-associated autoantibodies. In children born between the

beginning of 2003 and the end of the study, we have measured all four type 1 diabetes-associated autoantibodies in every sample taken.

6.5 Screening studies

Many opinions have been expressed in favor of screening for celiac disease and against it. Screening for celiac disease-associated antibodies among individuals with a known increased risk for celiac disease, e.g., patients with type 1 diabetes, is generally accepted, although unanswered questions remain (Freemark and Levitsky 2003). More open questions are raised when it comes to screening the unselected general population. Questions of the benefits of a gluten free diet for asymptomatic individuals, compliance and cost-effectiveness are especially controversial (Kumar 2003, Fasano 2003b, Hoffenberg 2005, Fasano 2009, Evans et al. 2009).

In the DIPP study, most parents were willing to participate in the celiac disease study and consented to the analysis of celiac disease-associated antibodies in their child's blood samples. Only a few parents of children with positive antibody values refused gastroscopy and biopsies. This has not been the case in other screening studies, where the fraction of parents not consenting to gastroscopy and small bowel biopsy of their child has been high (Hoffenberg et al. 2003, Hummel et al. 2007). In our study, ultimately only one family decided not to adhere to the gluten free diet after celiac disease was confirmed by duodenal biopsies. Only few children with celiac disease-associated antibodies had any symptoms. Some children had mild abdominal discomfort, but in most cases the symptoms were so mild that the family had not sought any medical help.

The results of the present studies have added to our understanding and knowledge about natural history of the development of antibodies associated with celiac disease. We have shown that TGA usually appears only after the first year of life, while gliadin antibodies appear soon after cereals have been added to the child's diet. We have also demonstrated that celiac disease-associated antibodies may disappear spontaneously, at least in children with genetic risk, and this emphasizes the significance of repeatedly confirmed seropositivity before proceeding to biopsy. The fluctuating nature of celiac disease-associated antibodies also stresses the importance of duodenal biopsy for the diagnosis of celiac disease. This information is helpful when designing screening of celiac disease either for at-risk groups or the general population.

7. SUMMARY AND CONCLUSIONS

The present studies have elucidated the natural history of the development of celiac disease associated antibodies and overt celiac disease in a cohort of children at genetic risk for celiac disease. Antibodies associated with celiac disease developed very early; AGA-IgG appear usually first, and this may occur when the child is only 6 months old, i.e. very soon after cereals are introduced to the diet. AGA-IgA is often the second antibody in order to appear, although AGA-IgA seroconversion is by no means very common. TGA, EMA and ARA appear almost simultaneously, at the earliest when the child is 1 year old.

In this study, 4.3% of children at risk for celiac disease were positive for celiac disease-associated antibodies at least in one sample. Celiac disease was confirmed in 2.1% of children with genetic risk when the children had a median age of 5.7 years.

Surprisingly, the seroprevalence of celiac disease-associated antibodies was not stable: almost 50% of the antibody positive children had the antibodies disappear with no dietary intervention. Obviously, the autoimmune process was aborted for unknown reasons. The same phenomenon was seen in all antibody species, and most children were positive for more than one antibody type simultaneously. Longer follow-up will reveal if children with transient antibodies develop celiac disease later in life.

The current study shows that celiac disease-associated antibodies often develop earlier than diabetes-associated autoantibodies or concomitantly with them in children with a HLA-conferred risk for both diseases. Seroconversion to positivity for the first-appearing celiac disease-associated antibody occurred at a median age of 1.5 years, i.e., among markedly younger children than the seroconversion to positivity for the first diabetes-associated autoantibody (which occurs at age 3.0 years). The children who developed both diabetes- and celiac disease-associated antibodies generated the two types of antibodies usually in a random order within a short time interval. Overt diabetes and celiac disease were ultimately diagnosed at approximately the same age.

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Satu Simell

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