DIAGNOSTICS IN CELIAC DISEASE

AMANI MUBARAK

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DIAGNOSTICS IN CELIAC DISEASE

DIAGNOSTIEK BIJ COELIAKIE

(met een samenvatting in het Nederlands)

Proefschrift

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door

Amani Mubarak

geboren 31 mei 1986 te Bagdad, Irak

Promotoren:	Prof.dr. E.E.S. Nieuwenhuis Prof.dr. F.J.W. ten Kate
Co-promotor:	Dr. R.H.J. Houwen

aan: dokter Mubarak
ik voel niet veel versgeel
alleen: toen ik gluten at had ik meer hoofpijn
en: elke morgen wel minder gezur
en: als ik poepte deet het pijn
en: buikpijn heb ik minder
en: toen moest ik meer poepen
en: ik ben meer mezelf
en: en ik eet meer
en: ik ben minder moe

Een brief van Sarah, een 7-jarig meisje met coeliakie, 6 weken nadat zij was begonnen met een glutenvrij dieet

to: doctor Mubarak
I don't feel a lot of difference
however: when I ate gluten I had more headaches
and: every morning there is less trouble
and: pooping did really hurt
and: I have less stomachache
and: at that time I had to poop more often
and: I'm more myself
and: and I eat more
and: I'm less tired

A letter from Sarah, a 7-year-old girl with celiac disease, 6 weeks after she started a gluten free diet

Voor mijn ouders

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PREFACE

Celiac disease (CD) is characterized by an inadequate immune response to gluten, a storage protein in wheat and the related grain species barley and rye. This immune reaction, giving intestinal inflammation and malabsorption, will only occur in genetically susceptible individuals carrying specific human leukocyte antigen (HLA) heterodimers, and consists primarily of a gluten specific T-cell response, although a specific antibody response is also present. The latter includes anti-gliadin antibodies (AGA) and the autoantibodies anti-tissue-transglutaminase (tTGA) and anti-endomysium (EMA). In most cases the inflammation is completely reversible upon withdrawal of gluten, which is, until now, the only method to treat the disease. In **Chapter 1** we give a comprehensive overview of CD, from pathophysiology to treatment, and including diagnostic methods, which is the topic of this thesis.

Currently the worldwide prevalence of CD is estimated to be around 1% although only 1 out of 8 patients truly gets diagnosed. This is because CD can present with a wide range of symptoms that frequently remain unrecognized. Classically, the disease manifests with a malabsorption syndrome characterized by diarrhea, steatorrhea, abdominal distention, and weight loss or failure to thrive. However, many more patients suffer from mild or unspecific abdominal symptoms without clear signs of malabsorption. In addition, CD may be diagnosed in patients with extra-intestinal manifestations such as fatigue, growth failure, anemia, osteoporosis, liver disease or reproductive problems in women. Moreover, a great number of patients do not have any symptoms and are only diagnosed because they belong to a group at risk for CD. With the increasing insight in these different faces of the disease, diagnosing CD has become a challenge.
Diagnostic methods in CD consist of measuring disease specific antibodies, which permits the selection of people at risk for the disease and who therefore require a small intestinal biopsy for histological confirmation. Additionally, HLA-typing can be used to support the diagnosis or make it highly unlikely. However, on each level several diagnostic problems are present.

The aim of this thesis was therefore to investigate invasive as well as non-invasive methods for the diagnosis of CD, and thereby to contribute to the development of diagnostic algorithms that are as accurate as possible but at the same time using the least invasive methods.

In the first part of the thesis we focused on the histological diagnosis of CD. Intestinal damage is the main feature of CD and is characterized by inflammation of the epithelium of the small intestine (intra-epithelial lymphocytosis), increased proliferation of the basal layer of the epithelium (crypt hyperplasia) and in most cases destruction of the villi (villous atrophy). Finding these features upon a small intestinal biopsy has been considered to be the gold standard for the diagnosis of CD for decades. However, besides being invasive, expensive and time-consuming, a small intestinal biopsy may not be as accurate as has always been thought. For example the biopsy specimen may be of poor quality or suboptimal orientation, potentially making evaluation less accurate. In addition, diagnostic difficulties may arise due to patchy lesions or early stage disease.

In **Chapter 2** we studied whether these problems result in a high inter-observer variability between pathologists. Subsequently, in **Chapter 3** we investigated whether performing CD3 stains, to detect the intra-epithelial inflammation, contributes to a more accurate diagnosis.

Nevertheless, even if a perfect histological evaluation can be reached, the ultimate goal is, of course, to reach a correct diagnosis by using less invasive methods. This was the topic of the second part of this thesis.
The best way to exclude CD is by performing HLA-typing, as virtually all patients with CD carry either HLA-DQ2.5 or HLA-DQ8. However other studies reported that CD patients can also carry the HLA-DQ2.2 heterodimer, but its frequency in CD remained unclear. Therefore, we tried to answer this question in **Chapter 4**, where the HLA distribution in pediatric patients with CD was investigated. Subsequently, in the following chapters, we studied the diagnostic value of the CD specific antibodies in the diagnosis of CD and evaluated whether a small intestinal biopsy can be avoided in specific cases.

Historically, AGA were the first antibodies to be used in CD diagnostics, but were disappointing in clinical practice as sensitivity and specificity were only in the order of 80%. By contrast, EMA and tTGA —both directed against the auto-antigen tissue-transglutaminase— performed much better, although they did not reach 100% accuracy, so a small intestinal biopsy was still considered obligatory to make a lifelong diagnosis of CD. In addition, in very young children these antibodies have been reported to be less sensitive. For the latter group it was even suggested that it would be more appropriate to use the generally poorly performing AGA, as they could have a better diagnostic performance. Therefore, in **Chapter 5**, we investigated the sensitivity and specificity of the newly developed antibodies against deamidated gliadin peptides, and specifically addressed their performance in the subgroup of very young children. In **Chapter 6** we studied whether a high cut off value (≥10 times the upper limit) for tTGA gives a better disease prediction and helps in avoiding a biopsy. In **Chapter 7** we repeated the study in a prospective setting in order to minimize the chance of selection bias and to strengthen the scientific evidence. Subsequently, to better understand the difference between CD patients with low and high tTGA levels, in **Chapter 8** we studied the difference in genotype and phenotype between these two groups. Finally, the thesis ends with a discussion (**Chapter 9**), where the results of the abovementioned chapters are discussed and future directions are given.

PART ONE


disease
directed
characterized
heterodimer
HLA-DQ8
research
basal
gluten
diagnostics
Classically
atrophy
negative
destruction
small
tTGA
diagnosis
susceptible
endomysium
barley
T-cell
measuring
leukocyte
villi
histological
transglutaminase
hyperplasia
non-invasive
positive
HLA-DQ2.2
value
syndrome
HLA
also
levels
undetected
failure
intestine
increased
distention
villous
antigen
invasive
lymphocytosis
specificity
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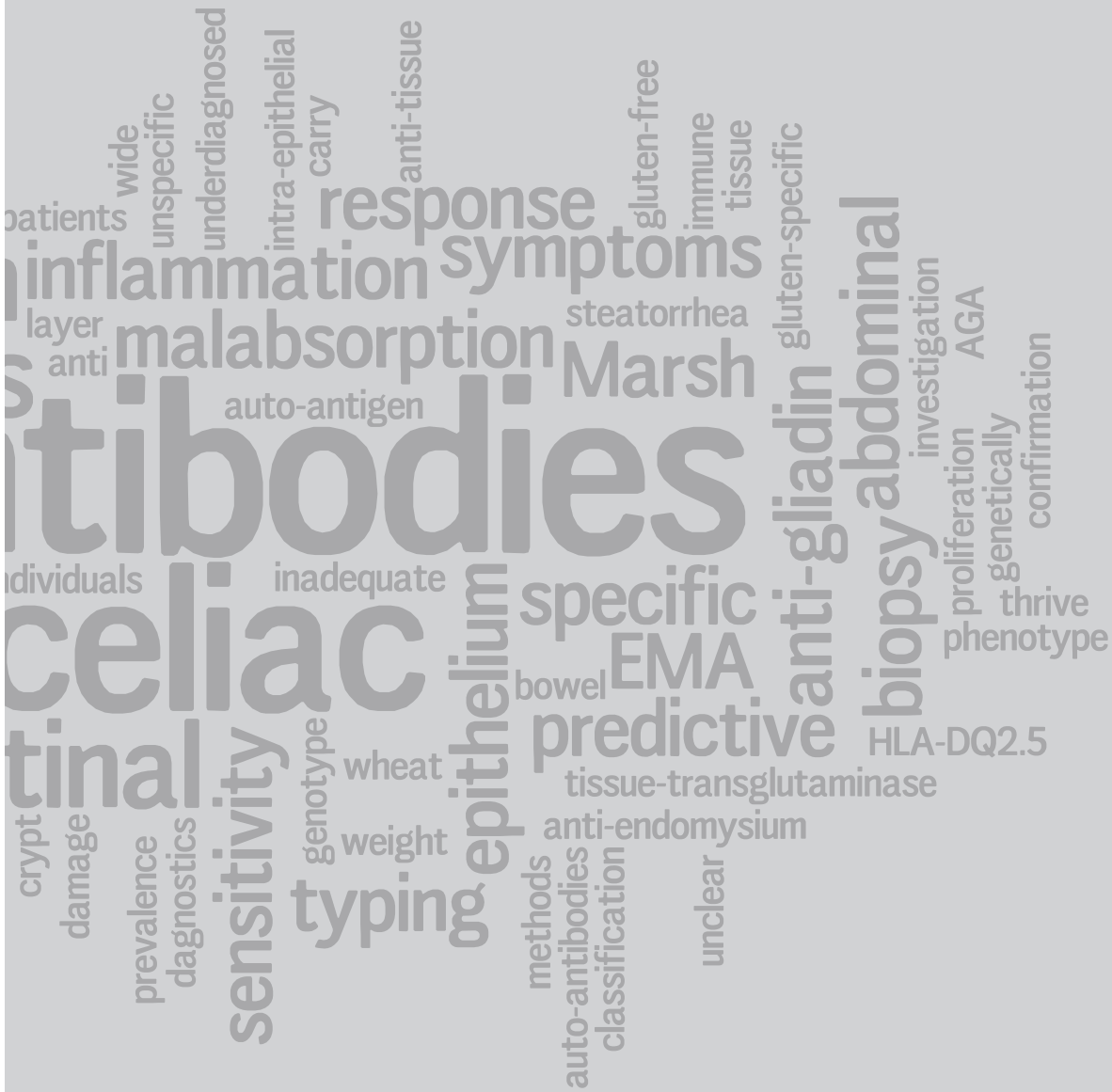
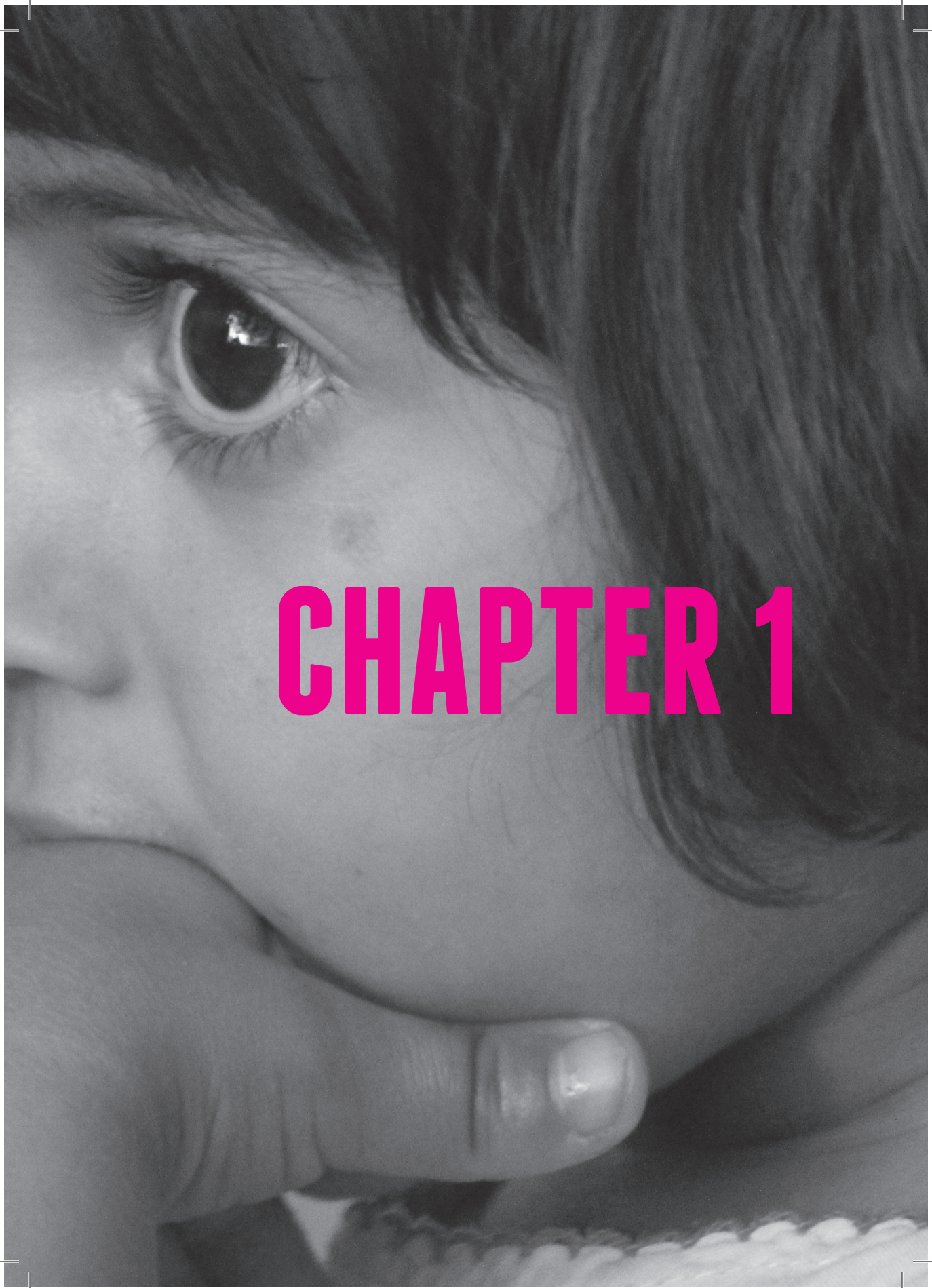
human
diet
heterodimers
loss
range
diarrhea
crypt



TOWARDS AN IMPROVED HISTOLOGICAL DIAGNOSIS


CHAPTER 1

CHAPTER 1

Celiac disease: an overview from pathophysiology to treatment

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R.H.J. Houwen
V.M. Wolters

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VM Wolters

Abstract

Celiac disease (CD) is one of the most common immune-mediated diseases with a worldwide prevalence of around 1%, although a couple of decades ago the disease was thought to be very rare. CD is characterized by an inadequate inflammatory response to gluten in genetically susceptible individuals. In this inflammatory response both the adaptive and innate immunity are involved. The clinical picture of CD is variable ranging from severe malabsorption syndrome to silent cases. Disease specific antibodies can aid in selecting patients for a small intestinal biopsy, which is thought to be the gold standard investigation to diagnose CD. However, in selected patients, serology can be sufficient to confirm the diagnosis and a biopsy is not needed. Hitherto, the only treatment for CD is adherence to a lifelong strict gluten free diet. The purpose of this review was to summarize current literature on the epidemiology and pathophysiology of CD and to discuss diagnostic and therapeutic approaches.

1

Introduction

The new definition of celiac disease (CD) as proposed by most recent ESPGHAN guidelines states that CD is an immune-mediated systemic disorder elicited by gluten in genetically susceptible individuals and characterized by the presence of a variable combination of gluten dependent clinical manifestations, CD-specific antibodies, HLA-DQ2.5 or HLA-DQ8 haplotypes and enteropathy.[1] This definition illustrates the complex interaction between immunological, genetic and environmental factors in the development of the disease, although the interplay between these factors has only been (partially) unraveled in recent decades.

However, the first signs of the possible existence of CD date back somewhere between the first and second century when Aretaeus described a disorder in adults with chronic diarrhea and several signs of starvation and called it the celiac (celiac from Greek κοιλιακός koiliakos, "abdominal") state.[2] He believed the disease to be caused by partial indigestion of food, which should initially be treated by relieving the bowel of stress by rest and fasting.[2] The first modern definition of CD was given by the British doctor Samuel Gee in the end of the nine-teenth century, who used the term celiac affection to describe a disease with classical symptoms of malabsorption syndrome.[3] He literary defined the disease as "a kind of chronic indigestion which is met within persons of all ages, yet especially occurs in children between one and five years old".[3] He also declared that regulation of the food is very important and suggested that if the disease could be cured at all, it must be by means of diet.[3] In the beginning of the twentieth century, just before the death of Gee, the American doctor Herter described a fat malabsorption in such patients and he noticed that they tolerated fatty foods more than carbohydrate rich products.[4] A decade later Still noticed that these patients have an intolerance to bread and in 1921 Howland confirmed an intolerance to carbohydrates.[5,6]

The real breakthrough in CD occurred in the end of the thirties of the last century when the Dutch pediatrician Willem Karel Dicke discovered that wheat and barley protein (gluten) but not wheat starch elicited fat malabsorption, which could be reversed after following a gluten free diet (GFD).[7,8] He, along with his colleagues Van der Kramer and Weyers, also discovered that it is the alcohol soluble fraction or the gliadin component of the water insoluble protein of wheat (gluten) that was responsible for the malabsorption in CD.[9] Later on, it was demonstrated that the malabsorption comes along with intestinal abnormalities that were also responsive to the diet.[10,11] Since then, research on CD has dramatically expanded, increasing our knowledge on the disease yet rising interesting controversies.

In this review, present epidemiological and pathophysiological views as well as current diagnostics and therapeutic approaches in CD will be discussed.

Epidemiology

General population

At the time of Dicke's discovery, CD was thought to be a rare disorder with one of the earliest reports showing incidence rates as low as ~1/6000 in Great Britain, although at that time disease detection was based mainly on the presence of typical symptoms.[12] However, after biopsy techniques became available and disease awareness increased, significantly higher prevalence rates (up to 1/450-500) in European countries have been described in the 1970s, 1980s and early 1990s.[13-18] In these studies however, a great selection bias existed, since only patients with classical symptoms underwent a small intestinal biopsy. Outside Europe, where disease awareness and diagnostic possibilities were way less than in Europe, CD was thought to be extremely rare.[19-20]

It was not until last two decades when case finding became more feasible with the introduction of CD-associated antibodies as routine screening tools: initially the anti-gliadin antibodies (AGA) and later on the anti-endomysium antibodies (EMA) and anti-tissue transglutaminase antibodies (tTGA). With these tests, the frequency of CD increased and a prevalence of ~1% was reported in the majority of European countries.[21-24] In addition, large studies also demonstrated that the prevalence in North and South America, the Middle East and North-Africa was equal to that of Europe and that CD was even common in East Asia.[24-32] An exception to this ~1% prevalence, is the Saharawi population in Algeria where the prevalence of CD is much higher than in any other population, reaching 5.6%.[33] Whether this overall rise in the worldwide prevalence of CD is only due to improved case finding or that CD incidence is actually increasing is still under debate.[21-34]

High-risk groups

Numerous diseases show an increased prevalence of CD in affected patients as compared to the general population. First of all, the disease frequently co-occurs with other autoimmune diseases of which diabetes mellitus type 1 (3-12%), autoimmune thyroiditis (up to 7%), autoimmune liver disease (12-13%) but also IgA nephropathy and juvenile chronic arthritis are the most important.[1] Additionally, patients with selective IgA-deficiency have a significantly increased risk to develop autoimmune diseases among whom CD (2-8%) is one of the most important.[1-35] Moreover, CD is related to a number of genetic syndromes, i.e., Down syndrome (5-12%), Turner syndrome (2-5%), and Williams syndrome (up to 9%).[1] Finally, first and second degree relatives of CD patients, are at an increased risk of developing the disease with a prevalence of 10-20% in first degree relatives.[1,25]

For other diseases, the association with CD is less obvious. Early studies show contradictory results with regard to the co-occurrence of CD and psoriasis. However, a recent large study conducted in almost 30,000 biopsy verified CD patients and almost 150,000 healthy controls showed the relationship between the two diseases to be unequivocally present.[36] Other autoimmune disorders such as alopecia areata, pernicious anemia, myasthenia gravis, and multiple sclerosis, and so on, may also hold an increased risk of CD but have not been studied extensively. Since these diseases are rare (in children) the pediatrician will rarely encounter them. The relationship between inflammatory bowel disease and CD is still unclear with studies being contradictory.[37,38] The co-occurrence of cystic fibrosis and CD has been reported, but with weak evidence due to methodical

weaknesses. However, a recent study demonstrated an increase in CD incidence in a non selected group of patients with cystic fibrosis as compared to controls.[39]
Associations between CD and epilepsy (with occipital calcifications) and autism are even less well defined, and probably very small if present at all.[1,40] The same was thought to be true for schizophrenia, yet a recent study in over 10,000 patients with schizophrenia, showed that these patients are at an increased risk for CD (OR 2.3, CI 1.12-5.27) but also for other autoimmune diseases.[41]

Pathophysiology

Environmental factors

The necessary triggering factor in the development of CD is gluten (from Latin for glue), a storage protein in wheat, barley and rye. Due to its unique absorption capacity, cohesivisity and visco-elasticity, gluten is essential for dough formation and contributes to bread's structure and taste.[42] These properties make gluten also usable as enhancers in different kinds of non-dough related products (hidden gluten), making it one of the most common ingredients in human nutrition. On average, the gluten intake in a Western country is 15 gram per day in children.[1]
Gluten consists of the alcohol soluble fraction gliadin in wheat, or related prolamines in barely (secalins) and rye (hordeins), and glutenins. It is high in glutamine and proline content, the latter making it resistant to degradation by gastro-intestinal enzymes.[42]
In CD, the amount and timing of gluten introduction in infants is thought to influence disease development. This was based on observations in England in the end of the 1970s showing that the incidence of CD decreased after recommendations to avoid gluten introduction before the age of four months.[43] Further studies confirmed this, but additionally showed that late introduction (≥7 months) also carries along an increased risk of developing the disease.[44]
It has been well established that breastfeeding protects against the development of CD.[44,45] However, the timing of weaning in relation to the timing of first gluten exposure and the amount of gluten intake also appear to influence CD development. Clues for this were first gained from the Swedish epidemic experience in which the annual incidence rate for CD increased fourfold between 1985 and 1987 and declined sharply to previous rates in 1995.[46] This change in incidence seemed to correspond well to the dietetic guidelines in 1982, which lead to a later introduction of gluten with consequently more children being weaned from the breast at the time of gluten introduction. In addition, larger amounts of gluten were given to infants at that time.[46] The decline in incidence was related to an increased proportion of infants still being breastfed at the age of six months and a decreased gluten intake in infants. Interestingly, at 12 years of age, children born during the epidemic, still carried a higher prevalence of the disease.[47] However, the prevalence of undiagnosed CD now detected by means of serological screening appeared to be no different in children born before and after the dietetic recommendations were changed again in the mid-1990s, suggesting that breastfeeding and amount of gluten perhaps mainly affect the development of symptomatic CD, but that it does not protect against subclinical forms of the disease.[48] In addition, it remains to be unclear whether the possible protective effects are long-lasting throughout life or that there is simply a

delay in onset of the disease.
However, until prospective and long term follow-up studies are available, ESPGHAN guidelines recommend to avoid both early (<4 months) and late (≥7 months) introduction of gluten, and to introduce gluten gradually while the infant is still being breastfed.[44]
Another important environmental factor suspected in the etiology of CD is the occurrence of (recurrent) abdominal infections in early childhood. Illustratively, it has been well demonstrated that a high frequency of Rotavirus infections increases the risk of CD.[49] It is even suggested that a great part of the protective effect of breast milk may be attributable to the associated protection against gastroenteritis.[50] Hypothetically, intestinal infection and inflammation may damage the small intestinal barrier thereby increasing the intestinal permeability to the toxic gluten components. In that way, exposure of the immune system to gluten is increased, making it more likely for an immune reaction to occur.[51] In addition, inflammation may cause up-regulation of inflammatory cytokines favouring antigen penetration and presentation, but also causes up-regulation of tissue-transglutaminase (tTG), an enzyme that is essential in the pathogenesis of CD.[50,52] Finally, molecular mimicry may explain the relationship between Rotavirus and CD.[53]
Alternatively, disturbances in the intestinal microbiotal environment could switch from gluten tolerance to an inflammatory response.[54-57] As intestinal microbiota play an important role in the establishment and maintenance of mucosal immune homeostasis, a shift in microbiota has been suggested to result in inadequate immune reactions, as is observed in inflammatory bowel disease.[50] Likewise, in CD an imbalanced intestinal microbiota has been reported in affected patients compared to healthy individuals.[54-56] Interestingly, a recent longitudinal study has shown that infants with genetic susceptibility to CD have a different development of microbiota composition compared to infants with a non-selected genetic background, in the period during and after gluten introduction.[57] Finally, the protective effect of breastmilk could also be partially attributed to intestinal microbiota differences between breastfed and formula-fed newborns.[50]

Genetics

In CD a strong genetic component exists, as illustrated by a 83-86% concordance of the disease in monozygotic twins, as compared to 17-20% in dizygotic twins, the latter being similar to the prevalence in other first-degree relatives (10-20%).[1,58,59] By far the most important contribution to this genetic predisposition to CD originates from the HLA class II genes, which are estimated to account for approximately 40% of the genetic risk.[60] Indeed, up to 90% of the CD patients carry the HLA-DQ2.5 heterodimer, traditionally called HLA-DQ2, which most commonly occurs in the cis form, encoded by the alleles DQA1*05:01 and DQB1*02:01. Alternatively, HLA-DQ2.5 can be expressed in the trans configuration (DQA1*05:05-DQB1*02:02), which occurs in individuals heterozygous for HLA-DQ7.5 (DQA1*05:05-DQB1*03:01) and HLA-DQ2.2 (DQA1*02:01-DQB1*02:02).[61-63] Almost all remaining patients have HLA-DQ8, formed by the DQA1*03:01-DQB1*03:02 alleles.[62-66]
However, since these HLA-types are also widely present in the general population (~30%) and only around 1% of the population develops CD, other factors must contribute to the development of the disease.[61,63,67] Many non-HLA loci have been identified in genome-wide association studies, although the effect size of each locus is very small. Interestingly, most non-HLA loci found to be associated with CD are linked to genes related to pro-

teins with an immunological function and genes possibly important in intestinal barrier function.[67] In addition, there is a great overlap in susceptibly loci between CD and other immune-mediated disorders, suggesting a common aetiology.[67]

Immunological factors

The immunological basis of CD lies in its association with HLA-class II molecules. Functionally, these molecules, present on antigen presenting cells such as dendritic cells, are heterodimers consisting of 2 α- and 2 β-chains. They present epitopes in their binding groove to CD4+ T-helper cells and thereby activate the immune system against the presented epitopes.

In CD the inappropriate immune reaction to gluten results in a characteristic enteropathy with intra-epithelial lymphocytosis, hyperplasia of the crypts and villous destruction.[68] In 1975 it was established that gluten peptides lead to a cell-mediated immune response in the small intestine.[69] Later on, it was discovered that gluten specific CD4+ T-cells can be isolated from the small intestine of CD patients but not in controls.[70,71] These T-cells are restricted to the CD-associated HLA-types, suggesting their important role in CD pathogenesis, although at that time the mechanism behind the HLA association was a mystery; HLA-DQ2.5 and HLA-DQ8 preferentially bind negatively charged peptides while native gluten virtually lack such properties.[42]

Along with this cellular response to gluten, a strong B-cell response is present in the form of anti-food antibodies and auto-antibodies. Initially the auto-antibody response was defined as anti-reticulin and then anti-endomysium to indicate a poorly defined reaction to an extra-cellular matrix component of the intestine.[72,73] However, in the end of the 1990s the auto-antigen triggering these antibodies was discovered to be the enzyme tTG.[74] This finding brought the cellular and serological responses in CD together and made us understand the HLA-mechanism behind the disease. Indeed, tTG appeared to be responsible for the deami-dation of gliadin.[75,76] In this reaction the glutamine in gliadin is transformed into glutamic acid by this means shaping negatively charged peptides that fit perfectly in the binding groove of HLA-DQ2.5 and HLA-DQ8 molecules thereby optimizing presentation to CD4+ T-cells which results in a stronger B- and T-cell response.[77-80]

The importance of deamidation and presentation is also illustrated by the strong HLA-DQ2.5 gene-dose effect. First of all, homozygosity for HLA-DQ2.5 is associated with superior antigen presentation compared to heterozygosity resulting in an increased magnitude of the T-cell response.[81] Indeed, individuals with homozygosity have a five-fold increased risk to develop CD and if they do, to develop severe complications.[66,82]

So tTG can generate a large repertoire of highly immunogenic gliadin epitopes that trigger the immune system. However, before this occurs, two things must happen. First tTG and antigen presenting cells must encounter gluten. Because the gluten content in our diet is high and since gluten peptides are highly resistant to degradation by intestinal enzymes, as a result of their high proline content, it is guaranteed that they are highly abundant in the small intestine. However, the peptides must cross the intestinal barrier to get in contact with tTG and dendritic cells. This was thought to mainly happen paracellulary via leaky tight junctions, as observed in patient with CD but not in controls.[83] Nevertheless, recent studies showed that transcellular transport contributes significantly in the crossover of gliadin.[84,85] Interestingly, the transcytosis occurs without degradation

of the gliadin peptides, by contrast to control peptides that do get degradated during transcytosis, suggesting a defective degradation mechanism in gliadin peptides.[85] In addition, a transport mechanism involving the retrotranscytosis of secretory IgA–gliadin peptide immune complexes via the transferrin receptor CD71, also known as an IgA1 receptor, has also been suggested because of its ectopic expression at the apical membrane of enterocytes in active CD.[84] Future studies are needed to explore whether these changes in gliadin transport are already present in at risk individuals before the onset of CD, or that they occur secondary to CD.

Secondly, for the important deamidation to take place, tTG must be released from the intracellular compartment and become activated, which occurs in case of tissue damage.[86] This damage could be initiated by small reactions to un-deamidated gluten; reactions to native gliadin, and also to glutenins.[87] Alternatively, the earlier described infections or disturbances in microbiota balance could potentially cause tissue damage and release of tTG.

Finally, although the CD4+ T cell response is essential for the development of CD, it does not elucidate the total phenotype of the enteropathy. For instance T-helper activation does not completely explain the presence of intra-epithelial lymphocytes, which produce interferon-Y and induce apoptosis and cytolysis, so eventually lead to tissue damage (villous atrophy).[88,89] From this perspective, it is thought that the innate immunity may play a role. In fact, some studies have suggested that a native gliadin peptide, can induce direct toxicity without the adaptive immune system being involved.[90] In such a case, the damage would help in up-regulating tTG and destroying the intestinal barrier leading to the same scenario, as described above.

However, innate immunity could also help in sustaining the adaptive response.[88,90,91] In this scenario, (native) gliadin peptides can induce IL-15 production by epithelial cells and dendritic cells.[91] On its turn, IL-15 acts on migration and expansion of intra-epithelial lymphocytes and promotes interferon-γ production by these cells.[92,93] Moreover, IL-15 was found to have apoptotic effect on enterocytes (villous atrophy), but an anti-apoptotic effect on T-cells.[92] In addition, IL-18, another cytokine of the innate immunity produced in the epithelium of the crypts, is thought to be involved in maintaining the interferon-γ production and consequently the inflammatory response.[94] Further studies are awaited to elucidate the exact mechanism between innate and adaptive immunity in CD.

Clinical presentation

Originally, it was thought that a CD patient ought to suffer from classical symptoms of malabsorption syndrome characterized by diarrhea, steatorrhoea, frequently also accompanied by abdominal distention, and weight loss or failure to thrive. However, it is now clear that these classical patients are only the tip of the iceberg and that the majority of patients suffer from (mild) intestinal symptoms without clear signs of malabsorption or even non-classical extra-intestinal symptoms.[95] Moreover, a great number of CD affected people are asymptomatic (silent CD) and have only been detected in population screening studies or upon screening of at-risk groups.[21-24] In fact, the majority of CD patients remain undiagnosed because they are unrecognized by physicians due to this diversity of symptoms.

Intestinal symptoms without clear signs of malabsorption include abdominal pain or discomfort, bloating, flatulence, diarrhea, constipation or irregular bowel habits.[1] In addition, upper gastrointestinal-tract symptoms such as vomiting and dyspepsia are also common as many patients also suffer from a decreased motility of the upper gastrointestinal system.[1,96] Extra-intestinal atypical signs can be the only symptoms and include chronic fatigue, irritability, (iron deficient) anemia, vitamin deficiencies (mainly vitamin B12 and folate), unexplained elevation of transaminases, short stature, ammenorhoea, delayed puberty, infertility and recurrent abortion in women, neurological symptoms, dental enamel hypoplasia, stomatitis and dermatitis herpatiformis.[1,97-100] The latter, is a skin manifestation of CD presenting with blistering and characterized by IgA deposits in the skin.[1,100] Just like many other immune-mediated diseases, CD occurs 2-3 times more common in females.[34]

Another change in disease presentation that has been unraveled in past decades is the age of onset of CD. Traditionally, CD was considered a disease of early childhood, but it is now widely accepted that this disorder is a disease of all ages, with even a trend towards older age at diagnosis over the past years.[34] Whether these patients have had CD since childhood but have only become symptomatic later on in life or whether they have actually developed CD in adulthood is still under debate. In favor of the first hypothesis, are studies showing that patients diagnosed at older age, are more at risk of developing complications such as refractory CD, a condition in which the intestinal inflammation becomes unresponsive to the diet which might lead to lymphoma.[101] This suggests that CD must have been active for a long time, as it is thought that a long period of uncontrolled inflammation must precede before refractory CD and lymphoma develop. On the other hand, it has also been shown that patients negative for CD-associated antibodies can develop these antibodies and the associated enteropathy later in life, suggesting that CD can also develop in adulthood.[102] Probably, both scenarios exist.

Finally, an important issue in the clinical picture of CD is our current understanding of the existence of potential CD, which is defined as the presence of CD-associated antibodies in the absence of the classical histological lesion.[1] Such patients may be symptomatic but may also lack any symptoms and may develop CD-associated enteropathy later in life. However, in some patients antibody levels decline over time and active CD does not develop while in others antibody levels remain fluctuating over years.[103-108] Interestingly, this seems to occur especially in children with an increased risk for CD, suggesting that there might be a way back to a beginning gluten intolerance. Unfortunately, predictors for the natural history of potential CD are still unknown.

Pathophysiological reasons for the described heterogeneous clinical presentation of CD are until now unclear. Some studies have investigated the HLA dose effect and the clinical presentation, but results seem contradictory.[110-116] A recent study compared genetic and expression markers and found slight differences between potential CD and CD.[109] Studies investigating this processes are highly awaited as this could lead to therapeutical interventions.

Diagnostics

Histology

The gold standard investigation to diagnose CD is considered to be a small intestinal biopsy and has been used to make the diagnosis of CD ever since the histological lesions of the disease were discovered in 1954.[10] The histological variability in CD, was first described by Marsh.[68] He classified the enteropathy to be gradual, starting from solely an increased number of intraepithelial lymphocytes (Marsh I), with later on also crypt hyperplasia (Marsh II) and eventually also accompanied by various degrees of villous atrophy (Marsh IIIA-C).

The first diagnostic criteria for CD, were established in 1969 and were based primarily on histology. To make the diagnosis of CD three criteria were required: histologically confirmed villous atrophy on a gluten containing diet, histological improvement on a GFD and deterioration of the mucosa after a gluten challenge.[13,117]
In 1990, these EPSGHAN criteria were revised making a gluten challenge unnecessary except for children under 2 years of age.[118] At that time, the diagnosis was still based on histology. However, the reliance on small intestinal biopsies for the definitive diagnosis of CD carries along a couple of disadvantages. To begin with, a small intestinal biopsy is invasive, time consuming, and also causes substantial distress to the child and its parents. In addition, it is now well recognized that even with this gold standard investigation several diagnostic difficulties may arise. First of all, the biopsy specimen may be of such poor quality that a diagnosis cannot be made.[119] Secondly, the classical histological lesion may not be abundant as CD often begins with minor intestinal changes that can easily be overlooked.[120-123] Finally, CD can be missed because the expression of the CD lesion may be very patchy.[119] Consequently, a high inter-observer disagreement among pathologists exists.[125] Therefore, in recent years, research attention was focused on finding non-invasive markers to diagnose CD as an alternative for a small intestinal biopsy. The most dramatic change in diagnostic criteria was made this year. First of all, according to the 2012 guidelines, a gluten challenge is not required anymore in children <2 years of age, but only needed under unusual circumstances, i.e., in case of doubt about the diagnosis.

In addition, until recently, villous atrophy (Marsh III) was required to set the diagnosis of CD. However, the new ESPGHAN guidelines have concluded that Marsh II is also sufficient to establish the diagnoses of CD, as it has been proven that these patients actually suffer from CD.[1] By contrast, a Marsh I lesion is still considered insufficient for the diagnosis of CD, as this nonspecific lesion can also be associated with other diseases, i.e. food protein hypersensitivities, giardiasis and other infections.[125] Some patients however, eventually develop CD making follow-up necessary. If severe symptoms are present, it is advisable to try a GFD, but the diagnosis should always be confirmed by a gluten challenge and a second biopsy, after symptoms have stabilized.[1]
The most important change in the new guidelines, is that the diagnosis of CD can be made without histological confirmation in a selected group of patients, which will be discussed below. However, in case a biopsy is needed, at least five samples should be collected, including one from the bulb, as this can be the only affected site.[1]

Human leukocyte antigen typing

The exclusivity of HLA-DQ2.5 and HLA-DQ8 in CD patients and the rarity of patients lacking them, makes HLA-typing also helpful in the diagnostic workup of CD. Indeed, most studies have shown that the sensitivity of HLA-DQ2.5 and HLA-DQ8 exceeds 96%.[1] However, because HLA-DQ2.5 and HLA-DQ8 are also commonly prevalent in the general population, the specificity is quite low. Therefore, in general, HLA-typing is only useful to exclude CD or to make it highly unlikely. This could be especially useful in patients with an increased risk for CD, such as first-degree relatives of CD patients and patients with autoimmune diseases or syndromes associated with CD, as they require repetitive testing for CD. Indeed, according to the new guidelines in asymptomatic patients in a high risk group, this is now the first-line test to be used.[1] In addition, HLA-typing could also be helpful in cases with doubtful histology, or in patients with serological evidence of CD but who lack histological confirmation. In such cases if a patient is negative for HLA-DQ2.5 and HLA-DQ8, CD can be lifelong excluded and no further investigations and follow-up are needed.

Occasionally, HLA-DQ2.5 and HLA-DQ8 negative patients are reported, although in some studies this is higher than expected. Mostly these negative patients are homozygous for the β-chain of HLA-DQ2 (DQ*B1:02) or carry the HLA-DQ2 variant HLA-DQ2.2.[126] Such findings are often not reported by laboratories, and even if reported, the significance of such results is still unknown. Therefore, studies investigating these rare HLA-types are still needed.

Serological markers

Over the last 50 years, the availability of CD specific serological tests has dramatically improved the diagnosis of CD. The first CD-associated antibodies were discovered in 1964 by Berger.[127] These anti-food antibodies against gliadin (AGA) initially seemed very promising, but turned out to be really disappointing in clinical practice, mainly because of a high false positive rate.[128] In the next decade anti-reticulin antibodies were discovered as the first auto-antibodies but also seemed to lack specificity in clinical practice.[129]

However, it was in the early 1980s when the highly specific EMA were discovered.[128,130] These antibodies are typically measured in IgA class, but can also be measured in IgG class in case of an IgA-deficient patient. Despite the high specificity of the EMA test, the search for new antibodies was not ceased, as EMA is measured by means of indirect immunofluorescence, which is a subjective semiquantative method that is despite rigorous quality control not easy to standardize. For that reason, in less trained hands, that is in routine clinical settings, the specificities might be not as high as reported in research laboratories.[131]

In the late 1990s, when tTG was discovered, new ELISAs were developed which initially used guinea-pig tTG and later on human recombinant tTG as an antigen substrate to measure antibodies against tTG (tTGA).[132] Just like EMA, tTGA can be measured in either IgA or IgG class, of which the latter is important in IgA-deficient patients.[133] The user friendly tTGA test is highly sensitive and specific and is therefore widely used a first screening tool for CD, although sensitivity nor specificity reach a 100%, making histological confirmation still required for the diagnosis in most cases.[128] Nevertheless, it seems that a high level of these antibodies is extremely predictive for CD. Illustratively, Barker

et al and Donaldson et al showed that 48 of 49 and 38 of 38 pediatric patients with a tTGA level ≥100 U/mL (≥10 x upper limit) had histological evidence of CD.[134,135] A subsequent study conducted in a mixed adult/pediatric population, showed that tTGA ≥100 U/mL almost exclusively occurs in the setting of CD (73/76 patients) and that the three cases without villous atrophy did have minimal histological changes suggestive of early CD.[136] Finally, a recent study showed (N=128) that all symptomatic patients with a tTGA≥100 U/mOL who also responded to the GFD had CD (N=111).[137] Thus, symptomatic patients with high tTGA levels, who also carry the disease associated HLA-types, and who respond to the diet, do not need a confirmatory small bowel biopsy, which is now also stated by current ESPGHAN guidelines.[1,137]

At last, special attention should be paid to the diagnostic accuracy of serology in children <2 years of age, where EMA and tTGA seem to be less sensitive.[138-140] Fortunately, in this age group newly developed ELISA tests, using deamidated gliadin peptides as an antigen substrate instead of the conventional gliadin peptides, seem to be useful. These antibodies against deamidated gliadin peptides (a-DGP) generally do not outperform EMA and tTGA, but in very young children the IgG subtype of a-DGP seems to be extremely accurate.[141-145] So in very small children, adding IgG a-DGP to the diagnostic work-up will improve the de-tection rate of CD.
Now, two issues remain to be discussed when considering non-invasive diagnosis in CD. First. the new ESPGHAN guidelines do not give a clear statement about IgA deficient children, where IgG tTGA might not perform as well as IgA tTGA.[146] Potentially, IgG a-DGP is a better marker in IgA-deficient children, although in IgA-deficient adults this was not the case.[147] Secondly, in all asymptomatic children and children with positive tTGA ≤100 U/mL histological confirmation is still needed. Therefore, new markers for this patient group need to be found. Potential candidates are plasma citrulline and plasma intestinal fatty acid binding protein, both markers for intestinal damage, and could have an added value when combined with current serology.[148,149] Likewise, measuring gluten specific T-cells in the blood of patients after a short gluten challenge, could improve diagnosis and should be further studied.[150]

Treatment and follow-up

Currently, adherence to a strict lifelong GFD is the only available treatment for CD. In the majority of cases symptoms quickly improve and the inflammation resolves as observed by serological and histological normalization.[1] Numerous therapeutic approaches are currently under development by targeting several pathological pathways, but it is not expected that such therapies will be clinically available on short term.
The individual sensitivity to maximum gluten intake in patients with CD is variable, but the threshold of 20 p.p.m by the Codex alimentatrius, is thought to be safe in the majority of patients.[151,152] However, a GFD is not easy to follow and many products are potentially contaminated with hidden gluten. In addition, patients should get clear guidance from a dietician, because patients on a GFD are more likely to suffer from nutrient and fiber deficiencies.[153] Also, patients must be aware that gluten free food tends to have a high fat and caloric content as an alternative to improve taste.

Measuring dietary adherence is very difficult in CD. In this respect measuring tTGA is the best available indicator but remains suboptimal since it can be negative despite minor (inadvertent) gluten intake.[128] In addition, it is unidentified how quickly antibodies should decline in case of very strict adherence to the diet. In a recent study, it was shown that tTGA levels declined for almost 75% 3 months after the diet and that 1.5 years after the diet the level of tTGA is expected to be below cut-off for negativity.[154] However, 20% of the children are still positive for tTGA two years after the diet, although the dietary compliance was not reported in this study. Moreover, in IgA-deficient there is an additional problem, as IgG tTGA positivity can even persist despite normalization of histology.[155] According to ESPGHAN guidelines tTGA should have normalized approximately one year after diet.[1] However, studies are still focusing on finding better markers to measure compliance. Detecting gluten peptides in the human feces correlates with the amount of gluten intake and seems to be a promising test to measure dietary compliance.[156] In addition, markers for intestinal damage could also be helpful.[148, 149]

Adherence to a GFD is determined by the relief of symptoms and the patient's knowledge about long term benefits of the diet.[157] In symptomatic patients, the benefit of a GFD on the health status and quality of life is clear.[158-169] In asymptomatic individuals however, it is unclear whether they would benefit from treatment, and thus whether they should be screened in the first place. Adherence to a GFD might load them with the burden of the diet and negatively affect their quality of life. On the other hand, screen detected patients might feel better on a diet and might become aware that they actually had symptoms before the diet. In such patients the quality of life could be improved. Unfortunately, studies investigating the quality of life are limited and contradictory.[160-169] In addition, randomized studies have not been performed. Finally, many studies that show a positive effect have been performed in countries, such as Finland, where the availability of gluten free products is excellent. Of course, this may be different in other countries where the GFD is more difficult to follow.

On the long term, the effect of a GFD is also debatable. First, it is believed that patients with untreated CD have an increased mortality risk, even patients with positive serology but normal small intestinal histology.[170-173] However, increased mortality has also been described in treated CD patients, so the true effect of the diet on mortality is uncertain.[173] In addition, early studies showed an increased risk of malignancy in untreated CD patients compared to the general population, while this was smaller in treated CD patients.[174,175] However later studies showed the risk to be much more modest, especially when looking at the absolute risk.[176]

Secondly, a protective effect of the GFD on the development of concomitant auto-immune diseases has been described, but again studies are inconsistent: two studies found that in non-compliant patients the risk of autoimmunity is increased, although statistical significance is only reported in one study.[177,178] Additionally, only three of the five studies that have used the age of diagnosis as a determinant demonstrated an increased risk of autoimmune diseases with increasing age.[178-182]

In conclusion, the benefit of screening for asymptomatic persons is still a subject of debate, because it is not clear if the increase in health status will outweigh the burden of the adherence to the GFD. When considering all these studies, it must be stressed that even when a positive effect was found, the risk was not stratified between symptomatic and asymptomatic individuals. In addition, the selection of truly asymptomatic patients is hard. Many apparently asymptomatic patients have underlying anemia or osteoporosis, on which a GFD has a positive effect. So, should all patients be screened for such from the outside invisible symptoms? These issues make the question of whether to screen or not screen asymptomatic individuals very hard.
In the future, this is one of the most important questions in the CD field that needs to be answered. Especially, because many asymptomatic individuals in the high risk groups are now being screened, as suggested by guidelines, but this is only on the basis of their increased risk for the development of CD. Therefore, long term follow-up and randomized trials are highly needed.

Conclusions

CD is an immune-mediated disorder with a worldwide prevalence of around 1%. The disease occurs in genetically susceptible individuals upon the ingestion of gluten and related prolamins in barley and rye. Gliadin, the alcohol-soluble fraction of gluten is the toxic agent leading to the classical combination of increased intra-epithelial lymphocytes, crypt hyperplasia, and in most cases villous atrophy in the small intestinal-mucosa of untreated CD patients. The pathophysiology of CD is complex, with environmental, genetic and immunological factors contributing to disease development. Historically, the disease was only diagnosed in children with gastrointestinal malabsorption syndrome, although nowadays in most cases CD is presenting with a wide variety of nonspecific signs and symptoms or no symptoms at all. Until now, the only available treatment for CD is a strict GFD, after which symptoms quickly disappear and the small intestinal lesions dissolve. Additionally, it has been suggested that the diet might be even protective against long term complications of CD such as intestinal malignancies and other autoimmune diseases. The best way to exclude the diagnosis of CD is by performing HLA-DQ2.5 and HLA-DQ8 typing, as CD patients lacking these types are thought to be extremely rare. Serologically, CD is characterized by several antibodies that are very accurate in disease prediction of whom EMA and tTGA should be used as first line screening tests for CD. However, the diagnostic accuracy is still not a 100% making a small intestinal biopsy still necessary to confirm the diagnosis in most cases. However, an exception to this rule can be made in symptomatic genetically predisposed (HLA-DQ2.5 or HLA-DQ8 positive) patients with a tTGA ≥100 U/mL who respond well to the diet.

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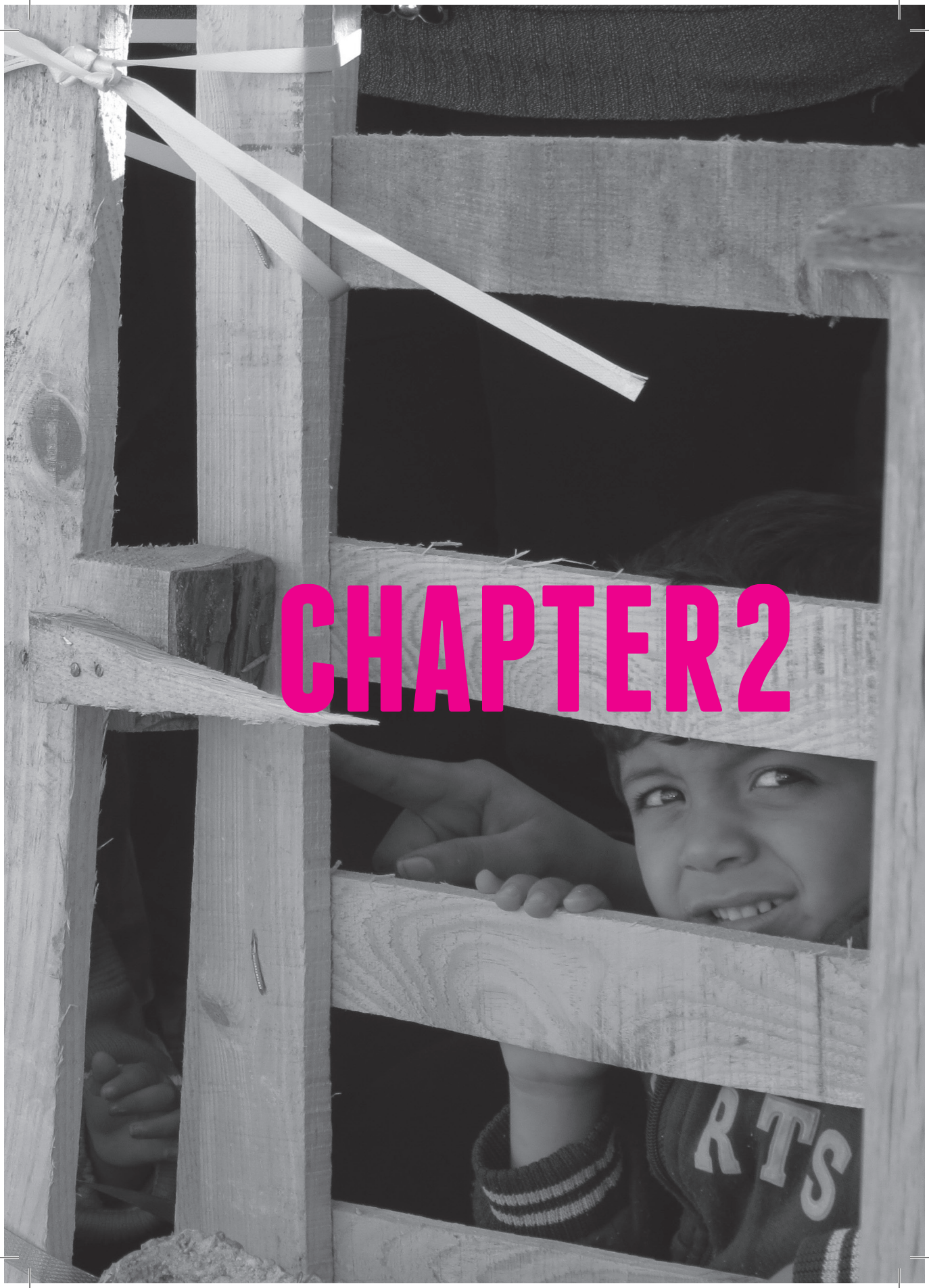
CHAPTER 2

CHAPTER 2

Reproducibility of the histological diagnosis of celiac disease

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Abstract

Objective A small intestinal biopsy is considered to be the gold standard for the diagnosis of celiac disease (CD). However, the assessment of small intestinal histology may vary between pathologists. Our aim was, therefore, to determine the interobserver variability in the histological diagnosis of CD.

Material and methods Biopsy specimens of 297 pediatric patients suspected of having CD were revised by a single experienced pathologist and compared to the original reports. Mucosal changes were scored using the Marsh classification. In patients with a discrepancy in diagnosis, clinical and serological data were used to determine the most probable diagnosis.

Results Although the interobserver variability for the Marsh classification was found to be moderate with a Kappa value of 0.486, the Kappa value for the diagnosis reached an almost perfect agreement (0.850). Nevertheless, in 22 patients a different diagnosis was made by the second observer. Interestingly, in this subgroup relatively more biopsies were classified to be of suboptimal quality. Based on clinical presentation, serology and follow-up, 19 of those patients truly had CD. In 14 of them the diagnosis was originally missed by the first observer while five cases were under-diagnosed by the second pathologist.

Conclusions CD can be missed histologically due to assessment variation between pathologists. A final diagnosis of CD should be based on histology, serology as well as response to the diet.

Introduction

Celiac disease (CD) is a common auto-immune disease characterized by a permanent intolerance to gluten in genetically predisposed individuals.[1,2] The disease usually becomes manifest in the small intestine where it classically leads to mucosal inflammation, destruction of the villi as well as crypt hyperplasia.[3] Although CD was originally known for the classical symptoms of a malaborption syndrome, that is, diarrhea, steatorrhea, and weight loss, nowadays the diagnosis is increasingly being made in asymptomatic individuals and in patients with atypical symptoms such as anemia, osteoporosis, or abdominal discomfort.[4-7] The diagnosis of CD is generally made using a two-step approach.[8–10]

Endomysium antibodies (EMA) and tissue-transglutaminase antibodies (tTGA) are the first diagnostic modality to be utilized when CD is suspected. These serological markers are highly sensitive and specific, but patients with false positive and negative test results are still reported. Therefore, until now, a small intestinal biopsy is still required to confirm the diagnosis.[11] However, even with this gold standard investigation several diagnostic difficulties may arise. First of all, the biopsy specimen may be of such poor quality that an accurate diagnosis is not possible.[12] Second, the classical histological lesions may not always be abundant as the development of CD is a dynamic process that often begins with minor changes that can easily be overlooked.[13–16] Also, the variability in expression of the histological lesions in CD may make diagnostic interpretation more difficult.[17,18] Finally, it should be kept in mind that, especially in children, small bowel mucosal atrophy can also be associated with other diseases that are sometimes difficult to differentiate from CD.[19] Taking all these difficulties into account, it can be anticipated that the rather subjective evaluation of biopsies may lead to variability in interpretation among different pathologists, which can hinder a correct diagnosis. This may be especially problematic in patients with a positive serology and a high clinical suspicion of the disease, but apparently normal small bowel histology.

Unfortunately, studies investigating the variability in histological diagnosis are rare and limited to small study populations. Therefore, we sought to determine the variability in almost 300 patients biopsied for CD based on more than 10 years of experience in our center.

Methods

Subjects

All patients referred between 1998 and 2009 to the Wilhelmina Children's Hospital, Utrecht, The Netherlands, with the suspicion of having CD were included in the study. Patients were referred due to CD associated symptoms or because they carried a risk factor for CD. Patients in whom serological tests were not performed were excluded from the study. A total of 297 patients (Figure 1) between 0.7 and 17.8 years (mean age 5.9 years) were eventually included in the study. In all, 131 (44.1%) were male and 166 (55.9%) were female.

Serology

Serum immunoglobulin A (IgA) EMA was measured by means of indirect immunofluorescence using sections of distal monkey esophagus mounted on glass slides (IMMCO Diagnostics Inc., Buffalo, NY, USA). Serum IgA tTGA was detected by means of ELISA using human recombinant tTG (ELiA Celikey IgA, Phadia AB, Uppsala, Sweden). As recommended by the manufacturer, the serum samples containing an antibody titer of more than 10 U/ml were considered positive. All samples were tested in accordance with the manufacturer's specifications.

Histology

In all patients, biopsies were taken from the distal duodenum by upper gastrointestinal endoscopy. First, the original histology reports of all patients were read by one of the authors (A Mubarak) and the severity of the lesion and diagnosis according to the initial pathologist were recorded. Second, all histological slides were revised by a single experienced gastro-intestinal pathologist (FJW Ten Kate) who was blinded to the original evaluation, clinical, and laboratory data. The original hematoxylin and eosin and, if available, the CD3 stained sections (n = 33) were used for the reevaluation (Figure 1).

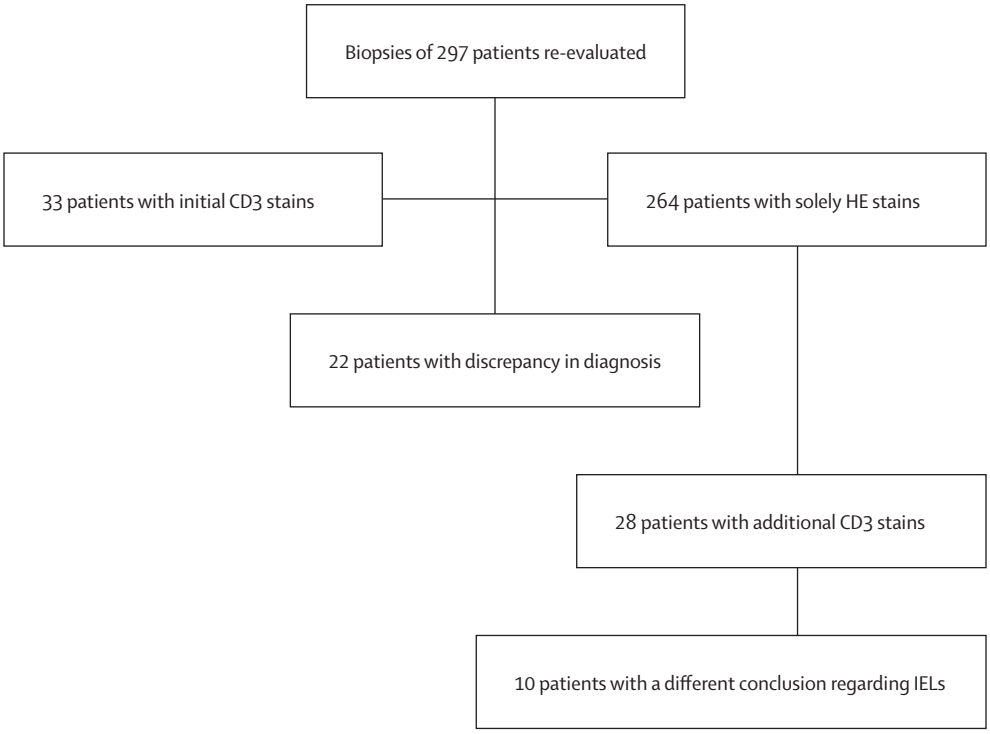

Figure 1 Overview of included patients and performed stains.
HE = hematoxylin and eosin; IELs = intraepithelial lymphocytes.

Before the revision, the quality of the biopsy specimen was classified as good, moderate, or poor. The degree of quality was based on the orientation of the slides and the presence of sufficient undamaged material, including mucosal as well as submucosal tissue. A biopsy was considered to be of poor quality in case of insufficient or damaged material and/or lack of submucosal tissue (Figure 2A). If only the orientation was affected, that is, in case of tangentially cut biopsies, the quality was classified as moderate (Figure 2B). Finally, a biopsy was considered to be of good quality when it was deep, undamaged, and well orientated, that is, when the crypts were perpendicularly positioned under the villi (Figure 2C). The overall quality in a patient was based on the best available biopsy in that patient. Subsequently, mucosal changes in each slide were scored using the Marsh criteria as modified by Oberhuber (0 = normal, I = increased intra-epithelial lymphocytes (by visual estimation); II=increased intra-epithelial lymphocytes and crypt hyperplasia; III = increased intraepithelial lymphocytes, crypt hyperplasia and partial (IIIa), subtotal (IIIb), or total (IIIc) villous atrophy).[3,20] If multiple changes were present within a single fragment or series of fragments in one patient, the most severe lesion was recorded. The overall grade assigned to each case was based on the highest Marsh lesion identified. A Marsh III lesion was considered to be diagnostic for CD. When the Marsh criteria could not be fulfilled in a patient, for example in case of solely villous atrophy or crypt hyperplasia, but no increased number of intraepithelial lymphocytes, biopsies were termed unclassifiable. Biopsies with such bad orientation that it was not possible to establish the Marsh classification were termed as undeterminable.

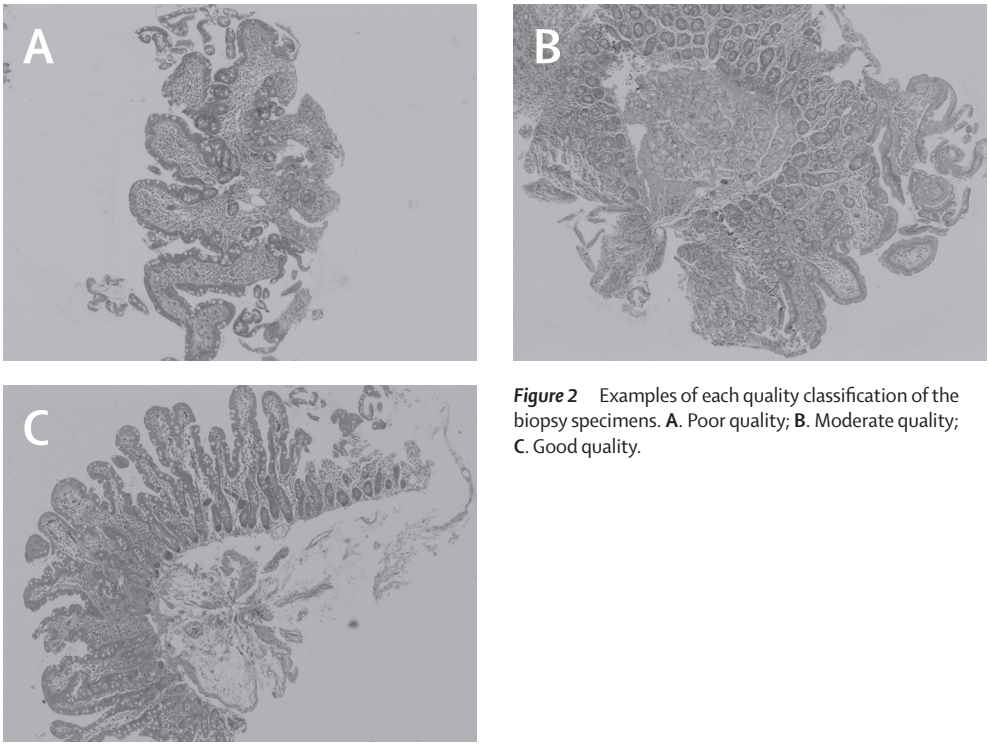

Figure 2 Examples of each quality classification of the biopsy specimens. **A**. Poor quality; **B**. Moderate quality; **C**. Good quality.

In addition, during the second evaluation the biopsy specimens were also evaluated for the presence of neutrophillic, eosinophilic and lymphoplasmocellular cell infiltration as well as the presence of gastric metaplasia. The infiltration was graded as normal or increased. Gastric metaplasia was defined as the presence of gastric epithelial cells containing periodic acid Schiff-positive neutral mucin together with the absence of a brush border. Subsequently, additional CD3 stains (n = 28) were performed whenever there was doubt about the number of intra-epithelial lymphocytes on the hematoxylin and eosin stained sections (visual estimation) (Figure 1). On the CD3 stains, a minimum of 30 intra-epithelial lymphocytes per 100 enterocytes was considered to be diagnostic for lymphocytic enteritis.[21] After evaluation of the CD3 stains, it was determined whether this led to a different conclusion regarding the number of intra-epithelial lymphocytes.

Finally, in patients with discrepancy in the diagnosis between the first and second observers, we aimed at making a final diagnosis which was based on symptoms, (clinical) response to the gluten free diet, serological results, and subsequent biopsies (during a gluten challenge). In patients with a positive serology and subsequent clinical and/or serological response to the diet, CD was considered as the final diagnosis. In case of negative serology and clinically no response to a strict diet, CD was considered to be highly unlikely. When the clinical and serological response could not be determined because a patient was asymptomatic, did not (strictly) adhere to the diet, or because no follow-up was available, the most probable diagnosis was considered to be the diagnosis correlating with the serology.

Statistical analysis

Descriptive analyses were performed using SPSS version 15.0 for Windows (SPSS Inc.). An interobserver variability analysis using the Kappa statistics was performed to determine consistency among the two assessments. Logically, the diagnosis made by the first pathologist was compared to the diagnosis made by the second observer before performing additional CD3 stains. According to Landis and Koch, the strength of agreement for the Kappa coefficient can be classified as poor when kappa values were ≤0, slight for values between 0.01 and 0.20, fair when they ranged from 0.21 to 0.40, moderate when values were between 0.41 and 0.60, substantial in the interval from 0.61 to 0.80, and Kappa values between 0.81 and 1.0 were termed as almost perfect agreement [22]. Finally, the Pearson Chi-Square test was used for statistical comparison of data (histological features and biopsy quality). A p-value of <0.05 was considered statistically significant.

Results

Between one (in four patients) and eight (in one patient) biopsies were obtained per person with a mean of 3.32 biopsies (standard deviation 1.12). The quality of the biopsy specimens was good in 210 (70.7%) patients, moderate in 78 (26.3%) patients, and poor in nine (3.0%) patients. Initial CD3 stains were performed in 33 (11.1%) patients.
In the first assessment, the biopsy slides of 123 (41.4%) patients were classified as Marsh 0 while only 106 (35.7%) patients had this classification in the second assessment (Table 1). In the first evaluation, 158 (53.2%) patients had a Marsh III lesion which is diagnostic

for CD while 14 (57.9%) more patients were classified with this lesion by the second observer. For the Marsh classification, the interobserver variability was found to be moderate with a Kappa value of 0.486.
A total of 160 (53.9%) patients were originally diagnosed with CD while 172 (57.9%) patients matched the diagnostic criteria for CD according to the second pathologist (Table 1). The Kappa value for the diagnosis was 0.850. Neutrophillic, eosinophilic, and lymphoplasmocellular cell infiltration in the lamina propria as well as gastric metaplasia were significantly ($p < 0.001$) more present in patients with CD (Table 2).

Classification / diagnosis	First assessment N (%)	Second assessment N (%)
Marsh 0	123 (41.4%)	106 (35.7%)
Marsh I	3 (1.0%)	2 (0.7%)
Marsh II	1 (0.3%)	2 (0.7%)
Marsh IIIa	42 (14.1%)	15 (5.1%)
Marsh IIIb	58 (19.5%)	66 (22.2%)
Marsh IIIc	58 (19.5%)	91 (30.6%)
Unclassifiable	10 (3.4%)	14 (4.7%)
Undeterminable *	2 (0.7%)	1 (0.3%)
Celiac disease	160 (53.9%)	172 (57.9%)
No celiac disease	137 (46.1%)	125 (42.1%)

Table 1 Frequencies of Marsh classification and diagnosis in the first and second assessment.
* Although the Marsh classification was undeterminable, those patients were diagnosed with CD

Histological features	Celiac disease, N=172	Non-celiac disease, N=125	P-value
Increased lymphoplasmocellular infiltrate	162 (94.2%)	87 (69.6%)	< 0.001
Increased eosinophilic infiltrate	90 (52.3%)	16 (12.8%)	< 0.001
Increased neutrophilic infiltrate	84 (48.8%)	6 (4.8%)	< 0.001
Gastric metaplasia	47 (27.3%)	9 (7.2%)	< 0.001

Table 2 Frequencies and statistical significance of several histological features in celiac disease versus non-celiac disease patients.

We also reviewed the results of serological testing in these patients. Of the patients originally diagnosed with CD (n = 160), two were solely negative for EMA, four patients were only negative for tTGA, and in one patient both EMA and tTGA were undetectable in the serum. On the other hand, of the patients in whom CD was originally excluded (n = 137), 30 patients had dual positivity for EMA and tTGA and 22 patients had solely increased EMA levels in the serum. After revision, 13 (43.3%) of those patients with apparently falsely increased EMA and tTGA turned out to have CD according to the second pathologist while only one (4.5%) patient with solely falsely increased EMA turned out to have CD. Additional CD3 stains were performed in 28 (9.4%) patients (Figure 1).

Patient	Age, gender	Symptoms	Dietary response	tTGA (U/ml)	EMA	First assessment	Second assessment	Challenge	Diagnosis
1*	6.0, M	No symptoms	Ud	>100	Pos	Marsh IIIa	Crypt-hyperplasia°	-	CD
2	12.0, M	Classical	No follow-up	29	Pos	Marsh IIIb	Clearly no CD, Marsh ud°	-	CD
3	15.3, F	Classical	Ud, no strict adherence**	131	Pos	Marsh IIIa	Marsh I	-	CD
4*	4.1, F	Classical	Good**	133	Pos	Marsh IIIa	Crypt-hyperplasia	-	CD
5	4.8, F	Classical	No response**~	38	Pos	Marsh IIIa	Marsh 0°°°	-	CD
6*	8.3, F	Bloating	Good**	>100	Pos	Marsh 0	Marsh IIIb	Marsh IIIb	CD
7*	4.4, M	No symptoms	Ud**	58	Pos	Marsh 0	Marsh IIIb°	-	CD
8*	9.2, F	No symptoms	Ud**	6	Pos	Marsh I	Marsh IIIa°	Marsh IIIa	CD
9	3.9, M	Classical	Good**	26	Pos	PVA	Marsh IIIb°	Marsh IIIa	CD
10	7.9, M	Classical & Dermatitis herpetiformis	Good	>100	Pos	PVA	Marsh IIIa°	-	CD
11	8.6, M	No symptoms, Down	Did not start	>100	Pos	Marsh 0	Marsh IIIb°°°	-	CD
12	6.4, F	Classical	No follow-up	>100	Pos	Marsh 0	Marsh IIIa°°°	Marsh IIIa	CD
13*	6.8, F	Classical	Did not start	17	Pos	PVA	Marsh IIIb	-	CD
14	9.1, F	Classical	No response, but IBS was also diagnosed	>100	Pos	Marsh 0	Marsh IIIa	-	CD
15	1.1, M	Classical	No response	0	Neg	Marsh II	Marsh IIIa°	Marsh 0	No CD
16	4.4, F	Classical, DM type 1 resistant to therapy	Good**	>133	Pos	Marsh 0	Marsh IIIb	Marsh 0	CD
17*	7.4, M	Classical	Did not start	0.9	Neg	Marsh 0	Marsh IIIb	-	No CD
18	2.4, F	Classical	Did not start	>133	Pos	PVA	Marsh IIIa	-	CD
19*	10.4, F	Bad-tempered	Did not start	99	Pos	PVA	Marsh IIIb°	-	CD
20	1.6, M	Classical	Did not start	0	Neg	Marsh 0	Marsh IIIb°°	-	No CD
21	15.7, M	Classical	Good **	45	Pos	Marsh 0	Marsh IIIa	Marsh IIIa	CD
22	1.6, M	Classical	Good**	23	Pos	PVA	Marsh IIIa	Marsh IIIa	CD

Table 3 Characteristics of patients with discrepancy in diagnosis between the first and second assessment. Classical: abdominal pain and/or diarrhoea and/or constipation and/or failure to thrive. tTGA, tissue transglutaminase antibodies; EMA, endomysium antibodies; M, male; F, female; DM, diabetes mellitus; ud, undeterminable; IBS, irritable bowel syndrome; pos, positive; neg, negative; PVA, partial villous atrophy; CD, celiac disease. * Positive family history. ** Serology normalised after initiation of the diet. ~Growth retardation eventually attributed to a syndromal disorder. ° CD3 stains were initially performed. °°Additional CD3 stains showed a normal number of Intra-epithelial lymphocytes. °°° Additional CD3 stains showed an increased number of Intra-epithelial lymphocytes

Five of the 12 patients who were concluded to have normal intra-epithelial lymphocyte counts on the hematoxylin and eosin stained slides had an elevated number of intraepithelial lymphocytes on the CD3 stained biopsies. Five of the 16 patients who appeared to have an elevated number of intra-epithelial lymphocytes on the hematoxylin and eosin stained slides had a normal count on the CD3 stain. Thus, in 10 (35.7%) of the 28 patients, performing CD3 stains led to a different assessment with regard to the number of intra-epithelial lymphocytes than was originally made based on the hematoxylin and eosin stained slides.

In 22 (7.4%) patients, a different diagnosis was made by the second observer (Table 3). Interestingly, in this subgroup relatively more biopsies were classified to be of moderate (n = 9, 40.9%) and poor (n = 2, 9.1%) quality while only 11 (50.0%) biopsies were considered to be of good quality. The difference in the quality of the biopsy specimen between the patients with discrepancy in diagnosis and the patients without discrepant diagnosis was statistically significant (p-value 0.027) when comparing the biopsies with good quality versus the biopsies with suboptimal quality.
A mean of 3.00 biopsies was taken (range 2–6, standard deviation 0.976) in these patients which did not differ significantly from the number of biopsies taken in the total study population. Most patients (n = 16) had the classical symptoms of CD, eight had a family history for CD, one patient had Down syndrome, and one patient had diabetes mellitus type I.

Interestingly, in five patients who were originally diagnosed with CD, the diagnosis was excluded by the second pathologist (Table 3). However, taking into account the clinical presentation, response to the diet, and serology (all positive for tTGA and EMA), these five patients are likely to have CD.
Likewise, 17 patients in whom the diagnosis of CD was originally excluded were diagnosed with CD during the second evaluation. Based on the clinical and serological data, 14 patients are considered to truly have CD. The remaining three patients, who did not start the diet, had negative serology which makes CD unlikely. In some patients, a gluten challenge (patient numbers 6, 8, 9, 12, 15, 16, 21, and 22) had been performed as serology and histological reports were discrepant. Interestingly, in five (patient numbers 6, 8, 12, 21, and 22) of the eight patients who had undergone subsequent biopsies during a gluten challenge, the Marsh classification of these subsequent biopsies, which were evaluated only by the original pathologist, were exactly the same as observed by the second pathologist. In patient number 9, a Marsh IIIb lesion was demonstrated by the second pathologist while a Marsh IIIa lesion was found during gluten challenge. In patient number 15, a Marsh 0 was found during the challenge while the first pathologist diagnosed a Marsh II lesion and the second pathologist a Marsh IIIa lesion. Based on the negative serology and lack of response to the diet, CD is highly unlikely in this patient. Surprisingly, patient number 16 was diagnosed with a Marsh 0 lesion during gluten challenge, in accordance with the first pathologist, while the second observer has classified the biopsy of this patient as Marsh IIIb. Nevertheless, this patient most likely has CD due to the excellent clinical and serological response to the diet.

In the 22 patients, initial CD3 stains had been performed in eight patients. Additional CD3 stains were performed in four patients due to a doubtful number of intra-epithelial lymphocytes on the hematoxylin and eosin stained sections. Interestingly, in two patients (patient numbers 5 and 20), the conclusion regarding intra-epithelial lymphocytes made by the second observer on the basis of the hematoxylin an eosin stains was contradicted, and was thus in accordance with the conclusion made by the first observer. In patient numbers 11 and 12, the intra-epithelial lymphocyte counts were not different from the counts on the hematoxylin and eosin stained slides by the second observer.

Discussion

Serological tests such as EMA and tTGA are reliable screening methods for CD and are widely used to select patients for small intestinal biopsy, the internationally accepted gold standard investigation for CD. However, it is now widely recognized that even a small intestinal biopsy may not always be 100% accurate. Indeed, this study, which is the first study in such a large pediatric cohort, showed discrepancy in the diagnosis of CD between two independent evaluations in 22 (7.4%) of the 297 patients. Although the Kappa value (0.850) indicated an almost perfect agreement, 14 of these patients were initially misdiagnosed as non-celiac. Therefore, some of these patients did not start the gluten-free diet which can lead to both short- and long-term complications.[23] Additionally, eight of those 14 patients unnecessarily underwent a subsequent biopsy after being challenged with gluten which also has several disadvantages: it can cause considerable symptoms and possibly has a negative effect on the child's growing potential.[24] Moreover, undergoing a small bowel biopsy is associated with substantial distress to the child and the parents and also carries along the potential risk of complications of general anesthesia or sedation.

Our study thus suggests that CD can be missed due to a difference in assessment between pathologists, which is in accordance with previous reports in which CD was only diagnosed after revision of initially normal biopsies or performing additional biopsies.[25,26] This disagreement most commonly occurs due to suboptimal quality of the biopsy specimen, as was demonstrated by the present study, but also due to patchiness of CD and the possible absence of the classical histology. Interestingly, and by contrast to the abovementioned studies, disagreement in our study did not only occur in case of atypical lesions, but also in patients with classical CD histology. This emphasizes the fact that biopsy interpretation is a subjective skill dependent on the pathologist. Interestingly, in the current study, no patients were originally misdiagnosed as celiacs. This is by contrast to a previous Hungarian study which showed that in a significant amount of patients the original diagnosis of CD was incorrect while no new CD patients were detected after revision.[12] A potential cause for this difference is that the Hungarian study, by contrast to our study, selected a group of patients in the era that EMA and tTGA were unavailable; selection of patients for small intestinal biopsy was thus solely based on clinical grounds. By contrast, our study population has a relatively high proportion of patients with positive serology, and thus CD, because patients with negative serology are less likely to be referred to our center for a biopsy.

All 14 patients who were missed during the first assessment had positive EMA while 13 of them had elevated levels of tTGA. Those patients were a large proportion of the 52 patients who were thought to have false positive serology. Especially in the 30 patients with combined positivity for EMA and tTGA but apparently normal histology, the initial diagnosis was likely to be revised by the second pathologist (43.3%). By contrast, in only one of the 22 patients with solely elevated EMA and apparently normal histology according to the initial assessment, the diagnosis was revised. This illustrates that the diagnosis of CD should not be solely based on histology, but serology, symptoms, and response to the diet should also be taken into consideration, which is in support of recent experts' suggestions (Table 3).[27] These experts also propose adding HLA DQ2.5 or DQ8 positivity to the diagnostic criteria; unfortunately, this information was unavailable for our study population due to the retrospective study design. Alternatively, a revision of the biopsy should be considered in case of discrepancy between serology and histology, especially when both EMA and tTGA are elevated while histology appears to be normal.

Of course, in case of disagreement between serology and histology, not only assessment variability, but also other causes of missing the diagnosis of CD should be kept in mind. For example, in patients using immunosuppressive therapy histological inflammation may be minimal or absent. In addition, the diagnosis can be missed if a patient is not consuming a significant amount of gluten, which is not uncommon in children. Another important cause for missing CD is sampling error: the patchiness in CD is not only witnessed within a single biopsy fragment, but extends throughout the whole gastrointestinal tract. In fact, it has recently been suggested to take at least 3–5 duodenal biopsies per patient, including bulb biopsies, as this site may be the only affected site in CD.[17,18,28] In the present study, due to the retrospective design, duodenal bulb biopsies were not taken routinely and not all patients had a minimum of 3–5 biopsies. Therefore we cannot exclude that CD has been missed in at least some of the patients in the current study. For example, this sampling error may have occurred in patient number 16 during the gluten challenge; although the most probable diagnosis was considered to be CD, this diagnosis was missed during the gluten challenge. Alternatively, the pathologist may have missed the diagnosis in this patient due to interpretation difficulties.

The Marsh classification is the most commonly used categorization method among pathologists for the diagnosis of CD. In this study, we also determined the interobserver reproducibility of this classification which, with a Kappa value of 0.486 (moderate agreement), was much lower than that of the diagnosis. This value is slightly higher than reported before by Corazza et al. who found a Kappa value of 0.35 (fair agreement) in a much smaller study performed in 60 patients (adults and children).[29] By contrast, another similar study, which used a two-steps semistructured method and not the original Marsh classification for the histological evaluation, showed comparable results in 73 children with Kappa values of 0.53–0.57 (moderate agreement).[30] Unfortunately, both studies did not determine the eventual disagreement for the diagnosis.

Histological features, other than the characteristics of the Marsh classification, were also evaluated by the second observer (Table 2). We found that CD is also associated with an increased infiltration of neutrophillic, eosinophilic, and lymphoplasmocellular cells in the lamina propria as well as the presence of gastric metaplasia. However, these features did not occur exclusively in CD which makes them aspecific. In fact, according to earlier reports, inflammation, or hypercellularity, of the lamina propria and gastric metaplasia are considered to be non-specific markers of mucosal injury that may thus also develop due to gluten toxicity.[31–34]

In the current study, a CD3 stain was not performed routinely. It was ordered by the first pathologist in case of doubt about the number of intra-epithelial lymphocytes (n = 33), which were measured by visual estimation. During the revision process, the same strategy was carried out and in 28 cases an additional CD3 stain was performed. In 10 patients, a different conclusion concerning the number of intra-epithelial lymphocytes was made. In our experience, most problems in evaluation occurred in patients with discontinuity in the intraepithelial lymphocytosis within the same biopsy specimen. Especially in the hematoxylin and eosin stained sections, the lack of contrast between the immunoreactive cells and the epithelial cell population may complicate exact counting of the lymphocytes which can lead to an underestimation of the number of intra-epithelial lymphocytes. We therefore stress for caution when interpreting the intra-epithelial lymphocytes and suggest, similar to other authors, that CD3 staining and counting should be made whenever intra-epithelial lymphocytosis is suspected on the hematoxylin and eosin stained slides by visual estimation.[21]

In conclusion, this study showed that CD can be missed by histological evaluation due to several causes. Because a missed diagnosis of CD can be potentially harmful, we suggest the following strategy. The pathologist who initially evaluates the slides should state the quality of the biopsies in the report. Further, it should be stated whether on the basis of histology, the diagnosis can be made with confidence. For this, we suggest the following definitions: (1) the histological diagnosis of CD is probable, (2) CD is histologically possible, and (3) CD is histologically not probable. We do believe that by applying this method, a more useful report will be available for the clinician. Finally, in case of a discrepancy between serology and histology, revision of the biopsy should be made by a (second) pathologist before considering subsequent biopsies.

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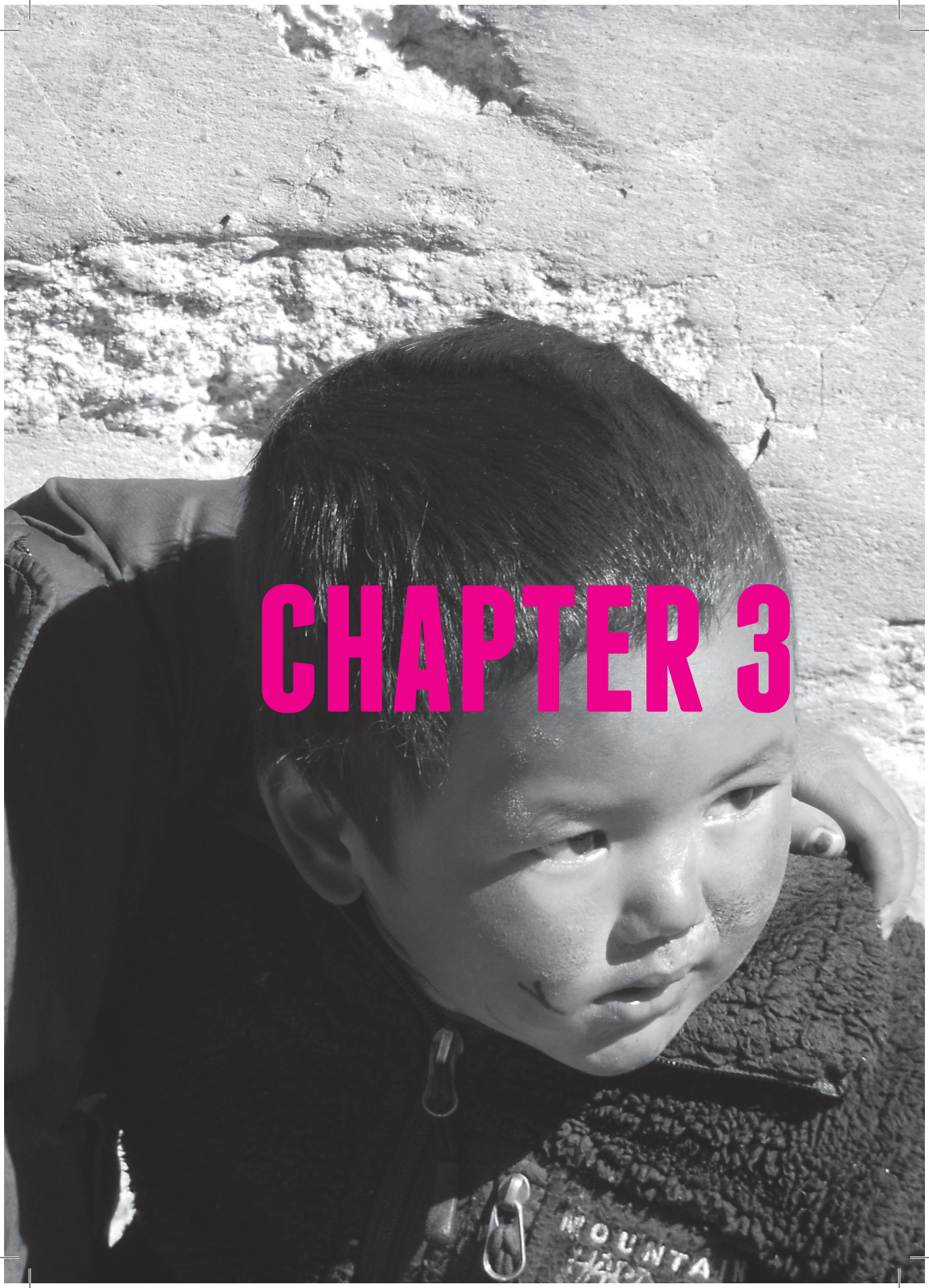

CHAPTER 3

CHAPTER 3

Immunohistochemical CD3 staining detects additional patients with celiac disease

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Abstract

Objective The aim of this study was to investigate whether performing immunohistochemical CD3 staining, in order to improve the detection of intra-epithelial lymphocytosis, has an additional value in the histological diagnosis of celiac disease.

Material and methods Biopsies, stained by hematoxylin and eosin (HE) of 159 children were evaluated using the Marsh classification. Subsequently CD3 stains were evaluated separately and independently.

Results A difference in evaluation between the routine HE sections and the CD3 stains was present in 20 (12.6%) cases. In 10 (6.3%) patients the diagnosis of celiac disease (Marsh II and III) changed upon examination of the CD3 stains: in 9 cases celiac disease had initially been missed on the HE sections while 1 patient had been over-diagnosed on the routine sections. In all patients the final diagnosis based on the CD3 stains was concordant with serological results, but was not so previously. In the other 10 (12.3%) patients the detection of sole intra-epithelial lymphocytosis (Marsh I) improved. Nine patients turned out to have Marsh I on CD3 sections, but this had been missed on routine sections. Interestingly, the only patient with negative serology had giardiasis. Finally, in 1 patient, with negative serology, in whom Marsh I was suspected on HE sections, this diagnosis was withdrawn after evaluation of the CD3 sections.

Conclusion Staining for CD3 has an additional value in the histological detection of celiac disease lesions, with CD3 stains to be performed whenever there is discrepancy between serology and the diagnosis made on HE sections.

Introduction

Celiac disease is a permanent intolerance to gluten, a storage protein in wheat and the related grain species barley and rye.[1] Ingesting these grain species in genetically susceptible individuals causes inflammation of the small intestine, which is reversible upon elimination of gluten from the diet.[2,3] To screen for celiac disease, highly specific and sensitive antibodies are available, but until now, in many cases a small intestinal biopsy is required for the diagnosis.[4,5]

Typically, the trias of an increased density of intra-epithelial lymphocytes (IELs), hyperplasia of the crypts and villous atrophy are observed in patients with celiac disease.[2] However, villous atrophy can also be found in various other diseases such as giardiasis, Whipple's disease, tropical sprue etc.[6] On the other hand according to most recent guidelines, villous atrophy is not necessary for the diagnosis of celiac disease, provided that intra-epithelial lymphocytosis and crypthyperplasia are present.[5] Crypt hyperplasia is a sign of increased intestinal turnover, and is thought to occur secondary to the villous destruction and inflammation. The presence of intra-epithelial lymphocytosis, although not pathognomonic for the disease, is considered to be the most important histological finding for celiac disease.[7,8] Therefore, in many cases detecting IELs provides the key to a correct diagnosis. The presence of IELs is usually evaluated on hematoxylin and eosin (HE) stains, but due to the lack of contrast between the cells, the presence of intra-epithelial lymphocytosis might not always be clear, especially when the number of IELs is only moderately increased. Because IELs are CD3 positive cells, performing immunohistochemical staining against CD3 might aid in estimating the number of IELs. The aim of this study was therefore to investigate whether CD3 staining should routinely be performed on all biopsies, or that it is only necessary in specific cases.

Methods

Patients

Pediatric patients (53 girls; 106 boys) suspected with celiac disease who had undergone a small intestinal biopsy between March 2009 and October 2012 in the Wilhelmina Children's Hospital, Utrecht, The Netherlands, were prospectively included in the study. Patients were referred to us because of celiac disease associated symptoms or because they carry a risk factor for celiac disease. Patients were between 0.9 years and 17.8 years at the time of the biopsy. When a patient had undergone more than one biopsy session, only biopsies from the first session were included in the study. The serological data of the patients were collected from the medical records. The study was performed according to the guidelines of the local medical ethical board.

Histology

Biopsies were obtained by upper endoscopy. Pediatric gastroenterologists were asked to take at least 4 biopsies from the distal duodenum and as of the end of 2009 at least 1 biopsy from the duodenal bulb. In reality, 0 (in 33 cases) to 5 biopsies were obtained from the duodenal bulb with an average of 2.0 biopsies. From the distal duodenum 3.1 (range

1-7) biopsies were acquired on average. Biopsies were fixed in formalin (10% neutral buffered formalin) and then embedded in paraffin, and 4-um-thick sections were stained with HE, Periodic acid-Schiff and CD3 (Dako, Glostrup, Denmark; batchnumber 81639; dilution 1:50; pretreatment with EDTA).
All biopsies were evaluated by an experienced pathologist, specialized in gastro-intestinal diseases, who was blinded to the clinical data of the patients. The pathologist first evaluated the HE stained sections. On a separate occasion the CD3 stains were evaluated independently from the HE stains.
Biopsy results were reported according to the Marsh classification, as modified by Oberhuber.[2,9] In case of patchy lesions, the final Marsh score was based on the worst affected site. Marsh I lesions are defined as an increased number of IELs. On the HE-stains this was determined by visual estimation. On the CD3 stains, ≥30 lymphocytes per 100 epithelial cells were considered as intra-epithelial lymphocytosis.[10,11] In Marsh II lesions crypt hyperplasia along with an increased number of IELs are found. Finally, Marsh III lesions include the findings in Marsh II, along with various grades of villous atrophy.
Marsh II and Marsh III lesion were considered to be diagnostic for celiac disease but were reported separately. Marsh I was reported as a separate entity. Celiac disease was excluded in patients with a normal small intestine (Marsh 0) or abnormalities not diagnostic for Marsh II or III (i.e. only crypt hyperplasia and/or villous atrophy without intra-epithelial lymphocytosis),

Data-analysis
Descriptive statistics using SPSS for Windows version 15.0 were used to compare the conclusion of the pathologist before and after performing the CD3 stains.

Results

A diagnosis of Marsh III, based on the HE stains, could be made in 87 patients, but celiac disease was rejected in 1 (1.1%) patient with negative celiac disease serology after examination of the CD3 stains (Table 1). Only 1 patient had a Marsh II lesion on the HE sections which was also recognized on the CD3 stains.

		Evaluation of CD3 stains			
		Positive			Negative
Evaluation of HE stains		Marsh III (N=93)	Marsh II (N=3)	Marsh I (N=13)	No CD (N=50)
	Marsh III (N=87)	86 (98.9%)	-	-	1 (1.1%)
	Marsh II (N=1)	-	1 (100%)	-	-
	Marsh I (N=6)	1 (16.7%)	-	4 (66.7%)	1 (16.7%)
	No CD (N=65)	6 (9.2%)	2 (3.1%)	9 (13.8%)	48 (73.8%)

Table 1 The Marsh classification of duodenal biopsies on HE stains versus CD3 stains.
HE, hematoxylin and eosin; CD, celiac disease. *≥30 intraepithelial lymphocytes per 100 epithelial cells

On the HE stains, 6 patients were considered to have a Marsh I lesion, but in 2 patients the diagnosis of Marsh I changed after assessment of the CD3 stains. In 1 (16,7%) patient with negative celiac disease serology a Marsh 0 was seen instead and in the other one (16.7%) a Marsh III lesion was present. In the latter patient, who had a positive tTGA and EMA, this could be explained by the fact that on the HE sections a Marsh I lesion was found in the bulb and crypt hyperplasia and villous atrophy (but without intra-epithelial lymphocytosis) were found in the distal duodenum. So, on the HE stains the most affected site seemed to be the duodenal bulb. However, on the CD3 stains an increased number of IELs was seen in both parts of the duodenum while the most affected site on the CD3 stains turned out to be the distal duodenum (Marsh III).

Celiac disease was excluded in 65 patients on the HE slides. However, celiac disease could be diagnosed after employing CD3 stains in 6 (9.2%) patients with Marsh III and 2 (3.1%) patients with Marsh II histology. All of them had positive celiac disease serology. Finally, after evaluation of the CD3 stains Marsh I lesions were identified in another 9 (13.8%) patients. Eight of them had positive celiac disease antibodies whereas 1 patient was negative for tTGA and EMA. Interestingly, the patient with negative serology and Marsh I had giardiasis.

In summary, a difference in assessment between the HE slides and the CD3 sections was found in 20 (12.6%) patients. In 9 (5.7%) patients a Marsh I was found and in 1 (0.6%) patient a Marsh I was rejected when evaluation the CD3 sections. Most importantly, in 10 (6.3%) patients the diagnosis of celiac disease (Marsh II and Marsh III) changed: on the CD3 stains 1 (0.6%) patient turned out to have no celiac disease, 2 (1.3) patients turned out to have Marsh II lesions and 7 (4.4%) patients Marsh III histology.

Discussion

Even after recent update of the ESPGHAN guidelines, for most patients a histological assessment of duodenal biopsies is still necessary for the diagnosis of celiac disease. In this respect, apart from grading villous atrophy and crypt hyperplasia, judging intra-epithelial lymphocytosis is essential.[5] We now evaluated whether performing CD3 stains improves the histological evaluation of celiac disease.

Our results show that compared to HE stains alone CD3 stains did lead to a different assessment in 12.6% (20/159) of the patients. More importantly, almost 10% (9/96) of the patients with celiac disease (Marsh II and III) in the current study would have been missed if a CD3 stain had not been performed. It is highly unlikely that these patients were over-diagnosed because all of them had positive celiac disease serology. They would probably not have started a gluten free diet or would unnecessarily have had subsequent biopsies. On the other hand, when the diagnosis of celiac disease is already made on the HE slides, the chance that celiac disease will be ruled out on subsequent CD3 stains is small. Yet, without a CD3 stain 1 of the 48 patients with apparently celiac disease on the HE stains would have been misdiagnosed with the disease, and would therefore unnecessarily have carried the burden of following the gluten free diet. Interestingly, in this

over-diagnosed patient, celiac disease serology was negative. Therefore, in order to catch all Marsh II and Marsh III lesions and at the same time not over-diagnose any patient with celiac disease, CD3 staining should be performed in all cases of villous atrophy and/or crypt hyperplasia, when the initial conclusion made on the HE stains is discrepant with the serology results.

In addition, performing CD3 staining, also leads to an improved detection of Marsh I lesions. In fact, in almost 14% (9/65) of the patients in whom on the HE slides celiac disease was excluded, a Marsh I lesion was found. Interestingly, only 1 of these 9 patients had negative serology, but the Marsh I in this patients could be explained by a giardiasis infection. In addition, without CD3 staining another patient, with negative serology, would have been over-diagnosed with Marsh I. Therefore, in order to catch al Marsh I lesions, that are unexplained by other conditions, and at the same time not over-diagnose any patient with Marsh I, CD3 staining should again be performed whenever there is discrepancy between serology and histology.

The implication of finding lymphocytic enteritis (Marsh I) is unclear however, because this lesion does not occur exclusively in celiac disease, as was also seen in our patient with giardiasis.[5,12,13] Nevertheless there is some evidence that a Marsh I lesion is clinically important and should therefore be detected, especially in patients with positive serology. First of all, Marsh I abnormalities may be an early stage of celiac disease and may thus develop in some patients into active celiac disease over time.[14-21] In addition, a gluten challenge seems to cause mucosal deterioration and a diagnosis of celiac disease in some patients with Marsh I.[22] Finally, various studies have shown that patients with Marsh I lesions might benefit from the gluten free diet, at least on the short term.[17-21]

In conclusion, immunohistochemical staining for CD3 has an additional role in the histological detection of celiac disease lesions. In order to make an appropriate diagnosis of the total spectrum of celiac disease associated lesions, CD3 staining should be performed in all cases of discrepancy between serology and the histological conclusion on the routine sections.

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PART TWO


disease
directed
characterized
heterodimer
HLA-DQ8
research
basal
gluten
diagnostics
Classically
atrophy
negative
destruction
small
tTGA
diagnosis
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endomysium
barley
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transglutaminase
hyperplasia
non-invasive
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HLA-DQ2.2
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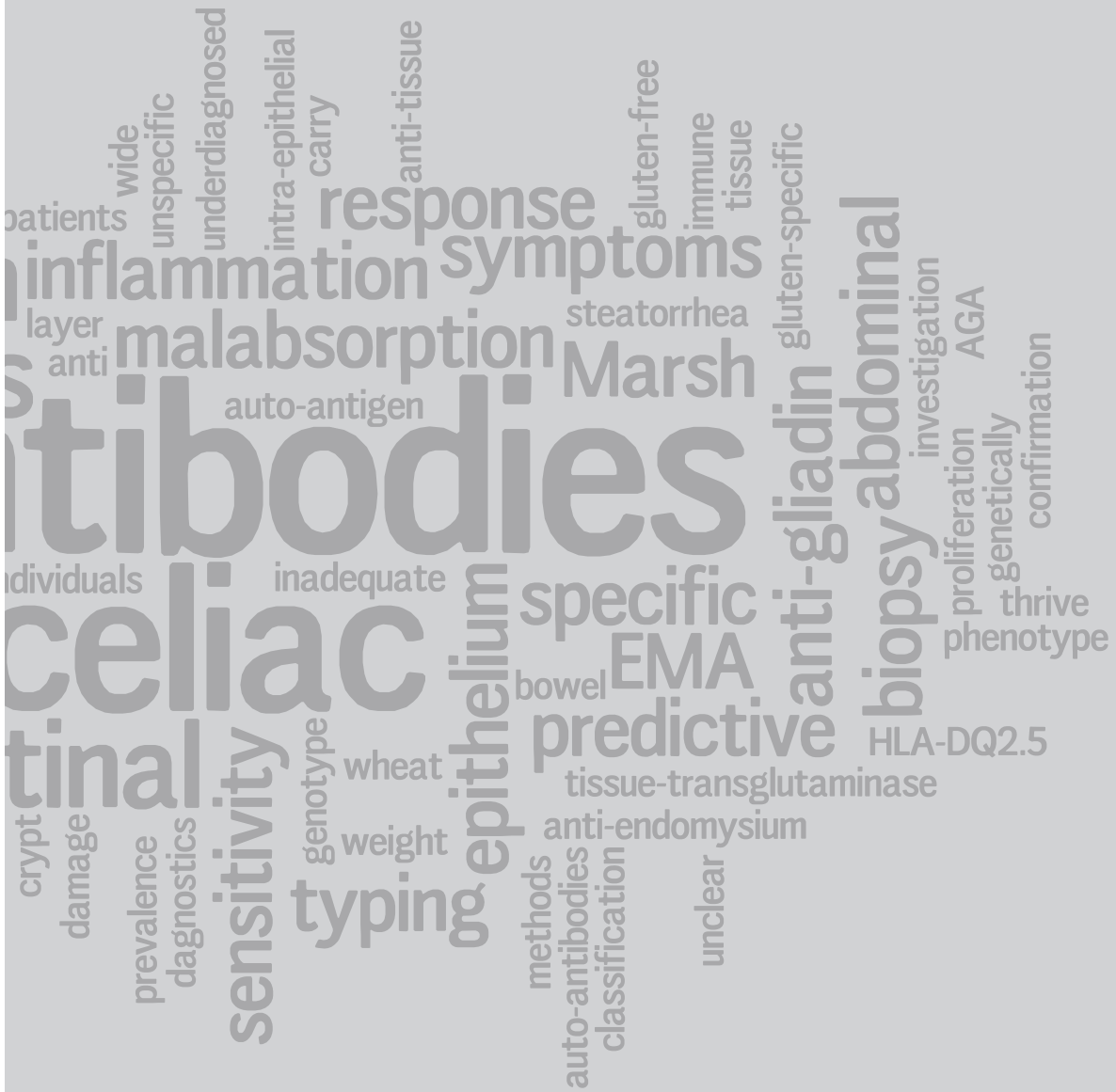
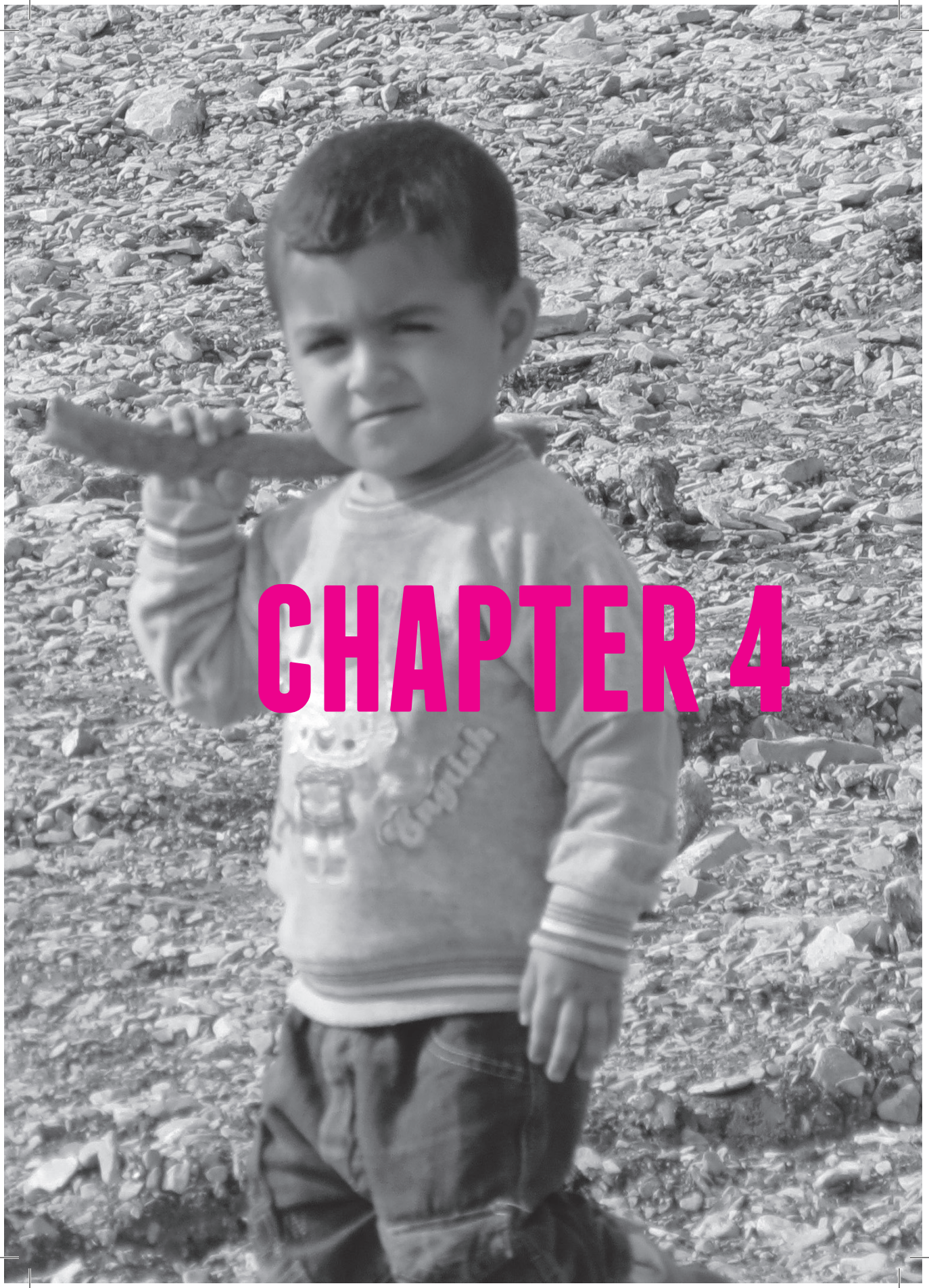


NON-INVASIVE STRATEGIES IN THE DIAGNOSIS OF CELIAC DISEASE



CHAPTER 4

CHAPTER 4

The human leukocyte antigen DQ2.2 and celiac disease

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Abstract

Patients with celiac disease (CD) lacking both human leukocyte antigen (HLA)-DQ2.5 in cis (DQA1*05:01, -DQB1*02:01) or trans (DQA1*05:05, -DQB1*02:02) configuration and HLA-DQ8 (DQA1*03:01, -DQB1*03:02) are considered to be rare. Therefore, absence of these genotypes is commonly used to exclude the diagnosis of CD. To investigate whether this approach is justified, the HLA-distribution in 155 children with CD was studied. A total of 139 (89.7%) patients carried HLA-DQ2.5. Of the remaining patients 7 (4.5%) carried HLA-DQ8. Interestingly, the 9 (5.8%) patients lacking HLA-DQ2.5 and HLA-DQ8, carried HLA-DQA1*02:01, -DQB1*02:02 (HLA-DQ2.2). Therefore, HLA-DQ2.2 should be included as an important HLA-type related to CD.

Introduction

Celiac disease (CD) has a strong genetic component mainly related to human leukocyte antigen (HLA) class II genes.[1] In fact, earlier studies indicate that CD can only occur in the context of 2 specific HLA-molecules: HLA-DQ2.5 and HLA-DQ8.[1-5] HLA-DQ is an αβ-heterodimer, of which the α- and β-chains are encoded by the HLA-DQA1 and HLA-DQB1 genes, respectively. HLA-DQ2.5 is either expressed in cis (encoded by HLA-DR3-DQA1*05:01, -DQB1*02:01) or trans configuration, encoded by HLA-DR11-DQA1*05:05, -DQB1*03:01 (HLA-DQ7.5) and HLA-DR7-DQA1*02:01, -DQB1*02:02 (HLA-DQ2.2). In the latter case the α-chain from the HLA-DQ7.5 (DQA1*05:05) and the β-chain from the HLA-DQ2.2 (DQB1*02:02) combine together to form DQA1*05:05, -DQB1*02:02, which is molecularly highly similar to HLA-DQA1*05:01, -DQB1*02:01 and therefore also called HLA-DQ2.5. HLA-DQ8 is encoded by HLA-DR4-DQA1*03:01, -DQB1*03:02.

The extremely high percentage of CD patients carrying either HLA-DQ2.5 or HLA-DQ8 has led to the common practise of excluding CD in patients without these HLA-types.[1-5] However, other reports describe CD patients who lack both HLA-DQ2.5 and HLA-DQ8, making the validity of this strong negative predictive value questionable.[6-8] Therefore, we set out to study the distribution of HLA subtypes in pediatric CD patients in 2 medical centers in the Netherlands.

Materials and methods

Study population

The study consisted of 2 parts. A retrospective study was carried out in the VU University Medical Center, Amsterdam, The Netherlands and included all biopsy proven (Marsh III) pediatric patients with CD (N=70; 50 females, 20 males) in whom HLA-typing was performed between 2003 and 2011. In this cohort HLA-typing had been performed without any prior selection. To avoid skewing of the study population, all patients were diagnosed independent from the HLA-typing. The average age at diagnosis in this group was 5.7 years and the average age at the time that HLA-typing was performed, was 7.6 years.

A prospective study was performed in the Wilhelmina Children's Hospital, Utrecht, The Netherlands. In this part of the study, HLA-typing was performed in all consecutive biopsy proven (Marsh III) pediatric patients with CD (N=85; 60 females, 25 males) in whom the diagnosis was made between December 2009 and June 2011. The average age at diagnosis was 6.2 years while HLA-typing was performed at an average of 6.5 years. In both centers, the study was carried out according to the guidelines of the local Medical Ethical Board.

HLA -typing

Genomic DNA was isolated from ethylenediaminetetraacetic acid-anticoagulated blood. In the VU University Medical Center, polymerase chain reaction (PCR)–amplified exon 2 amplicons were generated for low- to medium-resolution HLA-DQA1 and HLA-DQB1 genotyping in a combined, single-stranded conformation polymorphism–heteroduplex

assay by a semi-automated electrophoresis and gel-staining method on the PhastSystem (Amersham Pharmacia Biotech, Uppsala, Sweden).[9] In the samples from the Wilhelmina Children's Hospital, typing the HLA-DQA1 and HLA-DQB1 alleles was performed using the sequence-specific oligonucleotide Primed PCR (PCR-SSO) technique using the Luminex-based OneLambda LABType SSO Class II DQA1/DQB1 typing kit, following the recommendations of the manufacturer (One Lambda Inc., Canoga Park, CA). Descriptive statistics (SPSS for Windows, Version 15.0) were used to calculate the frequencies of the most common HLA-types.

Results

As expected the most prevalent HLA-type turned out to be HLA-DQ2.5. Remarkably, apart from HLA-DQ8, HLA-DQ2.2 (HLA-DQA1*02:01, -DQB1*02:02) was also found in a substantial number of patients.

In the retrospective cohort, the majority of the patients carried HLA-DQ2.5 (n=63, 90%), of whom 20 (28.6%) patients also had HLA-DQ2.2 and 6 (8.6%) HLA-DQ8, leaving 37 (52.9%) patients who solely had HLA-DQ2.5 (Table 1). Of these patients, 6 (8.6%) were homozygous for HLA-DQ2.5. No patient was seen with only HLA-DQ8, as the 2 (2.9%) patients with this HLA-type also carried HLA-DQ2.2. Finally, a total of 5 (7.1%) patients lacked the typical HLA-DQ2.5 and HLA-DQ8 genotypes. Interestingly, all of them carried HLA-DQ2.2, including 3 (4.3%) patients with homozygosity for the encoding genes. The 2 (2.9%) patients with heterozygous HLA-DQ2.2 also carried either HLA-DQ6.4 (HLA-DQA1*01:02, -DQB1*06:04) or HLA-DQ2.3 (HLA-DQA1*03:02, -DQB1*02:02).

In the prospective cohort, the distribution of HLA-types amongst the CD patients was virtually identical to the distribution in the retrospective cohort (Table 1). Most patients carried the typical HLA-DQ2.5 genes (N=76, 89.4%). A total of 23 (27.1%) also had HLA-DQ2.2 and 5 (5.9%) HLA-DQ8. Of the 48 (56.5%) patients who carried only the HLA-DQ2.5 genotype, 15 (17.6%) patients were homozygous. HLA-DQ8 was present in 10 (11.8%) patients, of whom 6 (7.1%) patients also had either HLA-DQ2.5 (n=5, 5.9%) or HLA-DQ2.2 (n=1, 1.2%). A total of 4 (4.7%) patients only had HLA-DQ8, of whom 2 (2.4%) patients were homozygous. In addition, 4 (4.7%) patients were negative for both HLA-DQ2.5 and -DQ8. All these patients had HLA-DQ2.2 in the heterozygous form (Table 1). The other HLA-DQ subtypes in these 4 patients were HLA-DQ5.1 (HLA-DQA1*01:01, -DQB1*05:01) (twice), HLA-DQ5.3 (HLA-DQA1*01:04, -DQB1*05:03) and HLA-DQ9.3 (HLA-DQA1*03:02, -DQB1*03:03).

In the combined cohorts the most common HLA-type amongst CD patients was HLA-DQ2.5 (N= 139, 89.7%), although a significant number of these patients also carried HLA-DQ2.2 (N=43, 27.7%) and less frequently HLA-DQ8 (N=11, 7.1%) (Table 1). Twenty-one patients were homozygous for HLA-DQ2.5. HLA-DQ2.2 was the second most common HLA-type being present in more than one-third of the patients (N=55, 35.5%), in most cases however combined with either HLA-DQ2.5 or HLA-DQ8. In 5.8% (N=9) of the patients no HLA-DQ2.5 or HLA-DQ8 was found. However in all those patients

HLA-DQ2.2 was present, of whom 3 were homozygous (Table 1). These 9 patients were all symptomatic, had positive CD serology, and showed a good clinical and serological response to gluten elimination. One of these patients was of Jewish origins; all others were of Dutch descent. Finally, HLA-DQ8 was the least frequent HLA genotype (N=18, 11.6%) and most commonly present in combination with either HLA-DQ2.5 (N=11, 7.1%) or HLA-DQ2.2 (N=3, 1.9%). In only 4 (2.6%) patients HLA-DQ8 was present without HLA-DQ2.5 or HLA-DQ2.2 (2 homozygous and 2 heterozygous patients; Table 1).

HLA type	Retrospective cohort (N=70)	Prospective cohort (N= 85)	All patients (N=155)	
HLA-DQ2.5, -DQX*	31 (44.3%)	33 (38.8%)	64 (41.3%)	
HLA-DQ2.5, -DQ2.5	6 (8.6%)	15 (17.6%)	21(13.5%)	
HLA-DQ2.5, -DQ2.2	20 (28.6%)	23 (27.1%) #	43 (27.7%)	→89.7%
HLA-DQ2.5, -DQ8	6 (8.6%)	5 (5.9%)	11 (7.1%)	
HLA-DQ8, -DQX *	0 (0.0%)	2 (2.4%)	2 (1.3%)	
HLA-DQ8, -DQ8	0 (0.0%)	2 (2.4%)	2 (1.3%)	→4.5%
HLA-DQ8, -DQ2.2	2 (2.9%)	1 (1.2%)	3 (1.9%)	
HLA-DQ2.2, -DQX *	2 (2.9%)	4 (4.7%)	6 (3.9%)	
HLA-DQ2.2, -DQ2.2	3 (4.3%)	0 (0.0%)	3 (1.9%)	→5.8%

Table 1 Frequencies of HLA-types in pediatric patients with CD. * any HLA-type other than HLA-DQ2.5, HLA-DQ8 and HLA-DQ2.2. # Two patients were HLA-DQ2.2 and HLA-DQ7.5 positive and were thus positive in trans configuration.

Discussion

A major proportion of the genetic predisposition to CD is derived from the HLA-complex. Indeed, up to 90% of the CD patients carry the HLA-DQ2.5 heterodimer, historically called HLA-DQ2, while most of the remaining CD patients are reported to express HLA-DQ8.[1-5] The finding that CD is virtually restricted to these HLA-heterodimers has led to the practise of considering HLA-DQ2.5 and HLA-DQ8 negative patients as being not at risk for CD.

However, in the current study, a different distribution of HLA-types was seen. Although almost 90% of the patients indeed carried the HLA-DQ2.5 genotype, of the 16 (10.3%) patients lacking this HLA-type only 7 (4.5%) had HLA-DQ8, while 9 (5.8%) patients were negative for both classical HLA-types and would therefore be missed if relying on current practise (Table 1). Interestingly, those 9 symptomatic patients, who all had positive CD-serology and quickly responded to the diet, possessed the HLA-DQ2.2 genotype. Of these 9 patients, 3 patients were homozygous and one patient also carried HLA-DQ2.3 (so homozygous DQB1*02), which is associated with a 5-fold increased risk of CD.[10] The remaining 5 patients carried various other HLA-types, so homozygosity for HLA-DQB1*02 cannot explain the development of CD in all patients lacking the typical HLA-types.

A similar prevalence of HLA-DQ2.2 in CD patients has been described before in a European study and more recently in a retrospective American study.[7,8] Both demonstrated a ~4% prevalence of CD patients who had HLA-DQ2.2, but no HLA-DQ2.5 or DQ8. In addition, a study performed in consecutive Spanish and Finnish CD patients showed that HLA-DQ2.2 was present in 3.2% of the Spanish patients, but in none of the Finnish patients.[6] Finally, Zubillaga et al too found a significantly increased prevalence of HLA-DQ2.2 in patients with CD.[11]

Many other studies have stated that the development of CD is almost restricted to individuals with either HLA-DQ2.5 or HLA-DQ8; however in some studies HLA-typing was limited to these 2 types, potentially missing patients with HLA-DQ2.2.[1-5, 12-16] In addition, some typing methods do not distinguish between HLA-DQ2.5 and HLA-DQ2.2.[17,18] The fact that HLA-DQ2.2 is only rarely mentioned in previous studies might also be explained by sampling errors, especially as the HLA-DQ2.2 prevalence in the general population may vary between different countries and most studies were performed in small cohorts, which increases the risk of sampling errors. Given this possible selection bias and/or incomplete typing in previous studies, and with 4 reports, including the current one, now describing a 3.2%-5.8% prevalence for HLA-DQ2.2 in CD, we propose that HLA-DQ2.2 should also be considered as a CD related genotype. This will have important clinical implications, as HLA-typing is an essential part of the new ESPGHAN guidelines for the diagnosis of CD.[10]

In summary the current study demonstrates that HLA-DQ2.2 is at least as frequent in patients with CD as HLA-DQ8. Consequently, in order to avoid missing CD patients, the HLA-DQ2.2 genotype should be considered as one of the HLA-types related to CD.

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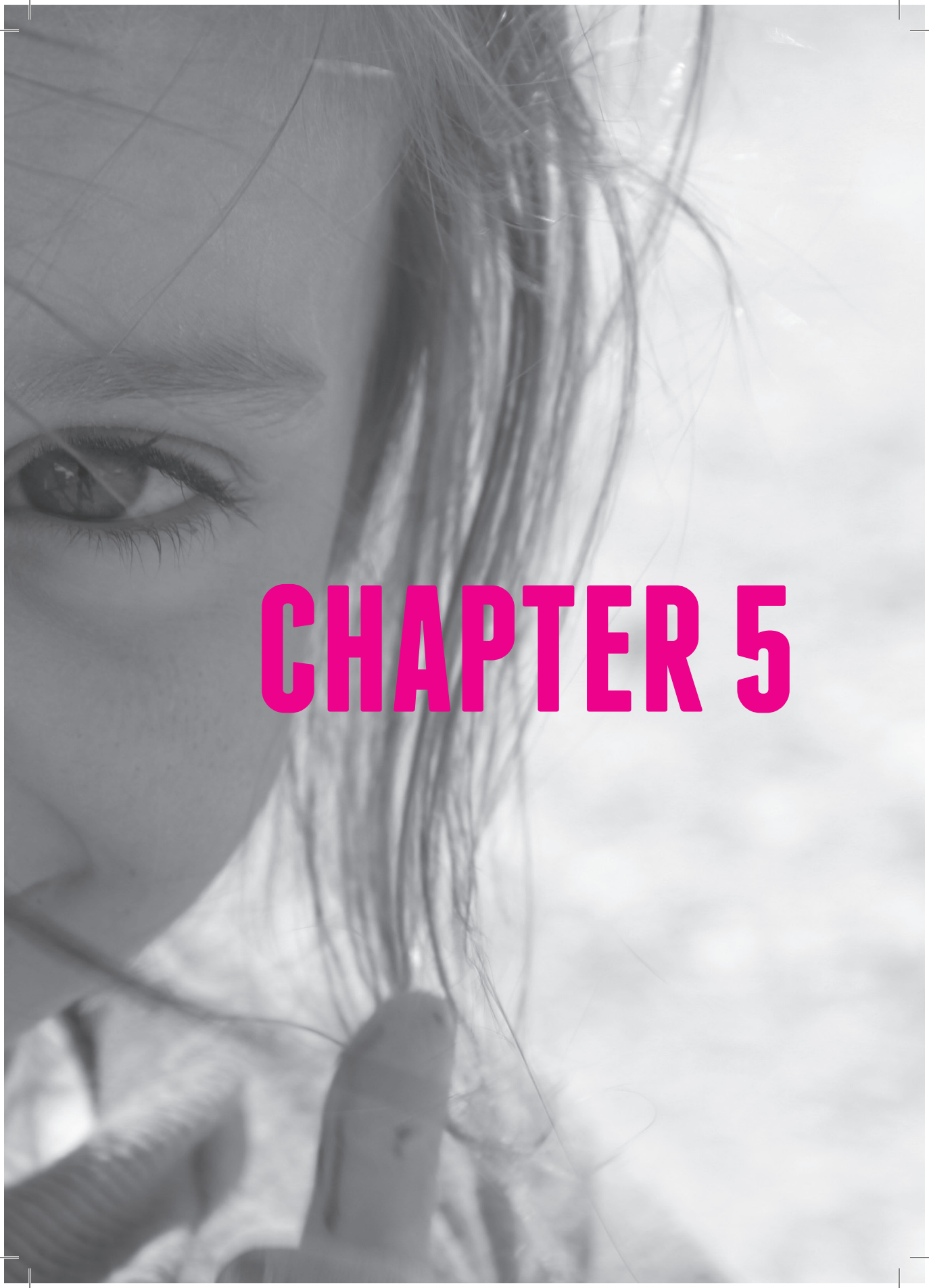

CHAPTER 5

CHAPTER 5

Immunoglobulin G antibodies against deamidated-gliadin-peptides outperform anti-endomysium and tissue transglutaminase antibodies in children <2 years age

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Abstract

To investigate the usefulness of deamidated-gliadin-peptides-antibodies in the diagnosis of celiac disease, serology was tested in 212 children suspected with celiac disease who had undergone a small-intestinal-biopsy. For deamidated-gliadin-peptides-antibodies, two kits were tested. Positive and negative predictive values for IgA deamidated-gliadin-peptides-antibodies using the Bindazyme-kit were 89% and 74%, while the Quanta-Lite-kit had values of 89% and 85%, respectively. For the IgG subtype using the Bindazyme-kit, these values were 85% and 89%, while they were 85% and 91% for the Quanta-Lite-kit. The positive predictive values for endomysium and tissue-transglutaminase antibodies were disappointing (77% and 87%), although the negative predictive values were better (97% and 96%). When the analysis was restricted to the 41 children aged <2 years, no misclassifications occurred with IgG deamidated-gliadin-peptides-antibodies giving 100% accuracy in both kits. The positive predictive value reached 100% for tissue-transglutaminase antibodies and both kits for IgA deamidated-gliadin-peptides-antibodies, while the negative predictive value was 94% in these assays. Positive and negative predictive values for endomysium antibodies were 96% and 93%, respectively. In conclusion, although deamidated-gliadin-peptides-antibodies do not outperform anti-endomysium antibodies in the total study population, the IgG subtype seems to be the best test in children aged <2 years, reaching 100% accuracy.

Introduction

Celiac disease (CD) is caused by the ingestion of wheat gluten, or related prolamins from rye or barley, in genetically susceptible individuals expressing the human leukocyte antigens (HLA) DQ2 or DQ8.[1, 2] Gliadin, the alcohol-soluble fraction of gluten is the toxic agent leading to the classical triad of villous atrophy, increased intraepithelial lymphocytes, and crypt hyperplasia in the small-intestinal-mucosa of untreated CD patients.[2] Serologically, CD is characterized by the presence of several antibodies that can be used to detect the disease. Immunoglobulin A (IgA) auto-antibodies against endomysium (EMA) and tissue-transglutaminase (tTGA), which both recognize the auto-antigen tissue-transglutaminase, are now widely used to detect CD and are highly sensitive and specific.[3, 4] The use of anti-gliadin antibodies has been abandoned mainly because of the relatively high false positive rate.[4–6] Interestingly, recent studies have shown that gliadin antibodies from sera of CD patients exhibit enhanced binding to deamidated-gliadin-peptides (DGP).[7] This emphasizes the important role of deamidation, catalyzed by tissue transglutaminase, in the pathogenesis of CD. These modified gliadin peptides show a greatly enhanced affinity to HLA DQ 2 or DQ 8 situated on the surface of antigen presenting cells, thereby initiating a stronger T-cell and antibody response.[8–10] Newly developed commercial ELISA tests, using DGP antigens as a substrate, have also been demonstrated to outperform conventional anti-gliadin antibody assays, and some studies even suggest that they may be of additional diagnostic value when compared with EMA or tTGA assays.[11–24] This may be especially true in very young children, where EMA and tTGA have shown to be diagnostically less accurate.[6, 25–28]

The aim of the present study was to determine the diagnostic accuracy of a new commercial ELISA kit and thereby determine whether antibodies against DGP (a-DGP) can be used in clinical practice to screen for CD, especially in young children.

Materials and methods

Study population

The study population included all patients referred to the Wilhelmina Children's Hospital in Utrecht, The Netherlands, suspected of having CD in whom both a small-intestinal-biopsy and serological testing (EMA and/or tTGA) were performed in the period 1998–2009. The patients were referred to us based on their symptoms (abdominal symptoms, growth retardation, fatigue, iron deficient anemia, behavioral changes) or because they belonged to a group at risk for CD (diabetes mellitus, Down syndrome, first degree relatives with CD). Any patient with abnormal serology, and also patients with negative serology and a high suspicion (CD-like symptoms) of the disease were biopsied. All patients were on a gluten-containing diet, had an IgA level of at least 0.08 g/L, and did not suffer from giardiasis. The study was performed according to the guidelines of the medical ethics board of the University Medical Center Utrecht, The Netherlands.

Serological assessment
All blood samples were stored at -80°C. In these samples, IgA and IgG a-DGP were determined using the following two methods: Bindazyme Human Anti-Gliadin EIA Kit IgA and IgG (The Binding Site, Birmingham, UK) and Quanta Lite® Gliadin IgA II and IgG II (Inova Diagnostics, San Diego, CA, USA). A cut-off value of 10 U/mL was used, as recommended by the manufacturer for the Bindazyme kit. Levels of at least 20 U/mL were considered positive as stated by the manufacturer for the Quanta-Lite-kit. This manufacturer also provides a combined kit for the detection of IgA and IgG a-DGP, as well as IgA and IgG tTGA in human serum (Quanta Lite®_h-tTG /DGP Screen), which was also used in all blood-samples. A cut-off value of 20 U/mL was employed, as recommended by the manufacturer.

In the same samples, IgA EMA was detected by indirect immunofluorescence using sections of distal monkey esophagus mounted on glass slides (IMMCO Diagnostics Inc., Buffalo, NY, USA). Serum IgA tTGA were measured by ELISA using human recombinant tTG (ELiA Celikey IgA, Phadia AB, Uppsala, Sweden). As recommended by the manufacturer, the serum samples containing an antibody titer of more than 10 U/mL were considered positive. All samples were tested in accordance with the manufacturer's specifications.

Histological evaluation
A mean of 3.2 biopsies per patient were obtained from the distal duodenum by upper endoscopy. For the purpose of this study, all original biopsies were revised by a single experienced pathologist who made the histological diagnosis using the Marsh modified classification. The pathologist had no knowledge of the clinical presentation and serological results of the patients. Marsh I (increased intraepithelial lymphocytes) and Marsh II (increased intraepithelial lymphocytes as well as crypt hyperplasia) were regarded as not conclusive for CD, whereas Marsh III, the presence of partial (Marsh IIIa), subtotal (Marsh IIIb) or total (Marsh IIIc) villous atrophy in addition to the histological findings in Marsh II, was considered diagnostic for CD.

Data analysis
The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of IgA a-DGP, immunoglobulin G (IgG) a-DGP, IgA EMA, and IgA tTGA along with their 95% confidence intervals (CI) were calculated using the histological evaluation as the gold standard. Sensitivity, specificity, and the predictive values were also specifically determined in different age categories (<2 and ≥2 years).

To determine the strength of agreement between the two kits for a-DGP, the Kappa value was calculated. Landis and Koch have proposed the following as standards for strength of agreement for the kappa coefficient: ≤ 0 = poor, 0.01–0.20 = slight, 0.21–0.40 = fair, 0.41–0.60 = moderate, 0.61–0.80 = substantial, and 0.81–1.0 = almost perfect.[29]

Results

Patient characteristics

Two-hundred and twelve patients suspected of having CD were included in the study. Of these, 98 (46.2%) were male and 114 (53.8%) were female with an age range between 0.7 and 17.8 years and a mean of 6.2 years. One-hundred and nine (51.4%) patients had a Marsh III lesion and were therefore diagnosed with CD. Of these 109 patients, nine had a Marsh IIIa, 44 a Marsh IIIb, and 56 a Marsh IIIc lesion. In the remaining (n = 103) patients, there was no histological evidence of CD, although two of them had a Marsh I lesion, and one patient had a Marsh II lesion. Of the total study population, 140 patients were positive for IgA EMA and/ or IgA tTGA, while 72 patients were negative for both antibodies. In this group, only one patient, 7 years of age, who was also negative for all other antibodies, turned out to have CD (Marsh IIIb).

Overall test performance

Ninety-eight of the 115 patients positive for IgG a-DGP Bindazyme indeed had CD (Table 1), giving a sensitivity of 90% (Table 2). The specificity, PPV, and NPV were 83%, 85%, and 89%, respectively. For IgG a-DGP Quanta Lite, these values were very similar with sensitivity, specificity, PPV, and NPV of 92%, 83%, 85%, and 91%, respectively.

The specificity for IgA a-DGP Bindazyme was higher (91%) than that of IgG a-DGP Bindazyme with only nine patients having false positive test results (Table 1). However, the sensitivity was as low as 70% (Table 2) with 33 of the CD patients being missed (Table 1). The PPV and NPV were 89% and 74%, respectively (Table 2). Interestingly, IgA a-DGP Quanta Lite performed much better than IgA a-DGP Bindazyme with the sensitivity, specificity, PPV, and NPV of 85%, 88%, 89%, and 85%, respectively. However, sensitivity remained lower than the IgG a-DGP.

The strength of agreement for IgA a-DGP between the two kits was moderate with a Kappa value of 0.58. This value was much higher (Kappa 0.82) for the two IgG a-DGP kits, indicating an almost perfect agreement.

Of all the serological tests, IgA EMA and IgA tTGA had the highest sensitivities, 98% and 96%, respectively (Table 2). NPVs were also high, reaching 97% for IgA EMA and 96% for IgA tTGA. By contrast, the specificities for IgA EMA and IgA tTGA were disappointing with values of 69% and 84%, respectively. The PPV was 77% for IgA EMA and 87% for IgA tTGA. The combined test for a-DGP/tTGA did not outperform IgA EMA or IgA tTGA with an equally high sensitivity of 98%, but with a rather disappointing specificity of 61%. The PPV and NPV were 73% and 97%, respectively.

For reasons of the high false positive rate for EMA in this cohort, we analyzed the patients with these false positive results. The clinical characteristics of these patients did not differ significantly from the total study population (data not shown). In addition, only one of these patients had a Marsh I duodenal histology, while the remaining patients had normal duodenal architecture.

		Biopsy data								
		Total patients N=212	CD N=109 (51.4%)	No CD N=103 (48.6%)	Patients <2 years N=41	CD N=26 63.4%	No CD N=15 36.4%	Patients ≥ 2 years N=171	CD N=83 48.5%	No CD N=88 51.5%
IgA EMA	Negative	73 (34.4%)	2 (1.8%)	71 (68.9%)	15 (36.6%)	1 (3.8%)	14 (93.3%)	58 (33.9%)	1 (1.2%)	57 (64.8%)
	Positive	139 (65.5%)	107 (98.2%)	32 (31.1%)	26 (63.4%)	25 (96.2%)	1 (6.7%)	113 (66.1%)	82 (98.9%)	31 (35.2%)
IgA tTGA	Negative	91 (42.9%)	4 (3.7%)	87 (84.5%)	16 (39.0%)	1 (3.8%)	15 (100%)	75 (43.9%)	3 (3.6%)	72 (81.8%)
	Positive	121 (57.1%)	105 (96.3%)	16 (15.5%)	25 (61.0%)	25 (96.2%)	0 (0.0%)	96 (56.1%)	80 (96.4%)	16 (18.2%)
IgA a-DGP Bindazyme	Negative	127 (59.9%)	33 (30.3%)	94 (91.3%)	16 (39.0%)	1 (3.8%)	15 (100%)	111 (64.9%)	32 (38.6%)	79 (89.8%)
	Positive	85 (40.1%)	76 (69.7%)	9 (8.7%)	25 (61.0%)	25 (96.2%)	0 (0.0%)	60 (35.1%)	51(61.4%)	9 (10.2%)
IgG a-DGP Bindazyme	Negative	97 (45.8%)	11 (10.1%)	86 (83.5%)	15 (36.6%)	0 (0.0%)	15 (100%)	82 (48.0%)	11 (13.3%)	71 (80.7%)
	Positive	115 (54.2%)	98 (89.9%)	17 (16.5%)	26 (63.4%)	26 (100%)	0 (0.0%)	89 (52.0%)	72 (86.7%)	17 (19.3%)
IgA a-DGP Quanta Lite	Negative	107 (50.5%)	16 (14.7%)	91 (88.3%)	16 (39.0%)	1 (3.8%)	15 (100%)	91 (53.2%)	15 (18.1%)	76 (86.4%)
	Positive	105 (49.5%)	93 (85.3%)	12 (11.7%)	25 (61.0%)	25 (96.2%)	0 (0.0%)	80 (46.8%)	68 (81.9%)	12 (13.6%)
IgG a-DGP Quanta Lite	Negative	95 (44.8%)	9 (8.3%)	86 (83.5%)	15 (36.6%)	0 (0.0%)	15 (100%)	80 (46.8%)	9 (10.8%)	71 (80.7%)
	Positive	117 (55.2%)	100 (91.7%)	17 (16.5%)	26 (63.4%)	26 (100%)	0 (0.0%)	91 (53.2%)	74 (89.2%)	17 (19.3%)
a-DGP/ tTGA	Negative	65 (30.7%)	2 (1.8%)	63 (61.2%)	14 (34.1%)	0 (0.0%)	14 (93.3%)	51 (29.8%)	2 (2.4%)	49 (55.7%)
	Positive	147 (69.3%)	107 (98.2%)	40 (38.8%)	27 (65.9%)	26 (100%)	1 (6.7%)	120 (70.2%)	81 (97.6%)	39 (44.3%)

Table 1 Results of small-intestinal biopsy, EMA, tTGA and a-DGP. CD, celiac disease; IgA, Immunoglobulin A; IgG, Immunoglobulin G; EMA, anti-endomysium antibodies; tTGA, anti-tissue transglutaminase antibodies; a-DGP, antibodies against deamidated gliadin peptides

	All ages N=212				< 2 years N=41				≥ 2 years N=171			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
IgA EMA	98% (93-100)	69% (59-77)	77% (69-84)	97% (90-100)	96% (78-100)	93% (66-100)	96% (78-100)	93% (66-100)	99% (93-100)	65% (54-74)	73% (63-80)	98% (90-100)
IgA tTGA	96% (90-99)	84% (76-91)	87% (79-92)	96% (89-99)	96% (78-100)	100% (75-100)	100% (83-100)	94% (68-100)	96% (89-99)	82% (72-89)	83% (74-90)	96% (88-99)
IgA a-DGP Bindayme	70% (60-78)	91% (84-96)	89% (80-95)	74% (65-81)	96% (78-100)	100% (75-100)	100% (83-100)	94% (68-100)	61% (50-72)	90% (81-95)	85% (73-92)	71% (62-79)
IgG a-DGP Bindayme	90% (82-95)	83% (75-90)	85% (77-91)	89% (80-94)	100% (84-100)	100% (75-100)	100% (84-100)	100% (75-100)	87% (77-93)	81% (71-88)	81% (71-88)	87% (77-93)
IgA a-DGP Quanta Lite	85% (77-91)	88% (80-94)	89% (81-94)	85% (77-91)	96% (78-100)	100% (75-100)	100% (83-100)	94% (68-100)	82% (72-89)	86% (77-92)	85% (75-92)	84% (74-90)
IgG a-DGP Quanta Lite	92% (84-96)	83% (75-90)	85% (77-91)	91% (82-95)	100% (84-100)	100% (75-100)	100% (84-100)	100% (75-100)	89% (80-95)	81% (71-88)	81% (71-88)	89% (79-94)
a-DGP/ tTGA	98% (93-100)	61% (51-70)	73% (65-80)	97% (88-99)	100% (84-100)	93% (66-100)	96% (79-100)	100% (73-100)	98% (91-100)	56% (45-66)	68% (58-76)	96% (85-99)

Table 2 Sensitivity, specificity, positive predictive value (PPV) and negative predictive value NPV) of different screening tests for celiac disease. The 95% confidence intervals are given in parenthesis. IgA, Immunoglobulin A; EMA, anti-endomysium antibodies; tTGA, anti-tissue transglutaminase antibodies; IgG, Immunoglobulin G; a-DGP antibodies against deamidated gliadin peptides.

Overall test performance in different age categories

To compare the performance of the serological tests in different age categories, subgroup analyses were performed in children aged <2 years (n = 41) and ≥2 years (n = 171). Remarkably, in children aged <2 years, no misclassifications occurred when using IgG a-DGP Bindazyme and Quanta Lite (Table 1). In addition, no false positive results were seen when using tTGA or IgA a-DGP Bindazyme and Quanta Lite. However, in both tests, patients with false negative results were found (one patient for IgA tTGA, one for IgA a-DGP Bindazyme, and one for IgA a-DGP Quanta Lite).

In children aged ≥2 years, none of the serological assays performed perfectly. The test with the lowest false negative classifications was IgA EMA (n = 1), and the one with the lowest false positive classifications was IgA a-DGP Bindazyme (n = 9).

Sensitivity, specificity, and the predictive values were also determined in the aforementioned age categories (Table 2). For all tests, specificities were significantly higher in children aged < 2 years reaching a 100% in all tests except for IgA EMA (93%) and a-DGP/tTGA (93%). Likewise, PPVs reached 100% in all assays except for IgA EMA (96%) and a-DGP/tTGA (96%).

Sensitivities for IgA EMA and IgA tTGA did not differ significantly between the two age groups. By contrast, all a-DGP tests were more sensitive when used in patients aged < 2 years as compared with patients aged ≥ 2 years.

As no test functioned perfectly in patients aged ≥ 2 years, it was determined whether combining serology could lead to a diagnostic accuracy of 100% in this age group. Unfortunately, no combination reached 100% accuracy as a 7-year-old CD patient was missed by all tests. Likewise, even with positivity for all antibodies (using the Inova kit for a-DGP), three patients would be incorrectly diagnosed with CD if no small-intestinal-biopsy is taken.

Discussion

The gold standard for diagnosing CD is histology, which typically shows villous atrophy, increased intra-epithelial lymphocytes, and crypt hyperplasia. Serological tests are generally used to select patients in whom a small-intestinal-biopsy is required. In this respect, tTGA and EMA are considered the most accurate, but recent studies have suggested that newly developed assays detecting antibodies against DGP might be of additional diagnostic value.[11–24] This might be especially so in children aged <2 years.[13, 18] Indeed, we can now confirm this last suggestion for this age group, as in our study, IgG a-DGP had 100% sensitivity and specificity, albeit with CI of 84–100% and 75–100%, respectively. In this specific age category, the overall performance of the other tests, IgA a-DGP, IgA tTGA, and IgA EMA, was lower. However, this difference was not significant, given the already very good performance of these tests.

Nevertheless, as IgA tTGA and IgA EMA will miss CD in children aged < 2 years, both in our and in other studies, it seems sensible to include IgG a-DGP when screening for CD in this age group.[6, 25–28] Unfortunately, in the older children, no test was 100% reliable, and even when combining tests, optimal reliability could not be reached.

This study also compared two kits for a-DGP, the Bindazyme Human Anti-Gliadin EIA kit and the Quanta Lite® kit. The IgG a-DGP of both kits performed similarly, which is also illustrated by a Kappa value of 0.82, indicating an almost perfect agreement. However, the sensitivity of the Bindazyme IgA a-DGP was lower, although statistically not significant, as compared with the Quanta Lite kit, while specificity was comparable, resulting in a Kappa value of 0.58 (moderate agreement).

Our results confirm and extend earlier pediatric studies using the Quanta Lite® kit, as overall performance (sensitivity, specificity, PPV, NPV) for a-DGP, both IgG and IgA, does not outperform IgA tTGA and IgA EMA in reliably detecting patients with CD, if no subdivision in age groups is made.[11, 13, 19, 23] In addition, sensitivities of IgG a-DGP and IgA a-DGP, using the Quanta Lite® kit in our study, were comparable to results described in previous studies, while specificities were slightly lower.

Finally, even the combined DGP/tTGA test did not outperform IgA EMA and IgA tTGA with a sensitivity reaching that of EMA (98%), but a lack of specificity with a value of only 61%. The sensitivity is comparable to values reported before, but the specificity is much lower.[11] In our study, the specificity of both IgA a-DGP and IgG a-DGP was higher than that of IgA EMA and IgA tTGA. However, the specificities of IgA tTGA and particularly IgA EMA in the present study were lower than values reported before for children.[25] This may have several explanations, the most important being the routine clinical setting in which this study was conducted.[6, 30, 31] In this situation, reported specificities vary between 65% and 87%.[30, 31]

Also, the present study was performed retrospectively, and patients with a positive serology were obviously more likely to be referred to our hospital, which may have affected the results. In addition, the relatively low specificity of IgA EMA may be partly due to some interobserver variation. The IgA EMA test is a semi-quantitative immunofluorescence method which, although subject to rigorous quality control, is yet not as easy to standardize as the IgA tTGA assay. Also, the composition of the team of technicians carrying out the IgA EMA tests has not been completely constant over the 10-year span of the current study, which may have caused some variability in interpretation.

Finally, it should be kept in mind that the false positive patients in this study may have latent CD, which can develop into overt CD at any point in their lives. Unfortunately, our study did not include follow-up of these patients.

In conclusion, a-DGP are good diagnostic markers for CD, but do generally not outperform IgA EMA and IgA tTGA. However, under the age of 2 years, the IgG a-DGP assay seems to be preferred above IgA tTGA and IgA EMA, as it was 100% accurate. By contrast, this value could not be reached in the older children, not even with any combination of tests.

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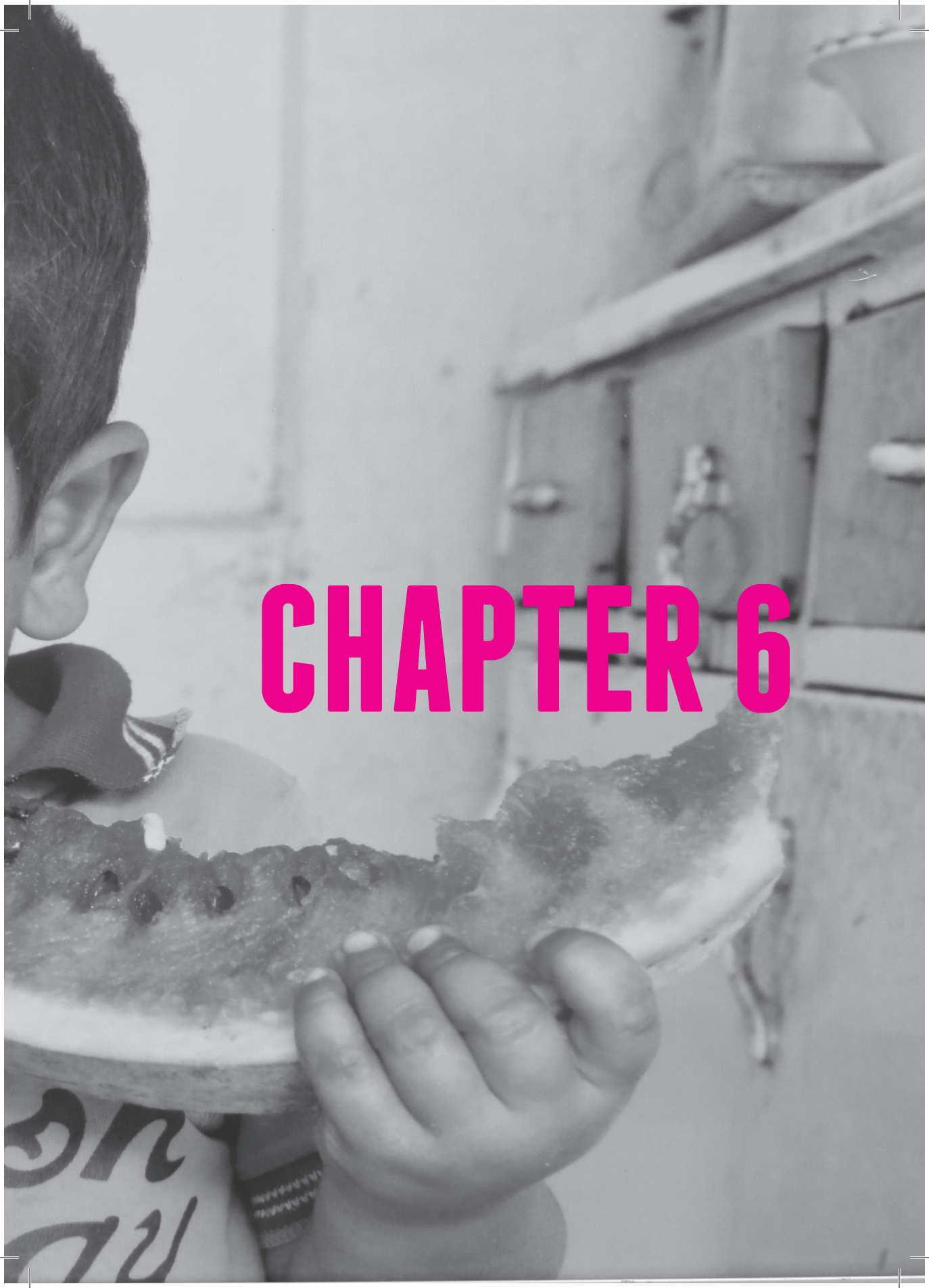

CHAPTER 6

CHAPTER 6

A biopsy is not always necessary to diagnose celiac disease

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Abstract

Objectives Small intestinal histology is the criterion standard for the diagnosis of celiac disease (CD). However, results of serological tests such as anti-endomysium antibodies and anti-tissue transglutaminase antibodies (tTGA) are becoming increasingly reliable. This raises the question of whether a small intestinal biopsy is always necessary. The aim of the present study was, therefore, to investigate whether a small intestinal biopsy can be avoided in a selected group of patients.

Patients and Methods Serology and histological slides obtained from 283 pediatric patients suspected of having CD were examined retrospectively. The response to a gluten-free diet (GFD) in patients with a tTGA level ≥100 U/mL was investigated.

Results A tTGA level ≥100 U/mL was found in 128 of the 283 patients. Upon microscopic examination of the small intestinal epithelium, villous atrophy was found in 124 of these patients, confirming the presence of CD. Three patients had crypt hyperplasia or an increased number of intraepithelial lymphocytes. In 1 patient no histological abnormalities were found. This patient did not respond to a GFD.

Conclusions Pediatric patients with a tTGA level ≥100 U/mL in whom symptoms improve upon consuming a GFD may not need a small intestinal biopsy to confirm CD.

Introduction

Celiac disease (CD) is a gluten-sensitive enteropathy characterized by small intestinal damage with loss of absorptive villi, classically leading to malabsorption, diarrhea, and failure to thrive.[1] The disease occurs in genetically susceptible individuals upon dietary ingestion of gluten, a storage protein in wheat, barley, and rye, and usually resolves upon its withdrawal.[1,2] Although the prevalence of CD may be as high as 0.5% to 1%, it is frequently not diagnosed because symptoms may be minimal or aspecific.[3,4]

In symptomatic patients serological tests are performed by measuring circulating disease-associated antibodies, particularly immunoglobulin A (IgA) auto-antibodies against endomysium, that is, anti-endomysium antibodies (EMA), and tissue transglutaminase (tTG), that is, anti-tissue transglutaminase antibodies (tTGA).[5–7] tTG, a calcium-dependent thiol enzyme, has been identified as the main, if not sole, autoantigen for both antibodies and is thought to play a major role in the pathogenesis of CD.[8–11]

To date, a small intestinal biopsy, which typically shows villous atrophy, increased intraepithelial lymphocytes, and hyperplastic crypts in patients with CD on a gluten-containing diet, is the criterion standard for the diagnosis of CD.[6] Considering the inconvenience and high costs associated with a biopsy, and because CD is a disease with a high prevalence, there is a growing call for less invasive tests to diagnose CD. Because both the sensitivity and specificity of the serological tests have increased to nearly perfect values, it is increasingly questioned whether these tests alone may be sufficient to confirm the diagnosis and thereby avoid the requirement for a biopsy in specific cases.[12–18] Nevertheless, the positive predictive value (PPV) of the serological tests, reflecting the probability that a patient with a positive test indeed has the disease, is far from ideal, especially in the general population.[12,19–21]

Consequently, if a biopsy is not performed in the workup of CD, a number of patients with falsely raised serological markers would unnecessarily follow a gluten-free diet (GFD), hitherto the only treatment available for CD. The aim of the present study was, therefore, to investigate whether specific factors may optimize the PPV and thus determine whether a small intestinal biopsy can be avoided in a selected group of patients.

Patients and methods

Study Population

The data of all of the patients who were referred between 1998 and 2009 to the Wilhelmina Children's Hospital in Utrecht, the Netherlands, suspected of having CD, were examined retrospectively according to the guidelines of the medical ethics board of the University Medical Center Utrecht, the Netherlands. Patients were referred to us because of symptoms that are associated with CD or because they belonged to a group at risk for CD.

All of the patients who had both a small intestinal biopsy and serological testing, including total serum IgA levels, were included in the study. Serological testing had been performed between 3 months before and 1 week after the initial small intestinal biopsy, whereas patients were on a gluten-containing diet; patients in whom the biopsy or serological testing was obtained during a GFD or a gluten challenge were excluded from the study. Patients with an IgA deficiency and patients with giardiasis were also excluded. For the patients with a tTGA level ≥100 U/mL, we retrospectively collected data regarding clinical presentation and responsiveness to the GFD.

Serological Assessment

IgA EMA were detected by means of indirect immunofluorescence using sections of distal monkey esophagus mounted on glass slides (IMMCO Diagnostics Inc, Buffalo, NY). Serum IgA tTGA were measured using the ELiA Celikey IgA kit (Phadia AB, Uppsala, Sweden). As recommended by the manufacturer, the serum samples containing an antibody titer of >10 U/mL were considered positive. Total IgA was measured in all of the patients, and a serum IgA concentration <0.07 g/L was regarded as IgA deficiency. All of the blood samples that were obtained between 1998 and 2009 had been stored at ≥80°C. To maximize experimental consistency, we retested all of the blood samples that had been investigated using other test versions than the ones described here. Because serological testing for tTGA was not available in our hospital before 2002, the serum tTGA of all of the patients who underwent biopsy before 2002 were measured using the stored blood samples.

Histological Evaluation

A minimum of 2 biopsies were taken from the distal duodenum by upper gastrointestinal endoscopy. Histological diagnosis for all of the patients was made by a single experienced pathologist using the Marsh classification. The pathologist had no knowledge of the serological results or the clinical presentation of the patients. An increased number of intraepithelial lymphocytes (Marsh I) and crypt hyperplasia (Marsh II), without villous atrophy, was considered insufficient for the diagnosis of CD. Only patients who had villous atrophy in addition to crypt hyperplasia and an increased number of intraepithelial lymphocytes (Marsh III) upon microscopic examination were diagnosed as having CD.

Data Analysis

The sensitivity, specificity, PPV, and negative predictive value (NPV) of the screening tests, along with their 95% confidence intervals (CI), were calculated using the histological evaluation as the criterion standard. Subsequently we determined whether a tTGA level ≥100 U/mL or dual positivity for tTGA and EMA could improve the PPV. Finally, in patients with a tTGA level ≥100 U/mL descriptive analysis of presenting symptoms and responsiveness to the GFD was performed.

Results

In 301 patients both a small intestinal biopsy and serological testing were performed. Four patients with giardiasis and 14 patients with an IgA deficiency were excluded, leaving 283 patients for analysis. Of those, 130 (45.9%) were boys and 153 (54.1%) were girls with an age range between 0.7 and 17.8 years and a mean age of 6 years.
A total of 163 (57.6%) patients had a biopsy diagnostic for CD (Marsh III), whereas a normal histology was found in 120 patients (42.4%) (Table 1). False-positive EMA were found in 41 patients (34.2%) and false-positive tTGA in 21 patients (17.5%). Twenty of these patients also had positive EMA. The clinical characteristics of these patients did not differ from the total study population (data not shown). False-negative EMA were found in 6 patients (3.7%) and false-negative tTGA in 7 patients (4.3%). In 4 patients with CD, EMA and tTGA were both undetectable. Three of these patients were younger than 2 years.

		Biopsy data		
		Patients N=283	Patients with CD N=163 (57.6%)	Patients with normal histology N=120 (42.4%)
IgA EMA	Negative	85 (30.0%)	6 (3.7%)	79 (65.8%)
	Positive	198 (70.0%)	157 (96.3%)	41 (34.2%)
IgA tTGA > 10	Negative	106 (37.5%)	7 (4.3%)	99 (82.5%)
	Positive	177 (62.5%)	156 (95.7%)	21 (17.5%)
IgA tTGA ≥ 100	Negative	155 (54.8%)	39 (23.9%)	116 (96.7%)
	Positive	128 (45.2%)	124 (76.1%)	4 (3.3%)

Table 1 Results of small-intestinal biopsy, anti-endomysium antibodies (EMA) and anti-tissue transglutaminase antibodies (tTGA) with a cut-off value of >10 and ≥100 U/ml. CD, Celiac disease; IgA, immunoglobulin A.

The resulting sensitivity was equally high for EMA and tTGA (96%) (Table 2); however, the specificity of EMA was as low as 66%, whereas specificity for tTGA was 83%. PPV for EMA was 79% and for tTGA 88%. Dual positivity for EMA and tTGA did not lead to a significant improvement in the diagnostic accuracy because PPV was 89% (CI 0.83–0.93) instead of 88% for tTGA alone. Combining negative EMA and tTGA to exclude CD resulted in a NPV of 95% (CI 0.87–0.98). A total of 49 patients (17.3%)had a tTGA level between 10 and 100 U/mL. Of those, 32 (65.3%) had CD, whereas the diagnosis could be histologically excluded in 17 (34.7%) patients. By contrast, of the 128 patients with a tTGA level ≥100 U/mL only 4 patients, all positive for EMA, did not have villous atrophy, and consequently did not have CD (Table 1). The corresponding PPV was 97% (Table 2). More important, of these 4 patients 3 had histological changes that are compatible with but not diagnostic for CD: 2 had crypt hyperplasia and 1 had an increased number of intraepithelial lymphocytes (Marsh I). Only 1 of the patients with a tTGA level ≥100 U/mL did not have any histological abnormality.

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
IgA EMA	96 (0.92-0.98)	66 (0.57-0.74)	79 (0.73-0.85)	93 (0.85-0.97)
IgA tTGA >10	96 (0.91-0.98)	83 (0.74-0.89)	88 (0.82-0.92)	93 (0.86-0.97)
IgA tTGA ≥100	76 (0.69-0.82)	97 (0.91-0.99)	97 (0.92-0.99)	75 (0.67-0.81)

Table 2 Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of different screening tests for celiac disease. The 95% confidence intervals are given in parenthesis. EMA, anti-endomysium antibodies; tTGA, anti-tissue transglutaminase antibodies

Presenting symptoms in the 128 patients with tTGA≥100 U/mL included growth failure, diarrhea, and abdominal pain, as well as various other symptoms, with most patients having more than 1 symptom (Table 3). Response to the GFD could be judged in 114 because 6 patients were lost to follow-up, 4 patients did not start or adhere to the GFD, and 4 patients were asymptomatic at diagnosis. One of them was diagnosed during routine screening in Down syndrome and the remaining 3 were identified during family screening after a sibling or parent was diagnosed as having CD. In all of the symptomatic patients clinical symptoms improved after the GFD started, with the exception of 3. One of these patients turned out to have an irritable bowel syndrome, which may explain her persisting symptoms of abdominal pain and constipation. The second was an 11.5-year-old girl presenting with short stature, who did not exhibit catch-up growth in the 3 years after diagnosis. The third and final patient, who did not respond, was the patient with a tTGA level ≥100 U/mL and a normal histology of the small intestinal mucosa upon microscopic examination.

Symptoms*	Response to the gluten free diet
Growth failure (n=72)	Responsive (n=111)
Abdominal pain (n=49)	Not responsive (n=3)
Diarrhoea (n=46)	No follow-up data (n=6)
Fatigue (n=36)	Did not start or adhere to the diet (n=4)
Bloating (n=34)	Asymptomatic (n=4)
Constipation (n=32)	
Anorexia (n=20)	
Vomiting (n=17)	
Behavioural changes (n=10)	
Asymptomatic (n=5)	
Nausea (n=3)	
Tooth enamel defects (n=2)	
Dermatitis herpetiformis (n=1)	

Table 3 Symptoms in 128 patients with tTGA ≥100 U/ml and response to the gluten free diet.
* Most patients had more than one symptom.

Discussion

Small intestinal histology is considered the criterion standard for the diagnosis of CD. However, with the advent of reliable serological tests, it is being questioned whether a biopsy is really necessary in all cases. Indeed, in patients with tTGA ≥100 U/mL there is increasing evidence that serology may be sufficient to diagnose CD.[22–24] Barker, et al showed that 48 of 49 pediatric patients with a tTGA level ≥100 U/mL had at least Marsh II duodenal histology.[22] A subsequent study, also in a pediatric population, showed that 38 of the 38 patients with tTGA ≥100 U/mL had Marsh III duodenal histology.[23] More recently, in a study conducted in a mixed adult/pediatric population, it was shown that tTGA ≥100 U/mL almost exclusively occurs in the setting of Marsh III (73/76 patients) and that the 3 cases without villous atrophy did have minimal histological changes (Marsh I and II) suggestive of early CD (24). By contrast, Freeman reported that 3 of 14 adult patients with tTGA ≥100 U/mL did not have CD.[25]

In the present study, 124 of 128 patients with a tTGA level ≥100 U/mL were shown to have a Marsh III lesion upon histological examination. Four patients with tTGA levels ≥100 U/mL did not match the classical diagnostic criteria for CD. However, only 1 of them had a normal biopsy. This patient also did not respond to a GFD. The remaining 3 patients had histological abnormalities, that is, crypt hyperplasia (Marsh II) or an increased number of intraepithelial lymphocytes (Marsh I), which suggests that these patients had in fact an early stage of CD. They are reminiscent of the patients who have abnormal serology but insufficient histological evidence of CD, but who later on develop more pronounced histological lesions, after which CD can be diagnosed.[26–32] In fact the diagnostic criteria, as recently issued by the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition, state that Marsh I and II along with positive tTGA is compatible with CD.[33] In addition, histological lesions in CD can be patchy and can therefore be missed sometimes; this may have been the case in at least some of the false-positive patients in the present study, for example the patient with Marsh 0 but a tTGA level ≥100 and positive EMA.[34] Similarly, CD may have been missed in some of these patients because duodenal bulb biopsies were not obtained routinely, whereas as has been described recently, this is sometimes the only site affected.[35] Finally, the patients with a false-positive serology may have potential CD and may thus develop CD at some point in their lives. Unfortunately, our study did not include follow-up of these patients to verify in how many patients CD eventually developed.

Even with the current caveats, our data imply that tTGA ≥100 U/mL is highly suggestive for CD because only 1 patient would have been misdiagnosed if a biopsy would not have been performed. Remarkably, this patient did not respond to the GFD, whereas almost all of the other symptomatic patients with tTGA ≥100 U/mL showed an excellent clinical response if they were compliant with the diet. Therefore, it can be considered to start a GFD in all symptomatic patients with a tTGA level ≥100 U/mL. If symptoms disappear the diagnosis is final, without a biopsy being required.

The present study suggests that by applying this strategy, no patients will be misdiagnosed as having CD, whereas the number of biopsies performed can be reduced significantly: of the 114 patients with a tTGA level ≥100 U/mL in whom the response to a GFD could be judged only 3 would have needed a biopsy. By contrast, of the 49 patients with a positive tTGA yet with <100 U/mL, 17 patients would have been misdiagnosed if a biopsy would not have been performed. Obviously, in these patients a biopsy is still necessary to confirm the diagnosis. Likewise, positivity for both EMA and tTGA did not guarantee the presence of CD. With a PPV of 89% this combination was only slightly more reliable than using tTGA alone (PPV 88%). When both EMA and tTGA are negative, CD is unlikely. However, a small intestinal biopsy should still be performed if CD is highly suspected on clinical grounds because 4 patients in the present study were negative for both EMA and tTGA (NPV 95%), but still had CD. This presence of seronegative patients with CD has been reported before, especially in children younger than age 2 years.[14,36–39] Indeed, in our study 3 of the 4 patients with false-negative results were younger than age 2 years.

The present study was performed retrospectively and patients with a positive serology were obviously more likely to be referred to our hospital for a small intestinal biopsy. This may have negatively affected the reliability of the serology in the present study. In addition, the specificity of EMA was substantially lower than the values that are generally reported.[12] This could be due to the routine clinical setting in which the study was performed; comparable low values for EMA have been reported from a similar setting.[40] Under these circumstances interobserver variability in judging the results of the semiquantative EMA immunofluorescence method is difficult to avoid, especially if a study is performed over a long period, such as ours.

In summary, no serological test was found to be 100% pathognomonic for CD. Histological confirmation is still needed in most cases. Nevertheless, in the present study all of the symptomatic patients with a tTGA level ≥100 U/mL in whom symptoms improved on a GFD had histological lesions compatible with CD. It can therefore be considered to omit a biopsy in this specific subgroup.

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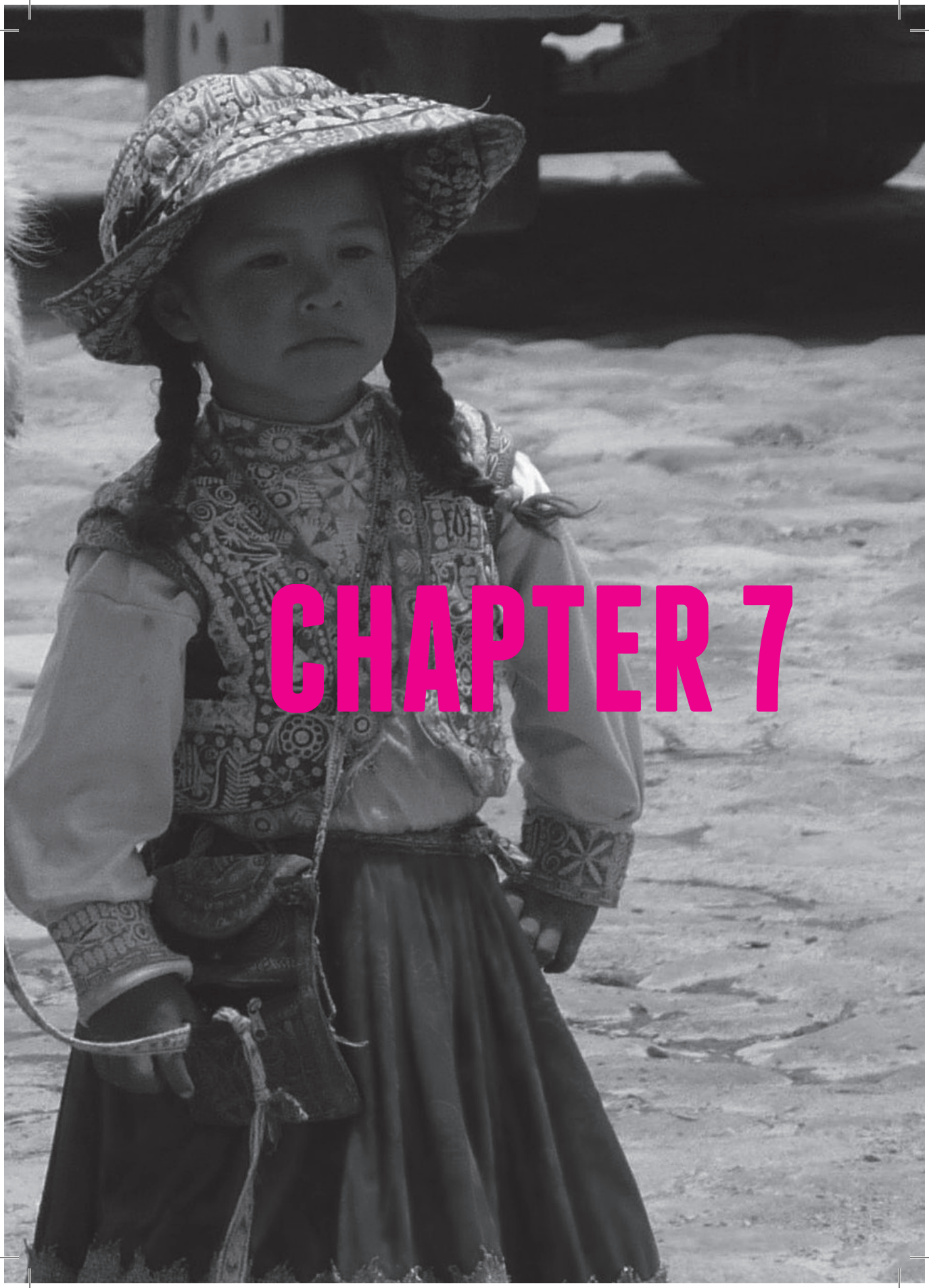

CHAPTER 7

CHAPTER 7

Tissue transglutaminase levels above 100 U/ml and celiac disease: a prospective study

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Abstract

Aim To investigate whether a tissue-transglutaminase antibody (tTGA) level ≥ 100 U/mL is sufficient for the diagnosis of celiac disease (CD).

Methods Children suspected of having CD were prospectively included in our study between March 2009 and September 2011. All patients with immune globulin A deficiency and all patients on a gluten-free diet were excluded from the study. Anti-endomysium antibodies (EMA) were detected by means of immunofluorescence using sections of distal monkey esophagus (EUROIMMUN, Luebeck, Germany). Serum anti-tissue-transglutaminase antibodies (tTGA) were measured by means of ELISA using human recombinant tissue transglutaminase (ELiA Celikey IgA kit Phadia AB, Uppsala, Sweden). The histological slides were graded by a single experienced pathologist using the Marsh classification as modified by Oberhuber. Marsh II and III lesions were considered to be diagnostic for the disease. The positive predictive values (PPVs), negative predictive values (NPVs), sensitivity and specificity of EMA and tTGA along with their 95% confidence intervals (CI) (for the cut off values >10 and ≥100 U/mL) were calculated using histology as the gold standard for CD.

Results A total of 183 children were included in the study. A total of 70 (38.3%) were male, while 113 (61.7%) were female. The age range was between 1.0 and 17.6 years, and the mean age was 6.2 years. One hundred twenty (65.6%) patients had a small intestinal biopsy diagnostic for the disease; 3 patients had a Marsh II lesion, and 117 patients had a Marsh III lesion. Of the patients without CD, only 4 patients had a Marsh I lesion. Of the 183 patients, 136 patients were positive for EMA, of whom 20 did not have CD, yielding a PPV for EMA of 85% (95% CI 78-90) and a corresponding specificity of 68% (95% CI 55-79). The NPV and sensitivity for EMA were 91% (95% CI 79-97) and 97% (95% CI 91-99), respectively. Increased levels of tTGA were found in 130 patients, although only 116 patients truly had histological evidence of the disease. The PPV for tTGA was 89% (95% CI 82-94), and the corresponding specificity was 78% (95% CI 65-87). The NPV and sensitivity were 92% (95% CI 81-98%) and 97% (95% CI 91-99%), respectively. A tTGA level ≥ 100 U/mL was found in 87 (47.5%) patients, all of whom were also positive for EMA. In all these 87 patients, epithelial lesions confirming CD were found, giving a PPV of 100% (95% CI 95-100). The corresponding specificity for this cut-off value was also 100% (95% CI 93-100). Within this group, a total of 83 patients had symptoms, at least gastrointestinal and/or growth retardation. Three patients were asymptomatic but were screened because they belonged to a group at risk for CD (diabetes mellitus type 1 or positive family history). The fourth patient who lacked CD-symptoms was detected by coincidence during an endoscopy performed for gastro-intestinal bleeding.

Conclusion This study confirms based on prospective data that a small intestinal biopsy is not necessary for the diagnosis of CD in symptomatic patients with tTGA ≥ 100 U/mL.

Introduction

Celiac disease (CD) is an immune-mediated enteropathy affecting approximately 1% of the worldwide population.[1-3] The immune reaction occurs when genetically susceptible individuals ingest gluten, which is a storage protein in wheat and the related grain species barley and rye, and this reaction is completely reversible upon gluten withdrawal, which is currently the only available treatment for CD.[3-5] The gold standard for the diagnosis of CD has been considered to be a small intestinal biopsy since the histological lesions of CD were discovered in 1954.[6, 7] However, a small intestinal biopsy is not only expensive, time-consuming and stressful for children and their parents but may also provide inconclusive or even false results, due to patchy disease or to inadequate quality or orientation of the biopsy specimen.[8-10] Therefore, there has long been research focused on finding non-invasive markers to diagnose CD. For this purpose, the disease-associated auto-antibodies, especially anti-endomysium antibodies (EMA) and anti-tissue-transglutaminase antibodies (tTGA), have proven to be highly sensitive and specific.[6, 11-14] In fact, according to the new ESPGHAN guidelines for the diagnosis of CD, a confirmatory small intestinal biopsy is no longer necessary in genetically predisposed individuals who are symptomatic and who have a tTGA of at least 10 times the upper limit of normal, a positive EMA and a good clinical response to the gluten free diet.[15] However, these new guidelines for children are mainly based on retrospective data.[16-18] Because such study designs are subject to selection bias, and because the diagnosis of CD implies a lifelong gluten free diet, the diagnosis of CD should be based on serology only when the chance of a false positive result is close to zero. The aim of the present study was therefore to evaluate prospectively whether the new diagnostic approach in patients with high tTGA levels is justified.

Materials and methods

Study population

All patients who were referred to the Wilhelmina Children's Hospital in Utrecht, the Netherlands with the suspicion of having CD were prospectively included in the study between March 2009 and September 2011. Patients were referred to us because of symptoms associated with CD (e.g., abdominal symptoms, growth retardation) or because they belonged to a group at risk for CD, e.g., patients with Down syndrome or Diabetes Mellitus and patients with a positive family history for CD. In this patient group, serology (both EMA and tTGA) was performed, and any patient with abnormal serology was biopsied, as were patients with negative serology but a high clinical suspicion of CD. Patients with immunoglobulin A (IgA) deficiency (n=8) and patients on gluten restriction during the diagnostic work-up were excluded from the study. The study was performed according to the guidelines of the local medical ethics board.

Serological assessment

IgA EMA values were detected by indirect immunofluorescence using sections of distal monkey esophagus mounted on glass slides (EUROIMMUN, Luebeck, Germany). Serum IgA tTGA values were measured using the ELiA Celikey IgA kit (Phadia AB, Upp-

sala, Sweden). As recommended by the manufacturer, serum samples containing an antibody titer greater than 10 U/mL were considered positive. Total IgA was measured in all patients, and a serum IgA concentration below 0.07 g/L was regarded as IgA deficiency.

Histological evaluation

Duodenal biopsies were obtained by upper gastrointestinal endoscopy. An average of 3.1 (with a range of 1 to 8) biopsies per patient were taken from the distal duodenum. Starting at the end of 2009, duodenal bulb biopsies were also routinely obtained during endoscopy, as recent studies suggested that this region could be the only affected site in CD.[19] On average, 1.9 biopsies per patient were taken from this location with a range of 0 to 5.

Histological diagnosis for all patients was performed by a single experienced pathologist using the Marsh classification as modified by Oberhuber.[20, 21] The pathologist had no knowledge of the serological results or of the clinical presentation of the patients. An increased number of intraepithelial lymphocytes (Marsh I) were considered not to be diagnostic for CD. By contrast, Marsh I combined with crypt hyperplasia (i.e., Marsh II) or findings with villous atrophy (Marsh III) were considered to be diagnostic for CD.

Statistical analysis

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the screening tests, which exhibited 95% confidence intervals (CI), were calculated using the histological evaluation as the gold standard. It was subsequently determined whether a tTGA level ≥ 100 U/mL is associated with a nearly perfect PPV.

Results

A total of 183 patients met the inclusion criteria of the study. Of those patients, 70 (38.3%) were male, and 113 (61.7%) female with an age range of between 1.0 and 17.6 years and a mean age of 6.2 years. A total of 120 (65.6%) patients had a biopsy diagnostic for CD, of whom only 3 patients had a Marsh II lesion. In the remaining 63 (34.4%) patients, the diagnosis of CD could be excluded. Of the patients without CD, only 4 patients had Marsh I histology.

Of the total study population, 138 patients had positive EMA and/or tTGA antibodies, while 45 patients were negative for both antibodies. The patients who were negative for both antibodies underwent a small intestinal biopsy because of a strong clinical suspicion of CD (CD-like symptoms). Three of these patients had a Marsh III lesion, and one patient had a Marsh II lesion, while the diagnosis of CD could be excluded in the remaining 41 patients.

A positive EMA was found in 136 (74.3%) patients; 20 (31.7%) of them did not meet the histological criteria for CD (Table 1), giving a specificity of only 68% (Table 2). The corresponding PPV was 85%. The specificity of tTGA was slightly better (78%), with 116 of 130 positive patients being correctly diagnosed (Table 1). The corresponding PPV was also better at 89% (Table 2).

EMA was undetectable in 47 (25.7%) patients, of whom 43 indeed showed normal histology (Table 1). Consequently, the sensitivity and NPV of EMA were high with values of 97% and 91%, respectively (Table 2). These values were equally high for tTGA, i.e., 97% and 92%, respectively. Illustratively, 49 of the 53 patients with negative tTGA did not have CD (Table 1).

		Biopsy data		
		Patients N=183	**Patients with CD N=120 (65.6)**	**Patients with normal histology N=63 (34.4)**
IgA EMA	Negative	47 (25.7)	4 (3.3)	43 (68.3%)
	Positive	136 (74.3)	116 (96.7)	20 (31.7%)
IgA tTGA >10	Negative	53 (29.0)	4 (3.3)	49 (77.8%)
	Positive	130 (71.0)	116 (96.7)	14 (22.2%)
IgA tTGA ≥ 100	Negative	96 (52.5)	33 (27.5)	63 (100%)
	Positive	87 (47.5)	87 (72.5%	0 (0.0%)

Table 1 Results of small-intestinal biopsy and serology n (%). CD, Celiac disease; IgA, immunoglobulin A; EMA, anti-endomysium antibodies; tTGA, anti-tissue-transglutaminase antibodies.

A total of 42 patients (23.0%) had tTGA levels between 10 and 100 U/mL. Of those patients, only 28 (66.7%) had CD, while the diagnosis could be histologically excluded in 14 (33.3%) patients. Of the latter group, 3 patients had a Marsh I lesion. By contrast, the 87 patients with a tTGA level ≥ 100 U/mL all met the histological criteria for CD (Table 1), yielding a PPV of 100% (Table 2). All were also positive for EMA. Among these 87 patients, only 4 patients were asymptomatic. Three patients were screened because they belonged to a group at risk for CD (diabetes mellitus type 1 or a positive family history for CD). The fourth patient who lacked CD-symptoms was detected by coincidence during an endoscopy performed for gastro-intestinal bleeding. All other patients (n = 83) had typical symptoms (at least gastro-intestinal symptoms and/or growth retardation). After the diagnosis of CD was made, all patients adhered to the gluten-free diet, and the vast majority showed clinical improvement.

	Sensitivity	**Specificity**	**PPV**	**NPV**
IgA EMA	97 (91-99)	68 (55-79)	85 (78-90)	91 (79-97)
IgA tTGA >10	97 (91-99)	78 (65-87)	89 (82-94)	92 (81-98)
IgA tTGA ≥ 100	73 (63-80)	100 (93-100)	100 (95-100)	66 (55-75)

Table 2 Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of EMA and tTGA (%). The 95% confidence intervals are given in parentheses. IgA, immunoglobulin A; EMA, anti-endomysium antibodies; tTGA, anti-tissue-transglutaminase antibodies.

Discussion

In patients with high tTGA levels, there is increasing evidence that a small intestinal biopsy is not needed to confirm the diagnosis of CD, as these increased levels are highly suggestive of the disease. This conclusion was also stated in the new ESPGHAN guidelines for the diagnosis of CD in the pediatric population.[15] Briefly, these guidelines suggest that in symptomatic individuals who have tTGA levels of at least 10 times the upper limit of normal and who respond well to the gluten free diet, histological confirmation is unnecessary. However, prospective studies are needed to confirm the applicability of these guidelines in clinical practice.

The sole reliance on serology for the diagnosis of CD is appropriate only if the PPV is close to 100%. In this study, it was prospectively shown that 87/87 patients with a tTGA of at least 100 U/mL did indeed suffer from CD, giving a PPV of 100%. However, in this cohort, most of the patients had typical CD symptoms and responded well to the diet, while only 4 patients lacked any CD associated symptoms. Therefore, due to the underrepresentation of asymptomatic patients in this cohort, it can be questioned whether this perfect PPV will also be observed in asymptomatic patients.

Comparable results were found in previous retrospective studies, showing that high tTGA levels are associated with histological lesions compatible with CD.[16-18] Barker et al. showed that 48 of 49 mostly symptomatic children with a tTGA level ≥100 U/mL had at least Marsh II enteropathy.[22] Comparably, Donaldson et al. showed that 38 of the 38 pediatric patients with tTGA ≥100 U/mL had Marsh III histopathology.[23] A subsequent retrospective study, also in a pediatric population, showed that all symptomatic patients with tTGA of at least 100 U/mL who responded well to the diet had CD (n = 111), thereby reaching a PPV of 100%.[24]

Similarly, in a study conducted in a mixed adult/pediatric population, it was shown that a tTGA ≥100 U/mL occurs almost exclusively in the setting of Marsh III (73 of 76 patients) and that the 3 patients without villous atrophy had either a Marsh II (n = 2) or a Marsh I (n = 1) lesion.[25] Likewise, a study performed in adults showed that 91 patients with a tTGA level of at least 10 times the upper limit all had at least Marsh II enteropathy.[16] By contrast, Freeman reported that 3 of 14 adult patients with tTGA ≥100 U/mL did not have CD.[26] Notably, in the latter 3 studies, an exact description of the clinical presentation of the patients was lacking.[16, 25, 26]

To the best of our knowledge, only one other prospective study has been performed in a mixed pediatric and adult population. This study showed that 1 of the 72 patients with a tTGA of at least 11.4 times the upper limit of normal had a normal small intestinal biopsy, yielding a PPV of 98.6%, which the authors considered to be insufficient for omitting a biopsy.[27] However, in this study, the presence of symptoms was not taken into consideration, which may influence the PPV. In fact, the patient with this high level of tTGA and a normal biopsy did have an excellent clinical and serological response to the diet, suggesting that CD may have been missed histologically.

In conclusion, the current study shows that 87/87 patients with tTGA ≥ 100 U/mL had CD, which confirms the new ESPGHAN guidelines and other retrospective studies. However, because almost all studied patients in this study were symptomatic, omitting a biopsy should only be considered in this group. By contrast, in asymptomatic individuals, a small intestinal biopsy should still be performed, at least until more studies become available studying this specific group.

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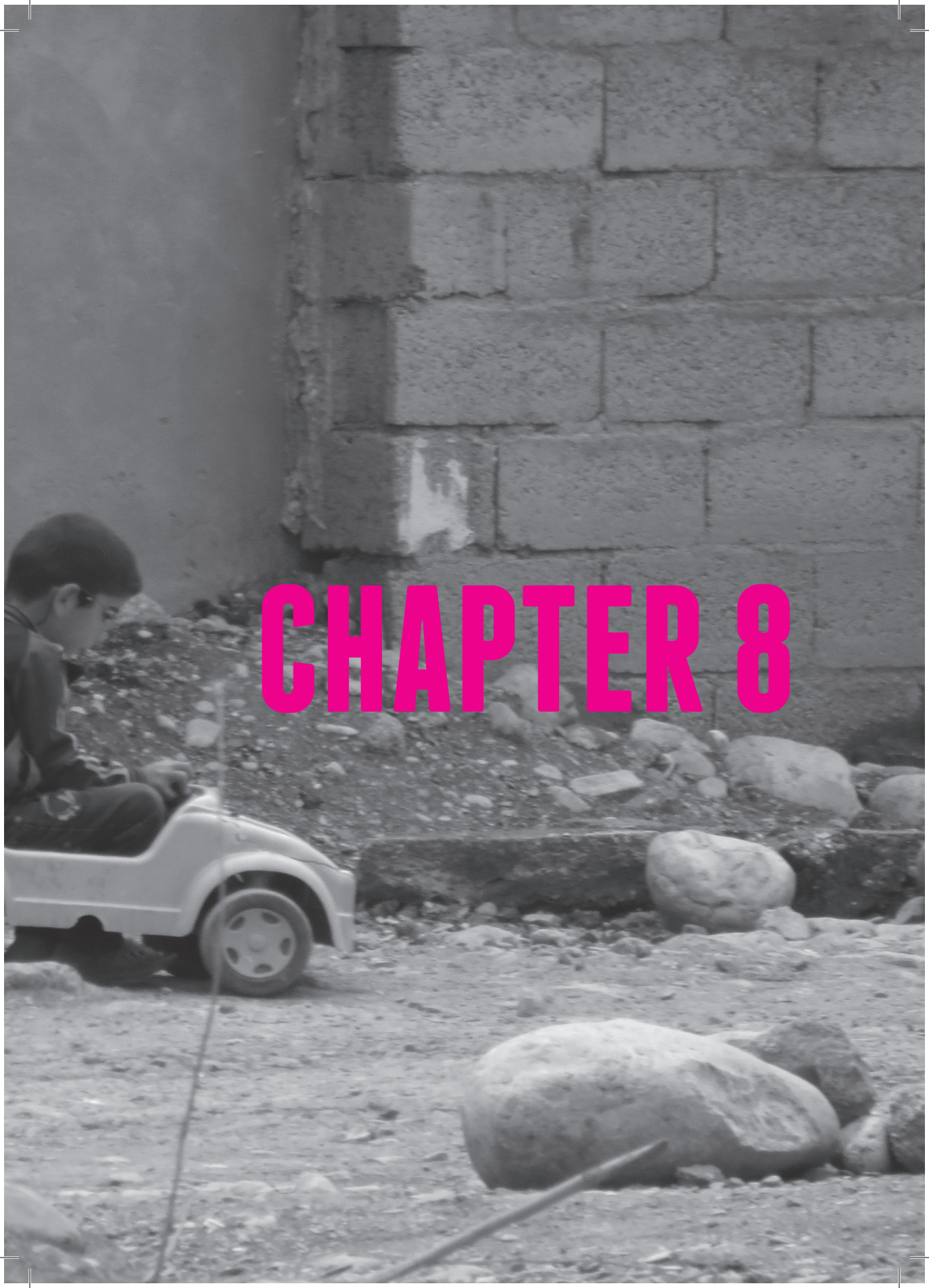

CHAPTER 8

CHAPTER 8

Children with celiac disease and high tTGA are genetically and phenotypically different

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Abstract

Aim To investigate whether celiac disease (CD) patients with tissue-transglutaminase antibody (tTGA) ≥100 U/ml are different from patients with lower tTGA levels.

Methods Biopsy-proven (Marsh III) pediatric CD patients (n=116) were prospectively included between March 2009 and October 2012. The biopsies were evaluated by a single pathologist who was blinded to all of the patients' clinical data. The patients were distributed into 2 groups according to their tTGA level, which was measured using ELISA: tTGA ≥100 U/ml and tTGA<100 U/ml. The patients' characteristics, symptoms, human leukocyte antigen (HLA) genotype and degree of histological involvement were compared between the 2 groups.

Results A total of 34 (29.3%) children had tTGA values <100 U/ml and 82 (70.7%) tTGA levels of ≥100 U/ml. Patients with high tTGA levels had lower average body weight-for-height standard deviation scores (SDS) than did patients with tTGA <100 U/ml (-0.20±1.19 SDS vs. 0.23±1.03 SDS; p-value 0.025). In the low tTGA group, gastrointestinal symptoms were more common (97.1% vs. 75.6%; p-value 0.006). More specifically, abdominal pain (76.5% vs. 51.2%; p-value 0.012) and nausea (17.6% vs. 3.7%; p-value 0.018) were more frequent among patients with low tTGA. In contrast, patients with solely extraintestinal manifestations were only present in the high tTGA group (18.3%; p-value 0.005). These patients more commonly presented with aphthous stomatitis (15.9% vs. 0.0%; p-value 0.010) and anemia (32.9% vs. 11.8%; p-value 0.019). In addition, when evaluating the number of CD-associated HLA-DQ heterodimers (HLA-DQ2.5, HLA-DQ2.2 and HLA-DQ8), patients with low tTGA levels more commonly had only 1 disease-associated heterodimer (61.8% vs. 31.7%; p-value 0.005), while patients with high tTGA more commonly had multiple heterodimers. Finally, patients with tTGA ≥100 U/ml more often had a Marsh IIIc lesion (73.2% vs. 20.6%; p-value <0.001) while in patients with low tTGA patchy lesions were more common (42.4% vs. 6.8%; p-value <0.001).

Conclusion Patients with tTGA ≥100 U/ml show several signs of more advanced disease. They also carry a larger number of CD associated HLA-DQ heterodimers.

Introduction

Celiac disease (CD) is a highly prevalent disorder with a strong genetic component. The disease has a complex and variable clinical presentation: some patients display symptoms ranging from severe malabsorption to vague intestinal or extraintestinal manifestations, while others have no symptoms at all.[1-3] The disease is caused by inappropriate immune responses to gluten, a storage protein in wheat and the related grain species barley and rye.[4] The immune reaction mainly affects the small intestine, where it typically causes lymphocyte invasion in the epithelium, hyperplasia of the crypts and various grades of villous atrophy.[5,6] These histological lesions can be patchily distributed throughout the small intestine and can even occasionally be localized exclusively in the duodenal bulb.[7,8] Serologically, signs of inflammation are also evidenced by the presence of disease-associated antibodies, including endomysium antibodies (EMA) and tissue transglutaminase antibodies (tTGA).[4,9,10]

Until recently, these serological and histological manifestations were used in combination to detect CD, with histological evaluation being essential for establishing the diagnosis in all cases.[9,11] However, given the excellent sensitivity and specificity of serology, the new ESPGHAN guidelines now indicate that a biopsy can be omitted in symptomatic children with tTGA levels ≥100 U/ml (≥10 times the upper limit) and positive EMA, provided the patient also carries a disease-associated human leukocyte antigen (HLA) type and responds well to the diet.[12] In contrast, in patients with a tTGA <100 U/ml, a biopsy is always necessary because a significant proportion of patients with these levels do not have CD.

It is unclear why patients with a tTGA ≥100 U/ml virtually always have CD. These high levels could be a sign of advanced disease. Patients with high serum tTGA may also have a different genetic risk profile. Because HLA genes make the greatest genetic contribution, the aim of this study was to assess whether patients with a tTGA ≥100 U/ml have a different HLA distribution compared with patients with lower tTGA levels.[13] We also investigated whether more advanced small intestinal histological lesions were present in patients with a tTGA ≥100 U/ml. In addition, as it remains to be resolved whether patients with tTGA levels ≥100 U/ml are phenotypically distinct from those with a tTGA <100 U/ml, we set out to detect differences in clinical presentation between both groups.

8

Materials and methods

Study population

Pediatric patients who had a histologically confirmed diagnosis of CD between March 2009 and October 2012 in the Wilhelmina Children's Hospital in Utrecht, The Netherlands, were prospectively included in the study. Patients were referred to us because of CD-associated symptoms or because they belonged to a group at risk for CD. Biopsies were collected from patients with abnormal serology. Biopsies were also collected from patients with negative serology but a strong clinical suspicion of the disease. Patients with immune globulin A (IgA) deficiency (N=3) were excluded from the study.

The clinical symptoms at presentation were collected from the medical records. The study was performed according to the guidelines of the local medical ethics board.

Histological evaluation

Biopsies were obtained using upper endoscopy. On average, 3.09 biopsies (range 1 to 5, SD 0.75) were obtained from the distal duodenum, and 2.41 (range 0 to 5, SD 1.03) were obtained from the duodenal bulb. The biopsies were evaluated by a single experienced pathologist who was blinded to all of the patients' clinical data and who used the Marsh classification, as modified by Oberhuber.[5,6] The duodenal bulb and the distal duodenum were scored separately, but the final Marsh score for each patient was graded according to the most affected site (highest Marsh score). Only Marsh III lesions (i.e., those characterized by an increased number of intraepithelial lymphocytes, crypt hyperplasia and villous atrophy) were considered diagnostic for CD. Patients with other histological findings were not included. Marsh III lesions were further classified according to the degree of villous atrophy: Marsh IIIa (partial villous atrophy), Marsh IIIb (subtotal villous atrophy) and Marsh IIIc (total villous atrophy).

Serological assessment

Serum IgA tTGA levels were measured using the ELiA Celikey IgA kit (Phadia AB, Uppsala, Sweden). Serum samples containing an antibody titer of more than 10 U/ml were considered positive, as recommended by the manufacturer. IgA EMA levels were detected via indirect immunofluorescence using sections of distal monkey esophagus mounted on glass slides (IMMCO Diagnostics Inc., Buffalo NY). Total IgA was measured in all patients, and a serum IgA concentration below 0.07 g/L was regarded as IgA deficiency.

HLA-typing

Genomic DNA was isolated from ethylenediaminetetraacetic acid-anticoagulated blood with a standardized DNAzol-based technique. The HLA-DQA1 and HLA-DQB1 alleles were typed using the sequence-specific oligonucleotide primed PCR (PCR-SSO) technique with the Luminex-based One Lambda LABType SSO Class II DQA1/DQB1 typing kit, following the recommendations of the manufacturer (One Lambda Inc., Canoga Park, CA, United states). Samples were analyzed on a LABScanTM 100 System (Luminex, Austin TX, United States), and data were interpreted using the HLA-Fusion 2.0 Software package (One Lambda).

HLA-DQ2.5 (DQA1*05:01, -DQB1*02:01 or DQA1*05:05, -DQB1*02:02), HLA-DQ2.2 DQA1*02:01, -DQB1*02:02) and HLA-DQ8 (DQA1*03:01, -DQB1*03:02 or DQA1*03:02, -DQB1*03:02) were considered CD-associated HLA-types. The patients were scored for the number of CD-associated heterodimers that they could form with their HLA-genotypes. For example, a patient who is homozygous for HLA-DQ2.5 (or HLA-DQ2.2 or HLA-DQ8) can form 4 different heterodimers that are associated with CD. The same is true of patients who are compound heterozygous for HLA-DQ2.5 and HLA-DQ2.2, because these patients can also make 4 different CD-associated heterodimers: HLA-DQA1*05:01, -DQB1*02:01; HLA-DQA1*02:01, -DQB1*02:02; HLA-DQA1*05:01, -DQB1*02:02 and HLA-DQA1*02:01, -DQB1*02:01 (the latter 2 of which are molecularly indistinguishable from the first 2).

Patients who are heterozygous for HLA-DQ2.5 and HLA-DQ8 or HLA-DQ2.2 and HLA-DQ8 can only form 2 CD-associated heterodimers. Finally, patients with only 1 CD-associated HLA genotype can only generate 1 CD-associated heterodimer.

Statistical analysis

The patients were divided in 2 groups: those with tTGA≥100 U/ml and those with tTGA<100 U/ml. Subsequently, the differences between the 2 groups in terms of gender, average age at diagnosis, average height and weight, the presence of a CD-associated disease, the presence of a first-degree relative with CD, symptoms, HLA-type, Marsh classification and histological differences between the duodenal bulb and the more distal duodenum were calculated using SPSS Version 20.0. To test for statistical significance, the chi-squared or Fisher exact test was used for nominal variables. For continuous variables, the independent t-test or the Mann-Whitney U-test were used. A p-value <0.05 was considered statistically significant.

Results

Patient characteristics

A total of 116 patients met the study's inclusion criteria. Of those, 34 (29.3%) patients had tTGA values <100 U/ml and 82 (70.7%) had a serum tTGA of at least 100 U/ml. Within the low tTGA group, 2 patients, a 10-month-old girl and a 2-year-old boy, had a tTGA level <10 U/ml and negative EMA, which is not an uncommon finding in very young children.[11,14-18] All of the remaining patients had positive EMA levels. Of the total study population, 32 (27.6%) were male and 84 (72.4%) female, with no difference in gender distribution between the high and low tTGA groups (Table 1).

	tTGA < 100 U/ml N=34	tTGA ≥ 100 U/ml N=82	*P* value
Gender (M)	8 (23.5)	24 (29.3)	0.529
Average age (yrs)	7.4 (SD 4.06)	6.1 (SD 3.82)	0.114
Average height in SDS	-0.60 (±1.15)	-0.83 (±1.22)	0.331
Average weight for height in SDS	0.23 (±1.03)	-0.20 (±1.19)	**0.025**
CD associated comorbidity	1 (2.9)	10 (12.2)	0.171
First degree relative with CD	9 (26.5)	14 (17.1)	0.248

Table 1 Characteristics of patients N (%). tTGA, anti-tissue transglutaminase antibodies; M, Male; yrs, years; SDS, standard deviation scores; CD, celiac disease.

The mean age of the included patients at diagnosis was 6.5 years, ranging from 0.9 to 17.7 years. The average age at diagnosis was slightly higher (7.4 years) in the low tTGA group compared with the high tTGA group (6.1 years), but this was statistically not significant. The patients in the high tTGA group were slightly shorter (-0.83 standard deviation score, SDS) compared with the low tTGA group (-0.60 SDS), but this difference was not significant.

In contrast, the average body weight-for-height was significantly lower (-0.20 SDS) in the high tTGA group compared with patients in the low tTGA group, who had an average weight of 0.23 SDS (p-value 0.025).

Regarding comorbidity, 5 (4.3%) patients had Down syndrome, and 1 (0.86%) of those also had hypothyroidism. Another 4 (3.4%) patients had diabetes mellitus Type I, 1 (0.86%) patient had juvenile rheumatoid arthritis and 1 (0.86%) patient had Graves disease. Remarkably, all but 1 of the patients with comorbidity had tTGA≥100 U/ml; however, this finding was not statistically significant. Finally, 9 (26.5%) patients in the low tTGA group had a first-degree relative with CD, compared with 14 (17.1%) patients in the high tTGA group; again, this difference was not statistically significant.

Symptoms

Only 5 (4.3%) patients were asymptomatic, 4 of which had a tTGA ≥100 U/ml and 1 of which had a tTGA <100 U/ml (Table 2). The other 111 (95.7%) patients had various gastrointestinal and extraintestinal symptoms. Interestingly, gastrointestinal symptoms were significantly (p-value 0.006) more common in the low tTGA group (N=33; 97.1%) compared with the high tTGA group, in which 75.6% (N=62) of the patients suffered from a gastrointestinal symptom. However, although patients with symptoms restricted to the gastrointestinal tract (without any extraintestinal manifestations) were also more common in the low tTGA group (23.5% vs. 9.8%, respectively), this difference was not statistically significant (p-value 0.074). In terms of specific gastrointestinal complaints, abdominal pain and nausea were significantly more common in the low tTGA group. Indeed, 76.5% (N=26) of the patients in the low tTGA group had abdominal pain, compared with 51.2% (N=42) in the high tTGA group (p-value 0.012). Similarly, in the low tTGA group, 17.6% (N=6) of the patients suffered from nausea, compared with 3.7% (N=3) in the high tTGA group (p-value 0.018). Moreover, there was a statistically non-significant trend (p-value 0.096) towards more constipation in the low tTGA group (N=14; 41.2%) compared with the high tTGA group (N=21; 25.6%). In contrast, diarrhea was more common in the high tTGA group (N=27; 32.9%) compared with the low tTGA group (N=8; 23.5%), but the difference was not significant (p-value 0.316). Similarly, a comparable trend (p-value 0.277) was seen for vomiting, which occurred more often in the high tTGA group (11.0% vs. 2.9%). Finally, the presence of bloating was comparable in both groups with more than 1/3 of the patients suffering from this symptom.

Extraintestinal symptoms occurred in 25 (73.5%) of the patients with low tTGA compared with 70 (85.4%) patients in the high tTGA group, but this difference was not statistically significant (p-value 0.132). However, patients with solely extraintestinal symptoms (i.e., without gastrointestinal symptoms) were only present in the high tTGA group (N=15; 18.3%), a finding that was statistically significant (p-value 0.005). Similarly, aphthous stomatitis only occurred in patients with high tTGA (N=13; 15.9%). This was statistically significant, with a p-value of 0.010. Likewise, anemia was significantly (p-value 0.019) more common in the high tTGA group: 27 (32.9%) of the patients with high tTGA had anemia, compared with 4 (11.8%) patients with low tTGA. There was also a trend towards more increased appetite (7.3% vs. 2.9%), joint pain (11.0% vs. 5.9%) and low weight (8.5% vs. 5.9%) in the high tTGA group, but these differences were not statistically significant (p-value >0.05).

Tooth enamel defects were more common in the low tTGA group (5.9% vs. 3.7%), but this was also not statistically significant (p-value 0.629). Finally, the presence of fatigue, irritability, anorexia and short stature was comparable in both groups.

Symptoms	tTGA <100 U/ml N=34	tTGA ≥100 U/ml N=82	*P* value
Asymptomatic	1 (2.9)	4 (4.9)	1.000
Gastrointestinal symptoms			
Any gastrointestinal symptom	33 (97.1)	62 (75.6)	0.006
Only gastrointestinal symptoms	8 (23.5)	8 (9.8)	0.074
Abdominal pain	26 (76.5)	42 (51.2)	0.012
Diarrhea	8 (23.5)	27 (32.9)	0.316
Constipation	14 (41.2)	21 (25.6)	0.096
Bloating	12 (35.3)	31 (37.8)	0.799
Nausea	6 (17.6)	3 (3.7)	0.018
Vomiting	1 (2.9)	9 (11.0)	0.277
Extraintestinal symptoms			
Any extraintestinal symptom	25 (73.5)	70 (85.4)	0.132
Only extraintestinal symptoms	0 (0.0)	15 (18.3)	0.005
Fatigue	16 (47.1)	35 (42.7)	0.666
Irritability	9 (26.5)	25 (30.5)	0.665
Anorexia	13 (38.2)	32 (39.0)	0.937
Increased appetite	1 (2.9)	6 (7.3)	0.672
Joint pain	2 (5.9)	9 (11.0)	0.504
Tooth enamel defects	2 (5.9)	3 (3.7)	0.629
Aphthous stomatitis	0 (0.0)	13 (15.9)	0.010
Anaemia	4 (11.8)	27 (32.9)	0.019
Short stature (height <-2 SDS)	4 (11.8)	11 (13.4)	1.000
Low weight (<-2 SDS)	2 (5.9)	7 (8.5)	1.000

Table 2 Symptoms in celiac disease patients N (%). tTGA, anti-tissue transglutaminase antibodies; SDS, standard deviation scores.

HLA-types

All of the patients carried at least one of the CD-associated HLA-types. In the high tTGA group, the patients more often carried multiple CD-associated heterodimers (p-value = 0.005; Table 3). Illustratively, in the low tTGA group, more than half of the patients (N=21; 61.8%) had only one CD-associated heterodimer, compared with 26 (31.7%) in the high tTGA group. Two patients (5.9%) with low tTGA had 2 CD-associated heterodimers, compared with 20 (24.4%) patients in the high tTGA group. Finally, 36 (43.9%) patients in the high tTGA group had 4 CD-associated heterodimers, compared with 11 (32.4%) patients with low tTGA.

Histology

In the low tTGA group, 5 (14.7%) patients had a Marsh IIIa lesion, 22 (64.7%) had a Marsh IIIb lesion, and only 7 (20.6%) had a Marsh IIIc lesion (Table 3). This was significantly different from the high tTGA group (p-value < 0.001). Illustratively, only 4 (4.9%) patients in the high tTGA group had a Marsh IIIa lesion; 18 (22.0%) had a Marsh IIIb lesion, and the largest proportion of the patients in the high tTGA group (N=60; 73.2%) had flat mucosa (Marsh IIIc).

In 106 patients, both duodenal bulb and distal duodenum biopsies were taken. To assess the presence of patchy lesions, the Marsh classification in both locations was compared. A patchy lesion was defined as the absence of villous atrophy in either the duodenal bulb or the distal duodenum. In 7 (6.6%) patients, a Marsh III lesion was only found in the duodenal bulb, while the distal duodenum was spared. In 12 (11.3%) patients, the distal duodenum was the only affected site. Interestingly, a discrepancy between the diagnosis in the distal duodenum vs. the duodenal bulb was more common in patients with low tTGA than in patients with high tTGA (42.4% vs. 6.8%; p-value <0.001). In addition, patchy lesions were more common in patients with Marsh IIIa (in 5 of 9 patients; 55.6%) than in patients with Marsh IIIb (in 13 of 35 patients; 37.1%) or IIIc lesions (in 1 of 62 patients; 1.6%; p-value <0.001).

	tTGA <100 U/ml N=34	tTGA ≥100 U/ml N=82	*P* value
HLA-score			
1 heterodimer	21 (61.8)	26 (31.7)	
2 heterodimers	2 (5.9)	20 (24.4)	0.005
4 heterodimers	11 (32.4)	36 (43.9)	
Marsh classification			<0.001
Marsh IIIa	5 (14.7)	4 (4.9)	
Marsh IIIb	22 (64.7)	18 (22.0)	
Marsh IIIc	7 (20.6)	60 (73.2)	
	N=33[1]	N=73[1]	<0.001
Patchy lesions[2]	14 (42.4)	5 (6.8)	

Table 3 Human leukocyte antigen distribution, Marsh classification in celiac disease patients. [1] Only 106 patients out of the total study population also underwent duodenal bulb biopsies; [2] Discrepancy in the diagnosis based on histology in the duodenal bulb *vs* in the distal duodenum. HLA: human leukocyte antigen; tTGA: anti-tissue-transglutaminase antibodies.

Discussion

CD is defined as a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals.[19] Patients with tTGA levels ≥100 U/ml (>10 times the upper limit) virtually always have CD, whereas the disease can be histologically absent in a significant number of patients with a lower serum tTGA level. In the present study, we show in a pediatric population that patients with a tTGA level ≥100 U/ml also have a different HLA-pattern and a more severe histological lesion and seem to be phenotypically different, with more extraintestinal symptoms and a lower body weight.

Patients with high tTGA levels are more likely to have 2 and 4 CD-associated heterodimers compared with patients with lower tTGA levels, who more often only have 1 CD-associated heterodimer (Table 3). This seems pathophysiologically logical. In CD, HLA-molecules on antigen-presenting cells in the lamina propria present gluten peptides to CD4+ T-cells, which in turn further activate the immune system, including B-cells.[20-22] Thus, increased cell-surface expression of CD-associated heterodimers will lead to more antigen presentation and therefore more T- and B-cell stimulation, which will eventually generate a stronger antibody response. However, because not all patients with multiple heterodimers had a tTGA ≥100 U/ml, and some patients with a single HLA-heterodimer also had tTGA levels ≥100 U/ml, other factors, such as non-HLA genes or environmental factors, are likely to contribute to the tTGA-level response. This finding is in line with a previous study showing a correlation between antibody level and HLA-dose; patients homozygous for HLA-DQB1*02 had significantly higher tTGA levels compared with patients with a single dose of HLA-DQB1*02 and to patients not carrying any HLA-DQB1*02.[23] In the current study, a comparable HLA-DQB1*02 correlation was found, but the difference was not significant (p-value 0.101; data not shown).

The current study also provided evidence that patients with high tTGA levels have more advanced mucosal lesions compared with CD patients with lower tTGA levels. First, patients with tTGA levels ≥100 U/ml had a more severe grade of villous atrophy, in line with previous studies showing an increasing tTGA titer with increasing villous atrophy.[24,25] However, we also showed that patchy lesions, defined as the absence of villous atrophy in either the duodenal bulb or the distal duodenum, were more common in patients with low tTGA than in patients with high tTGA, suggesting that in patients with high tTGA, the total area of mucosa involved is larger. In addition, patients with a lesser degree of villous atrophy, which is more common in the low tTGA group, also had a higher chance of patchy lesions, providing more evidence that the disease in these patients is truly less advanced.

Interestingly, we also found significant differences in clinical presentation between patients with high tTGA and those with levels <100 U/ml. The group with high tTGA levels had lower body weight and more extraintestinal complaints than did patients with low tTGA (Table 2). This suggests that patients with high tTGA levels have more advanced or generalized disease. Other studies investigating the relationship between antibody levels and symptoms are rare. Dahlbom and colleagues found that children with an onset of CD in early childhood and/or severe malabsorption had higher tTGA levels than did patients with a late childhood onset of disease and/or moderate symptoms, and also when compared with patients presenting in adulthood.[24] Taavela et al. also showed that the serum levels of antibodies associated with CD correlated with gastrointestinal symptoms.[26] None of these two studies specifically investigated the differences in intestinal and extraintestinal symptoms, so their results cannot be directly compared with our study. However, in both studies, a relationship between antibody levels and symptom severity was observed, once again suggesting that patients with a high tTGA have more advanced disease.

Finally, we showed that patients in the low tTGA group more often have a positive family history for CD (26.5% vs. 17.1%), although this difference was not statistically significant. This difference could have resulted because patients with a positive family history are detected earlier than those without a positive history, before a very high tTGA level is reached. Conversely, patients with comorbidity were found more frequently (although statistically not significant) in the high tTGA group (12.2% vs. 2.9%), which might be due to a more advanced disease progression in this group.

Our combined data confirm, in a pediatric population, the hypothesis that patients with tTGA ≥100 U/ml have more advanced disease, given the more severe histological involvement and the increased incidence of extraintestinal manifestations and lower body weight. Pathophysiologically, these patients also express more CD-associated HLA-heterodimers on their cells. These findings should also be investigated in adults.

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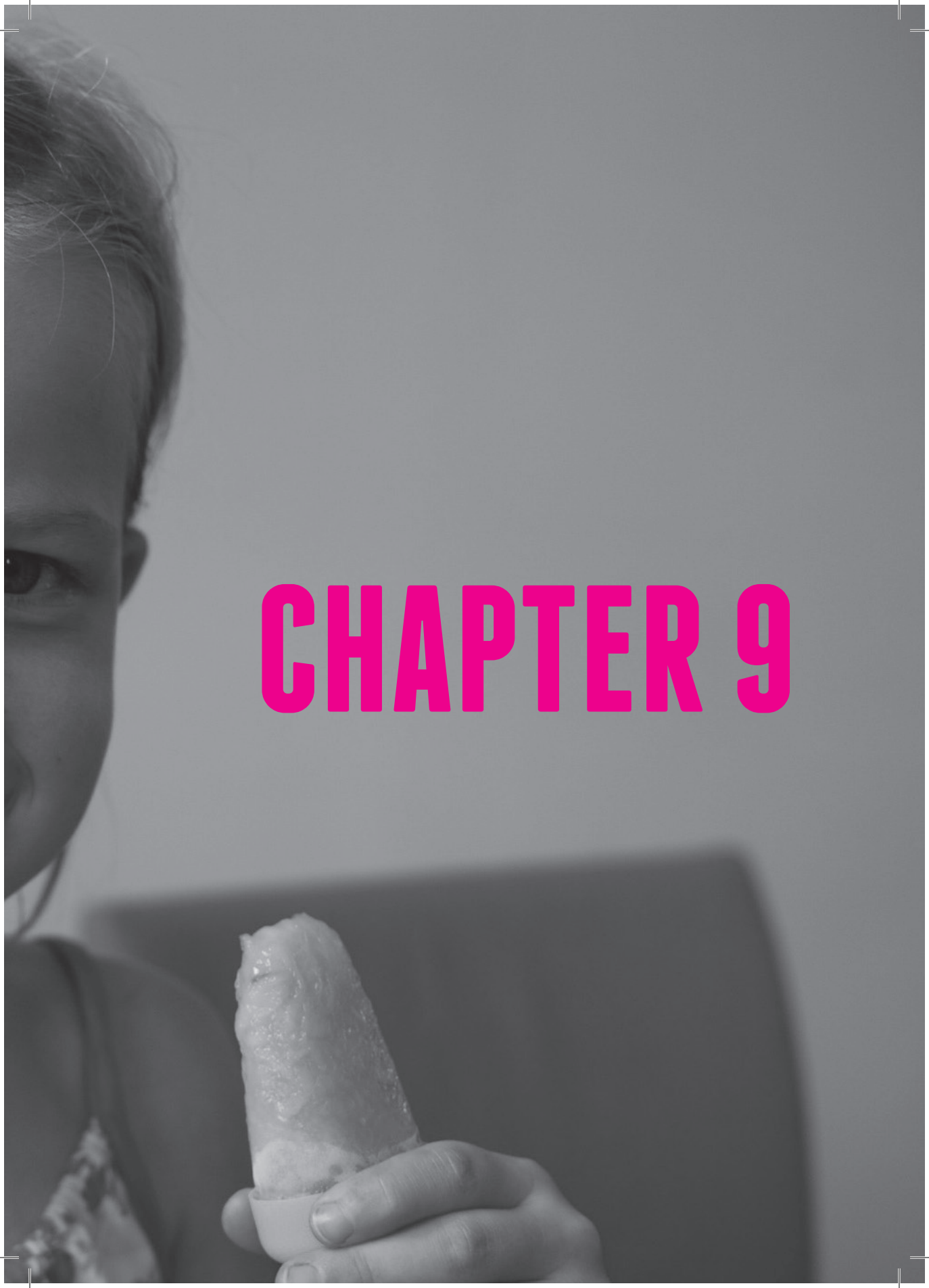

CHAPTER 9

CHAPTER 9

Summarizing discussion

A. Mubarak

The prevalence of celiac disease (CD) is around 1% worldwide.[1] Even with this high frequency the diagnostic methods used in these patients are not totally satisfactory. The aim of this thesis was therefore to improve diagnostics in CD.

Towards improved histology

The main feature of CD is inflammation of the small intestine, which in classical cases consists of intra-epithelial lymphocytosis, hyperplasia of the crypts and various degrees of villous atrophy (grade III enteropathy according to Marsh).[2] Finding these lesions upon small intestinal microscopy was, until recently, the gold standard investigation for the diagnosis of the disease. In fact, the first diagnostic guidelines for CD required even 3 small intestinal biopsies: a biopsy on a gluten containing diet showing the typical CD lesions, followed by a biopsy providing evidence of mucosal recovery on a gluten free diet, and finally a biopsy showing histological deterioration after gluten challenge.[3,4] However, a subsequent study showed that in only a small percentage of children a different diagnosis was made after a challenge, and that the vast majority of the cases with non-confirmed CD were very young children.[5] Therefore, the diagnostic criteria were changed in 1990 and required a gluten challenge only in children below the age of 2 years.[6] Later on it was shown, that even in these very young children a gluten challenge is not necessary.[7] This was a first step towards a less invasive diagnosis, but at least 1 biopsy was still needed.

A small intestinal biopsy is obtained by endoscopy, under general anesthesia in children. This is invasive, expensive, time-consuming, has health risks, and in addition a substantial emotional impact in children. Moreover, due to its invasive character it is not suitable for evaluation of diet effects or compliance. Additionally, a small intestinal biopsy may not be as accurate as was once thought. First of all, the biopsy specimen may be of such poor quality that a correct assessment is impossible.[8] Moreover, even if the quality is good, often the biopsies are cut obliquely, hindering an accurate evaluation of the crypt villous ratio, which is essential for a correct diagnosis. Moreover, the histological lesions in CD can be patchy in nature, thus the diagnosis may be missed due to sampling error.[9,10] In order to minimize this chance, at least 5 biopsies should be taken from the duodenum, including at least 1 biopsy from the duodenal bulb.[11] Finally, additional interpretation difficulties may arise if villous atrophy is not present, but only an increased amount of intra-epithelial lymphocytes (IELs; Marsh I) is found. This finding is however not diagnostic for CD, but when found along with hyperplasia of the crypts (Marsh II)

this becomes diagnostic for the disease, just like a Marsh III lesion.[11-15] However, the amount of IELs can easily be misinterpreted and the difference between Marsh I and II can be very subtle, potentially leading to a missed or incorrect diagnosis of CD. So when taking these difficulties into account, a small intestinal biopsy is a less than optimal gold standard investigation.

To further study the diagnostic reliability of the small intestinal biopsy in CD, in **Chapter 2** we studied the inter-observer variability between 2 pathologists and found that for the Marsh classification the agreement between pathologists is only moderate with a Kappa value of 0.486.[16] More importantly, when considering Marsh III as diagnostic for CD, in 22 (7.4%) of the 297 patients a different diagnosis was made by the second pathologist. Interestingly, in the 22 patients with discrepancy in diagnosis the quality of the biopsy specimen was significantly more often suboptimal. Based on serology, dietary response and follow-up biopsies 14 CD patients were missed by the first pathologist while the second pathologist missed 5 CD patients and over-diagnosed 3 patients. In fact the 14 patients initially missed were a large proportion of the patients who were thought to have false positive CD serology. These results prove that biopsy interpretation is a subjective skill, dependent on the pathologist, and that CD can easily be missed if relying solely on histology. A pathology report should therefore also include a detailed statement about the quality of the biopsy specimen and the certainty of the histological diagnosis. In addition, whenever serology and histology are discrepant, revision of the biopsies should be performed in order to avoid unnecessary subsequent biopsies. Finally, in the same chapter it was shown that performing CD3 staining in order to detect IELs could lead to a different diagnosis. However, CD3 stains were not performed in all patients but only when the number of IELs on the routine stains was unclear.

Therefore in **Chapter 3** we prospectively investigated the additional value of immunohistochemical staining for CD3 and found that in almost 13% (20/159) of the patients a different histological evaluation was made after performing CD3 stains; 9 (45%) patients turned out to have CD (Marsh II/III), but the diagnosis had initially been rejected on HE slides, 9 (45%) patients were classified as Marsh I, while this had also been missed on initial evaluation of the slides, and 2 (10%) patients turned out to be over-diagnosed with either CD or Marsh I on routine sections.[17] Interestingly, in all but one the change in evaluation after CD3 was seen in patients who had a discrepancy between serological results and the conclusion based on routine sections. This patient had negative serology and a Marsh I lesion on CD3 sections, but the intestinal inflammation seen was probably not CD related, but caused by giardiasis. Therefore, in order to make an appropriate diagnosis of all CD-related lesions, CD3 staining should be performed whenever there is discrepancy between serology and the conclusion of the pathologist on routine sections. However, while finding Marsh II and III lesion is clearly important, the relevance of finding Marsh I lesions is less obvious as this is not always a CD related finding.[11]

Non-invasive strategies

Human leukocyte antigen (HLA) typing

So histology is not an ideal gold standard investigation, but reliability may improve with CD3 staining. However, being able to omit a biopsy in the diagnosis of CD would be a major leap forward. To this aim several non-invasive methods could be employed, as discussed below.

To exclude the disease, the best test to use is HLA typing, as virtually all patients with CD carry either HLA-DQ2.5 or HLA-DQ8.[18-22] Therefore, negativity for these heterodimers was used in clinical practice to exclude the diagnosis of CD. However, some studies reported CD patients who were HLA-DQ2.2 positive, without having HLA-DQ2.5 or HLA-DQ8.[23-25] So it is not impossible to develop CD when HLA-DQ2.2 is present. However, it was unclear whether the frequency of this HLA type is sufficient to include it, together with HLA-DQ2.5 and HLA-DQ8, as a firmly established CD associated haplotype. We addressed this question in **Chapter 4** where we showed, in a combined cohort of retrospectively and prospectively included patients, that 9 (5.8%) of the 155 patients with Marsh III lacked the regular HLA types HLA-DQ2.5 or HLA-DQ8, but that all of them carried HLA-DQ2.2.[26] Interestingly, the frequency of HLA-DQ2.2 even surpassed that of HLA-DQ8, which occurred in only 7 (4.5%) patients who were negative for HLA-DQ2.5.

This distribution of HLA heterodimers in patients with CD can be understood pathofysiologically very well. Gluten derived peptides, especially after enzymatic deamidation of gliadin by the enzyme tissue transglutaminase 2, bind preferentially to HLA-DQ2.5 on the surface of antigen presenting cells in the lamina propria, which stimulates the proliferation of gliadin specific CD4+ T cells in the muscosa.[27, 28] The HLA-DQ2.2 molecule is homologous to HLA-DQ2.5 and has an almost identical peptide binding motive, although it is unable to bind gluten peptides with a proline at position 3.[29, 30] Therefore HLA-DQ2.2 can only bind a subset of the epitopes bound by HLA-DQ2.5. In addition, HLA-DQ2.2 preserves the gluten peptide less efficiently in its binding groove as compared to HLA-DQ2.5, making CD4+ T-cell stimulation through HLA- DQ2.2 less efficient.[31] Moreover, it was recently shown that patients with HLA-DQ2.2 have reactive T-cells that do not respond to the common HLA-DQ2.5 restricted epitopes, but to a distinct epitope that is not recognized by HLA-DQ2.5 positive patients. This immunodominant HLA-DQ2.2 epitope requires a serine residue at position 3 for stable binding, so fewer gluten peptides can bind stably to this heterodimer.[32] These differences together seem to explain the lower prevalence of HLA-DQ2.2 in CD patients as compared to HLA-DQ2.5.[33] Similar factors will reduce the efficiency of the T-cell response to gliadin derived peptides in patients with HLA-DQ8. The immunodominant epitope for this molecule is not rich in proline and thus more likely to be degraded by the intestinal enzymes as compared to the proline rich HLA-DQ2.5 restricted epitopes, making HLA-DQ8 restricted epitopes less abundant in the intestinal mucosa.[34, 35] In addition, for HLA-DQ8 deamidation at 2 positions is required for optimal binding of the epitopes whereas deamidation at only 1 position is needed to elicit a HLA-DQ2.5 restricted T-cell response.[33-35]

Serology

Subsequently we focused on the CD associated antibodies in the diagnostic work-up for CD. Since anti-gliadin antibodies (AGA) were already shown to be inaccurate we firstly compared, in **Chapter 5**, the widely-used immunoglobulin A (IgA) anti-endomysium anti-bodies (EMA) and IgA tissue-transglutaminase antibodies (tTGA) with the newly developed antibodies against deamidated gliadin peptides (a-DGP) which can be detected both as IgA and immunoglobulin G (IgG) antibodies.[36-45] We showed that a-DGP , as measured by 2 kits (Bindazyme and Quanta-Lite) did not outperform EMA and tTGA, which is in agreement with previous studies.[11, 46] However, in very young children (< 2 years), although EMA and tTGA performed better than could be expected from previous literature, IgG a-DGP was superior showing a positive and negative predictive value of 100% in our cohort of 55 children < 2 years of age.[46-49] Although these results have to be validated in a larger study with a prospective design, diagnostic accuracy of serology in this group seems to improve when implementing a-DGP.

In **Chapter 6** it was shown in a retrospective design that all 111 symptomatic patients with a tTGA of at least 100 U/ml (10 times the upper limit of normal), who all also had positive EMA and responded well to a gluten free diet, had histological lesions compatible with CD (Marsh III).[50] Therefore this subgroup of patients might not need a biopsy for confirmation of the disease. This was studied again with a prospective design (**Chapter 7**) where it was shown that the positive predictive value of tTGA was indeed 100% if a cut-off value of 100 U/ml (10 times upper the limit of normal) was used.[51] The patients with tTGA ≥100 U/ml all had positive EMA and the majority were symptomatic and responded well to the gluten free diet. Therefore, it seems sensible to omit a biopsy in symptomatic patients who show a good dietary response. The results of these 2 chapters are also supported by other studies.[52-56] With this strategy, which is now implicated in the latest ESPGHAN guidelines, a biopsy is not necessary anymore in a large proportion of patients with CD.[11] In fact a biopsy could have been omitted in almost 40% (111/283) of the patients included in the studies described in **Chapter 6** and **7**.

So in our studies patients with tTGA≥100 U/ml virtually always have CD, but this is not true for patients with lower levels. The question now was, are these 2 groups of CD patients different? We tried to answer this in **Chapter 8** where we prospectively investigated the genetic and phenotypic differences between the 2 groups of CD (Marsh III) patients and found that patients with high tTGA more often carry multiple CD-associated heterodimers compared to patients with lower levels.[57] In addition these patients have more advanced mucosal lesions which are also less patchy. Phenotypically, they have a lower body weight and more often present with extra-intestinal symptoms than patients with lower levels of tTGA who more often have intestinal symptoms. These results provide further evidence that patients with tTGA ≥100 U/ml are truly a distinct group with more advanced disease, probably due to a more severe genotype.

Future directions

In this thesis a first step towards a biopsy free diagnosis was made as it was shown that symptomatic patients with tTGA of at least 100 U/ml (10 times the upper limit of normal), positive EMA and a good clinical response to the diet did not a need a biopsy. However, a biopsy is still needed in patients with positive tTGA, but below <100 U/ml (10 times the upper limit of normal) and in all asymptomatic patients, as is summarized in the new ESPGHAN guidelines. In the near future the aim is to further reduce the necessity for a biopsy, using strategies as outlined below.

Studies until now have focused on measuring the B-cell response (by measuring specific antibodies) as a marker for CD, but measuring the T-cell response could also be useful, especially because a characteristic feature of CD is the presence of a highly specific HLA restricted CD4+ T-cell reaction against gluten.[60-65] Although labor-intensive, the gluten specific CD4+ T-cells, which are directed against a variety of epitopes consisting of α-, γ- and ω-gliadin and glutenin components, can be isolated from the small intestine of affected patients. More interestingly, recent studies have shown that these gut homing T-cells can also be demonstrated in the peripheral blood of adults with CD after a short-term gluten challenge.[66-69] Presumably, these T cells can be detected in the blood due to antigen driven expansion in the intestine and subsequent spillover into the systemic circulation. Larger studies in children need to show us whether these mini challenges followed by T-cell isolation from the blood can be of diagnostic value and replace a biopsy in the remaining patients. Also, this method could be useful in patients who present to the physician while following a gluten free diet, but do not wish to undergo a formal gluten challenge because of possible symptoms. Additionally, measuring the T-cell response in the small intestine might also be useful in patients who still require a small intestinal biopsy but in whom the biopsy turns out to be inconclusive.

Implementing recent genetic findings might also improve diagnostics. Apart from the HLA-genes, which constitutes the largest part of the genetic predisposition in CD, at present almost 40 non-HLA genes, mainly related to the immune system, were also found to be associated with CD.[70] Developing a risk model for CD by using these genes might be helpful in disease prediction. Illustratively, a recent study showed that genetic and expression markers can help to differentiate patients with CD from cases with positive serology but rather normal small intestinal morphology.[71]

At present patients with normal intestinal morphology upon biopsy (Marsh 0), but who nevertheless have CD associated antibodies will pose a diagnostic problem. Some of them will have potential CD, i.e. are at risk to develop overt CD in due course. Indeed the presence of CD associated antibodies is a good predictor for developing CD.[13, 72-81] However, in some patients active CD does not develop, although it is unknown whether CD would eventually have developed if the follow-up was long enough, or that serology in these patients was truly false positive. In addition, in other patients increased serology may clearly only be transient, which can occur in other conditions such as infectious disease, and can even sometimes be found in patients not carrying the disease associated HLA types.[13, 72, 75-77, 80, 82- 85] Usually such cases only have increased tTGA and not EMA. Finally, in a number of patients antibodies fluctuate over time, which especially is seen in patients with an increased risk for CD.[76-78, 80, 86, 87] The latter finding supports the hypothesis that a genetically predisposed individual must pass a threshold by an additional, perhaps environmental factor, before the disease truly develops.

The same problem is also present in patients with Marsh I lesions, which may be a step further towards overt CD. However, intra-epithelial lymphocytosis can also occur in various other diseases or may be a self limiting process.[88-93] Depending on the population studied, the gluten sensitivity of this lesion seem to vary and may even be below 10%.[11] However, it is very difficult to determine whether a Marsh I lesion in a patient is gluten dependent as neither symptoms nor clinical response to the gluten free diet are specific for CD, so we cannot completely rely on those.[94-96] Support for gluten sensitivity might be obtained by gluten challenge, which may worsen the histology to Marsh II/III, although this has only been studied in a small group of adults.[97] Of course, having positive serology is a reliable marker for the development of full blown CD and some patients with Marsh I and increased antibody levels seem to respond (clinical, serological, histological) to gluten elimination, at least on the short term.[13, 72, 73, 78, 79, 81, 92, 98-104] By contrast, some patients with Marsh I and negative serology may also respond to the diet or develop increased levels of antibodies and subsequently Marsh III lesions.[12, 105, 106] It could be possible that such patients had very low levels of antibodies that were only measurable on the intestinal level.[73, 78, 98, 106-109]

So, both potential CD and Marsh I abnormalities give an unclear diagnosis for patients; both findings may develop into overt CD, yet the diagnosis of CD cannot be made in this stage. New studies need to teach us more about the natural history of both conditions, as previous studies were not always transparent. In fact, in older studies sampling error, inadequate gluten intake prior to biopsy and a missed CD diagnosis by the pathologist were not always specifically ruled out, so it cannot be excluded that a proportion of the patients who in time developed CD already had CD in the first place. In addition, it is of great importance to rule out technical issues in measurement of the antibodies, which most often occur when using EMA, because its method is highly observer dependent. Illustratively, in our studies (**Chapter 5-7**), performed in an excellent clinical routine laboratory, we found a significantly higher false positive rate for EMA as compared to studies where EMA was done in research laboratory.[37, 110]

In addition, it would be interesting to know if the development of overt CD can be predicted. Genetics and gluten specific T-cells, but also measuring tTG 2 specific IgA deposits in the lamina propria might be useful for this purpose.[78, 98] If studies would show that not all patients with signs of a beginning gluten intolerance will eventually develop CD, it would be extremely important to know which factors are responsible for pushing the patients over the CD threshold or, on the other hand, which factors might keep them (permanently) from passing this threshold. This knowledge would be extremely useful in developing prevention strategies. Finally, it should be studied if patients with positive serology and Marsh 0 or Marsh I benefit from gluten withdrawal at this stage or that initiating the gluten free diet can be postponed until at least Marsh II histology develops. Again, different rules may apply for different subsets of patients, which we will have to learn from future studies.

Up to 10% of the patients with IgA deficiency develops CD.[11] The new ESPGHAN guidelines do not give a clear statement on the diagnosis in patients with IgA deficiency. As IgG tTGA might be less accurate, than IgA tTGA, it seems inappropriate to rely solely on serology for the diagnosis of CD in patients with IgA deficiency.[111] This is further complicated by the fact that IgG tTGA positivity can persist despite histological improvement, making it difficult to study the effect of the gluten free diet through serology.[112] IgG a-DGP was suggested to be a good alternative for IgG tTGA, but this was not studied yet in children, and results in adults were disappointing.[113]

Finally, and perhaps most importantly, an increasing number of patients with CD is diagnosed without any symptom. These patients are mostly detected when screening high risk individuals.[11] Although a gluten free diet in patients with CD is highly beneficial almost all studies showing these benefits were done in patients with symptoms, simply because before the era of serology asymptomatic patients were not identified. In patients without symptoms the long term benefits are unclear at present. The clinical practice of screening for CD in high risk individuals is only based on their increased risk and not on their potential benefit, although some "asymptomatic" patients report to feel better when a gluten free diet is implemented.[11, 114] Therefore it is urgently needed to investigate the health benefit of a gluten free diet in asymptomatic individuals diagnosed with CD. Of course inclusion in such a study should be very strict: many "asymptomatic" patient have symptoms that are not obvious initially, such as anemia or osteoporosis.[11]

Conclusions and clinical recommendations

The aim of this thesis was to improve diagnostics in CD through evaluating minimal invasive tests. We showed that a small intestinal biopsy is not an ideal gold standard, but this might be improved with more detailed reporting by the pathologist on the quality of the biopsy specimen and certainty of the diagnosis. In addition, the detection rate of CD associated lesions can be improved if immunohistochemical staining for CD3 is performed whenever a discrepancy is detected between serology and the conclusions made on routine sections. Furthermore, when a discrepancy between histology and serology is observed the best way to avoid subsequent biopsies is to revise the original biopsy, as this will lead to the diagnosis of CD in a significant number of patients.

In addition, we contributed to developing a biopsy free diagnosis by demonstrating that a biopsy can be omitted in symptomatic patients who have tTGA levels of at least 100 U/ml, positive EMA and respond well to the diet. Of course, they must also carry a disease associated HLA type, of which HLA-DQ2.2 was also demonstrated to be one. Interestingly, these patients with tTGA ≥100 U/ml show a more severe genotype and evidence of more advanced disease (more significant histological involvement and a worse phenotype). Moreover, we showed that diagnostics can be improved in very young children by measuring IgG a-DGP.

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SUMMARY

ENGLISH | DUTCH | ARABIC

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CURRICULUM VITAE

PUBLICATIONS

The prevalence of celiac disease (CD) is approximately 1% worldwide. The disease is characterized by an immunological response to gluten, the storage protein in wheat, barley and rye. This response causes intestinal inflammation but can also be detected serologically by measuring disease specific (auto-)antibodies. Detecting this immunological response against gluten through histological and/or serological methods is used in the diagnosis of CD. However, none of these methods is perfect yet, so in this thesis we aimed at improving diagnostic strategies in CD.

Histological lesions in CD are graded using the Marsh classification. Finding an increased number of intra-epithelial lymphocytes (IELs) and crypt hyperplasia (Marsh II), generally with villous atrophy (Marsh III), was considered to be the gold standard for the diagnosis of CD. However, several factors might make evaluation of small intestinal histology suboptimal. In **Chapter 2** we therefore studied the inter-observer variability in the histological diagnosis of CD and found that the agreement between 2 pathologists was only moderate, with a Kappa value of 0.486. More importantly in 7.4% of the cases a discrepancy in the diagnosis of CD between both pathologists was found, which occurred most commonly when the quality of the biopsy specimen was suboptimal. So, we cannot totally rely only on histology when diagnosing CD. In order to support the pediatric gastroenterologist in making the correct diagnosis, a pathology report should therefore include a detailed statement about the quality of the biopsy samples and the extent to which the pathologist is confident with the diagnosis. In addition, when a discrepancy between serology and histology is found, the first step should be to revise the biopsies.

In **Chapter 2** we also showed that performing CD3 staining in order to detect IELs could lead to a different diagnosis. Because CD3 staining was not done systematically, we studied the additional value of this immunohistochemical staining in **Chapter 3**, where we concluded that CD3 staining should be performed whenever there is a discrepancy between serology and the conclusion of the pathologist based on the routine sections. In our study this strategy resulted in an additional diagnosis of Marsh I in 5.0% of the studied patients, while in 0.6% of the cases a Marsh I lesion could be withdrawn after assessment of CD3 stains. More importantly, in 5.7% of the patients the diagnosis of CD was missed on routine stains. Finally, in 0.6% of the cases the diagnosis of CD could be rejected after evaluation of the CD3 sections.

Secondly, we studied methods to improve non-invasive tests in the diagnosis of CD. HLA typing is the best available test to exclude CD, because virtually all patients with CD are either HLA-DQ2.5 or HLA-DQ8 positive. However, the heterodimer HLA-DQ2.2 has also been detected in patients with CD. In **Chapter 4** we therefore studied the frequency of HLA-DQ2.2 in CD. We found that the 5.8% of CD patients, who lacked both HLA-DQ2.5 and HLA-DQ8, were all HLA-DQ2.2 positive. This heterodimer should therefore also be considered as positive when screening for CD.

Subsequently, we focused on serological tests as markers for CD. Immunoglobulin A (IgA) antibodies against tissue-transglutaminase (tTGA) or endomysium (EMA) are considered to be specific and sensitive screening tools for the disease, although they do not reach 100% accuracy. In **Chapter 5** we found that the newly developed antibodies against deamidated gliadin peptides (a-DGP) in general do not outperform EMA and tTGA. Because both EMA and tTGA have been reported to be less sensitive in children <2 years of age, we also studied their performance in this specific subgroup. Surprisingly both EMA and tTGA performed better than was expected from previous literature, but still the Immunoglobulin G class a-DGP were superior, showing 100% accuracy. Adding Immunoglobulin G a-DGP to the diagnostic work-up of CD might therefore be beneficial in young children.

In **Chapter 6** we studied in a retrospective design whether a tTGA of at least 10 times the upper limit of normal (100 U/ml) can be used to make the diagnosis of CD without needing a biopsy. We showed that all symptomatic patients with a tTGA of at least 100 U/ml, who all also had positive EMA and responded well to a gluten free diet, indeed had histological lesions compatible with CD. In **Chapter 7**, we subsequently confirmed these findings in a prospective design, adding more strength to this approach. By applying these criteria, which are now implemented in the new ESPGHAN guidelines for the diagnosis of CD, a small intestinal biopsy will not be needed in almost 40% of children suspected to have CD. Finally, in **Chapter 8** we showed that patients with very high tTGA also have a more severe disease: they have more extra-intestinal symptoms and more histological involvement. In addition they more often carry multiple disease associated HLA-types.

Coeliakie is een immuun-gemedieerde ziekte die voorkomt bij 1% van de bevolking. Deze immuunreactie ontstaat als reactie op gluten, een belangrijk bestandsdeel van tarwe, rogge en gerst, en is histologisch zichtbaar in de darmen. Daarnaast kan deze ontstekingsreactie serologisch worden geobjectiveerd door het meten van voor coeliakie specifieke antistoffen. Zowel histologie als serologie worden gebruikt als diagnostische hulpmiddelen om coeliakie vast te stellen. Geen enkele methode is echter optimaal. Het doel van dit proefschrift was daarom bij te dragen aan een verbetering van de diagnostiek van coeliakie.

Voor de gradatie van de histologische afwijkingen in de darm bij coeliakie wordt de Marsh classificatie gebruikt. Een toename van intra-epitheliale lymphocyten (IELs) en crypthyperplasie (Marsh II), meestal in combinatie met vlokatrofie (Marsh III), wordt beschouwd als de gouden standaard om coeliakie te diagnosticeren. Om verschillende redenen is een optimale beoordeling van de histologische coupes echter niet altijd mogelijk. Dit zagen we ook in **Hoofdstuk 2** van dit proefschrift, waar bleek dat de variatie in de histologische beoordeling (Marsh classificatie) tussen twee pathologen matig was (Kappa waarde 0.486). Wat betreft de diagnose coeliakie was er zelfs in 7.4% van de gevallen een verschil in de conclusie van de pathologen. Deze discrepantie kwam vaker voor wanneer er sprake was van een suboptimale kwaliteit van de biopten. In het rapport van de patholoog moeten daarom zowel de kwaliteit van de biopten, als de mate waarin de patholoog zeker is van de bevindingen worden vermeld.

In **Hoofdstuk 2** lieten we ook zien dat het verrichten van een immunohistochemische CD3 kleuring, welke wordt gebruikt om IELs beter te detecteren, de conclusie van de patholoog met betrekking tot de diagnose coeliakie kan doen veranderen. In deze studie werden CD3 kleuringen echter niet standaard verricht. De toegevoegde waarde van CD3 kleuringen werd daarom in **Hoofdstuk 3** van dit proefschrift onderzocht. De conclusie van deze studie was dat CD3 kleuringen moeten worden verricht wanneer er een discrepantie is tussen de conclusie van de patholoog op basis van standaard coupes en de serologische bevindingen bij de patient. Door middel van deze strategie kon bij 5.0% van de patiënten alsnog een Marsh I worden vastgesteld, terwijl in 0.6% van de gevallen een Marsh I werd uitgesloten na beoordeling van de CD3 kleuring. Daarnaast kon bij 5.7% van de patiënten, bij wie coeliakie met behulp van standaard coupes was uitgesloten, deze diagnose alsnog gesteld worden na toepassen van een CD3 kleuring. Verder kon in 0.6% van de gevallen de diagnose coeliakie na beoordeling van de CD3 coupes worden teruggetrokken.

In de volgende hoofdstukken van dit proefschrift hebben wij niet-invasieve methoden voor de diagnostiek van coeliakie onderzocht. Humaan leukocyt antigeen (HLA) typering is de beste methode voor het uitsluiten van coeliakie. Dit komt omdat bijna alle patiënten met coeliakie HLA-DQ2.5 of HLA-DQ8 positief zijn. Er zijn echter ook patiënten beschreven die het heterodimeer HLA-DQ2.2 dragen. In **Hoofdstuk 4** hebben wij daarom de frequentie van HLA-DQ2.2 onderzocht. Het bleek dat 5.8% van de coeliakie patiënten negatief waren voor HLA-DQ2.5 en HLA-DQ8. Al deze patiënten hadden echter het heterodimeer HLA-DQ2.2. Dit heterodimeer dient derhalve ook als positief te worden beschouwd, wanneer er op coeliakie wordt getest.

Vervolgens hebben we ons gericht op serologische markers voor de diagnostiek naar coeliakie. Immuunglobuline A antistoffen tegen tissue-transglutaminase (tTGA) en endomysium (EMA) zijn specifieke en sensitieve methoden methoden om coeliakie vast te stellen. Echter geen van beiden zijn 100% betrouwbaar. In **Hoofdstuk 5** onderzochten we daarom de nieuw ontwikkelde antistoffen tegen gedeamideerd gliadine (a-DGP). Over het algemeen bleken deze niet betrouwbaarder te zijn dan EMA en tTGA. Omdat EMA en tTGA volgens de literatuur niet erg gevoelig zijn bij jonge kinderen, is de betrouwbaarheid van a-DGP ook specifiek bekeken bij patiënten <2 jaar. In deze subgroep bleken EMA en tTGA betrouwbaarder dan in de literatuur is beschreven, doch onjuiste diagnoses kwamen nog steeds voor. De sensitiviteit en specificiteit van Immuunglobuline G a-DGP was echter 100%. Het testen van Immuunglobuline G a-DGP bij kinderen <2 jaar kan dus van toegevoegde waarde zijn.

In **Hoofdstuk 6** werd retrospectief onderzocht of een tTGA dat tenminste 10x verhoogd is gebruikt kan worden om de diagnose coeliakie te stellen zonder een dunnedarmbiopt te doen. Alle symptomatische patiënten met een tTGA van minimaal 100 U/ml en een positief EMA, die ook op het dieet reageren, hadden inderdaad coeliakie. In **Hoofdstuk 7** lieten we in prospectieve setting dezelfde resultaten zien. Met behulp van deze niet invasieve methoden, die ook in de nieuwe ESPGHAN richtlijn zijn geïmplementeerd, zal bij bijna 40% van de kinderen met een verdenking op coeliakie geen biopt meer nodig zijn. Tot slot werd in **Hoofdstuk 8** duidelijk dat kinderen met een tTGA ≥100 U/ml inderdaad een ernstigere ziekte hebben dan kinderen met coeliakie en lagere tTGA waardes. De groep met een tTGA ≥100 U/ml heeft vaker extra-intestinale manifestaties en uitgebreidere histologische afwijkingen. Bovendien hebben ze ook een ernstiger genotype met meer met coeliakie geassocieerde HLA heterodimeren.

امتلكوا بالفعل تقرحات نسجية متجانسة مع الداء البطني. في **الفصل السابع** , أكدنا هذه النتائج في تصميم استطلاعي، مضيفين المزيد من القوة لهذا النهج. من خلال تطبيق هذه المعايير، والتي يتم تنفيذها الآن في المبادئ التوجيهية الجديدة في الجمعية الأوروبية لأمراض الجهاز الهضمي الكبد والتغذية للأطفال لتشخيص الداء البطني، لن تكون هناك حاجة لخزعة الأمعاء الصغيرة في ما يقارب 40٪ من الأطفال الذين يحتمل اصابتهم بالداء البطني. أخيرا، في **الفصل الثامن**, أظهرنا أن المرضى الذين يعانون من tTGA عالي جدا يكون عندهم المرض بشدة أكثر: لديهم أعراض أكثر خارج الامعاء, و تضرر نسيجي أكبر. بالإضافة الى ذلك, فأنهم في كثير من الأحيان يحملون عدة أنواع من مستضدات الكريات البيضاء البشرية المرتبطة بالداء البطني.

بدراسة وتيرة HLA-DQ2.2 في الداء البطني في **الفصل الرابع**. وجدنا أن 5.8٪ من مرضى الداء البطني، الذين يفتقرون إلى كل من HLA-DQ2.5 وHLA-DQ8، كانوا جميعا يحملون HLA-DQ2.2. ولذلك ينبغي أيضا الأخذ بعين الاعتبار HLA-DQ2.2 كنتيجة ايجابية عند فحص الداء البطني.

بعد ذلك، ركزنا على الاختبارات المصلية كمؤشرات للداء البطني. تعتبر الأجسام المضادة لغلوبولين المناعي أ ضد الأنسجة EMA و tTGA أدوات فحص محددة وحساسة لهذا المرض، على الرغم من أنها لا تصل إلى 100٪ من الدقة. في **الفصل الخامس**, وجدنا أن الأجسام المضادة الحديثة a-DGP, بشكل عام, لا تتفوق على EMA و tTGA. لأن الاثنان ذُكر انهما أقل حساسية لدى الأطفال دون السنتين، درسنا أيضا أدائهما في هذه المجموعة الفرعية المحددة. من المستغرب على حد سواء ان EMA و tTGA كان أدائهما أفضل مما كان متوقعا من الدراسات السابقة، ولكن لا تزال الطبقة a-DGP من فئة الأجسام المضادة لغلوبولين المناعي ج متفوقة، والتي تبين 100٪ من الدقة. لذا اضافة a-DGP إلى تشخيص الداء البطني قد تكون مفيدة في الأطفال الصغار.

في **الفصل السادس**, درسنا في تصميم رجعي , ما إذا كانت tTGA التي لا تقل عن 10 أضعاف الحد الأعلى للطبيعي (100 U / مل) يمكن استخدامها لتشخيص الداء البطني دون الحاجة إلى الخزعة. أظهرنا أن جميع المرضى العرضيين مع tTGA التي لا تقل عن 100 U / مل، الذين أيضا يحملون EMA, استجابوا بشكل جيد للحمية الخالية من الغلوتين، وانهم

ومن أجل دعم طبيب أمراض الجهاز الهضمي للأطفال في التشخيص الصحيح, يجب أن يتضمن تقرير التشريح المرضي بيانا مفصلا عن نوعية عينات الخزعة وإلى أي مدى الطبيب واثق في التشخيص. بالإضافة إلى ذلك، ينبغي أن تكون إعادة النظر في الخزعات الخطوة الأولى ان تم العثور على التناقض بين نتيجة فحص الأمصال و فحص الأنسجة.

في **الفصل الثاني** أظهرنا أيضا أن اجراء تلوين CD3 من أجل الكشف عن الخلايا الليمفاوية داخل الظهارة قد يؤدي الى تشخيص مختلف. لأنه لم يتم التلوين بشكل انتظامي، درسنا القيمة الإضافية من هذا التلطيخ الكيميائي الهيستولوجي المناعي في **الفصل الثالث** ، حيث وصلنا الى خلاصة أن تلوين CD3 يجب أن يتم كلما وجد تناقض بين الأمصال وخلاصة الاختصاصي بناءًا على الأقسام الروتينية. في دراستنا, أسفرت هذه الاستراتيجية في تشخيص 5.0٪ اضافين من الخاضعين للدراسة مارش الاول، بينما 0.6٪ من ضرر مارش الاول يمكن سحبه بعد تقييم تلوين CD3. والأهم من ذلك، ان 5.7٪ من مرضى الداء البطني لم يُكتشفوا من خلال التلطيخ الروتيني. أخيرا، في 0.6٪ من الحالات , يمكن رفض تشخيص الداء البطني بعد تقييم اقسام CD3.

ثانيا، قمنا بدراسة طرق لتحسين الاختبارات غيرالخزعية في تشخيص الداء البطني. مستضدات الكريات البيضاء البشرية هو أفضل اختبار متاح لاستبعاد الداء البطني، وذلك لأن جميع مرضى الداء البطني يحملون إما HLA-DQ2.5 أو HLA-DQ8. ومع ذلك، لقد تم الكشف عن HLA-DQ2.2 في بعض المرضى الذين يعانون من الداء البطني. ولذا قمنا

الخلاصة

ان انتشار الداء البطني يقارب 1٪ في جميع أنحاء العالم. ويتميز هذا المرض بطريقة الاستجابة المناعية للغلوتين، البروتين المخزن في القمح والشعير والجاودار. هذه الاستجابة تسبب التهاب في الأمعاء ولكن تكشفه مصليا ايضا من خلال قياس الاجسام المضادة الخاصة في المرض. الكشف عن هذه الاستجابة المناعية ضد الغلوتين من خلال وسائل النسيجية و / أو المصلية تستخدم في تشخيص الداء البطني .ومع ذلك، فإن هذه الأساليب غير دقيقة بشكل كامل لحد الان. نهدف في هذه الرسالة الى تحسين استراتيجيات التشخيص في الداء البطني.

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لذلك في **الفصل الثاني** قمنا بدراسة التباين في التشخيص النسيجي للداء البطني، ووجدنا أن الاتفاق بين أخصائيين علم الامراض لم يكن سوى اتفاق عاديّ، مع قيمة كابا تبلغ 0.486. والأكثر اهمية هو التباين الموجود في 7.4٪ من حالات تشخيص الداء البطني بين كل من أخصائيين علم الامراض ، التي تحصل عادة عندما تكون نوعية الخزعة دون المستوى الأمثل. لذلك، لا يمكننا الاعتماد على علم الأنسجة فقط عند تشخيص الداء البطني.

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Amani Mubarak was born the 31st of May in 1986 in Bagdad, Iraq. At the age of 9 she moved with her family to the Netherlands.

Professional education and research projects

2013	Clinical Traineeship in Pediatrics | Wilhelmina Children's Hospital Utrecht
2010-2012	PhD thesis: Diagnostics in Celiac Disease
2011-2012	Graduate School of Life Sciences PhD Programme Infection & Immunity | Utrecht University
2010-2012	Celiac Disease Clinic | Wilhelmina Children's Hospital, Utrecht
2010	Alexandre Suerman Grant
2010	Medical Doctor degree (MD) | Utrecht University
2009	Grant by University Utrecht for an extracurricular research program
2008-2009	Honors Program Master | Utrecht University
2009	Social Medicine Training: Health education for children in underprivileged areas | Amel Association, Beirut, Lebanon
2008	Surgery Training | Hurghada General Hospital, Hurghada, Egypt
2008	Neonatology Training | Wilhelmina Children's Hospital, Utrecht
2006-2009	Regular Clinical Trainings | Utrecht University
2005	Propedeutical Exam Medicine | Utrecht University
2004	Atheneum | Koningin Wilhelmina College, Culemborg

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THE END...


disease
directed
characterized
heterodimer
HLA-DQ8
research
basal
gluten
diagnostics
Classically
atrophy
negative
destruction
small
tTGA
diagnosis
susceptible
endomysium
barley
T-cell
measuring
leukocyte
villi
histological
transglutaminase
hyperplasia
non-invasive
positive
syndrome
HLA-DQ2.2
value
specificity
cases
human
diet
heterodimers
loss
levels
undetected
failure
HLA
also
lymphocytosis
intestine
increased
distention
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antigen
invasive
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diarrhea
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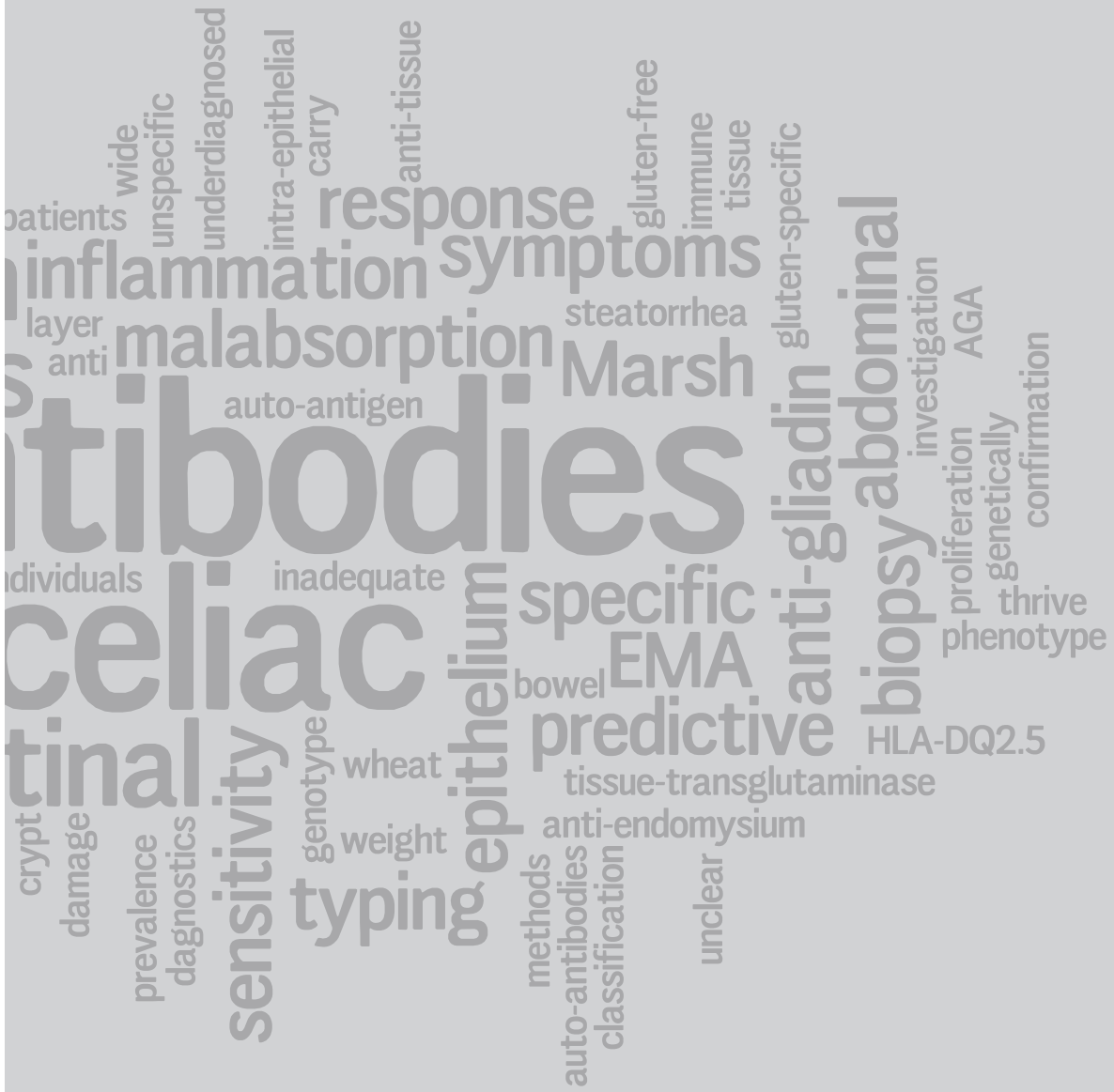

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