Linköping University Medical Dissertations
No. 965

Coeliac Disease in Childhood

On the Intestinal Mucosa and the Use of Oats

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Linköping 2006

The work in this thesis was supported by grants from the Swedish Research Council (VR:M and VR:NT), the King Gustaf V:th 80-year Foundation, The Health Research Council in the South-East of Sweden, the County Council of Östergötland, the Vårdal Foundation, the Swedish Nutrition Foundation, the Faculty of Health Sciences at Linköping University, the Lisa and Johan Grönberg Foundation, the Julin Foundation, Stiftelsen Samariten, Svenska Celiakiförbundet, FORMAS, and Lions Research Foundation.

Cover: Manipulated scanning electron microscopy image of small intestinal mucosa from a child with coeliac disease on gluten-free diet.

ISSN: 0345-0082

ISBN: 91-85643-60-2

Printed in Sweden by LiUTryck, Linköping 2006

Till Karolina

Once upon a time there was a little baby girl who had troubles with her stomach. Her cheeks were glowing, her belly was swollen, and her arms and legs were so very thin. The baby girl's parents were worried and they took their daughter to see the doctor. The doctor examined her small intestine and he finally gave the diagnosis: the baby had coeliac disease and she could not eat anything that contained wheat, rye, barley, or oats. The parents felt relieved knowing what was wrong with their little girl and they learned how to cook gluten-free food. Very soon the little baby girl began to grow, smile, and her stomach problems were all gone. And the family lived happily ever after…

ABSTRACT

Coeliac disease (CD) is one of our most common chronic diseases in childhood. The disease causes an intense inflammation in the small intestinal mucosa after ingestion of gluten-containing cereals in genetically predisposed individuals. The mucosal lesion in CD is characterised by villous atrophy and crypt hyperplasia, and both the absorptive and the barrier functions of the enterocytes are disturbed. The treatment of CD is a life-long adherence to a gluten-free diet (GFD). The toxic fraction of wheat gluten is gliadin, and there are similar proteins in rye, barley and oats. In oats this protein is called avenin, and it is proposed to be less toxic than the others. The use of oats in CD has been debated, but it is now considered safe for the majority of both children and adults with CD.

The aims of this thesis were to investigate the humoral and inflammatory reactions to oats in children with CD, and also to study the intestinal mucosa at different stages of the disease.

In a retrospective study we found that children with CD had antibodies to oats avenin, and that the levels were significantly higher than in controls. The levels were attenuated during GFD, and we also showed that there was no cross-reactivity between antibodies to oats and gliadin.

We then used our method for measuring antibodies to avenin in a randomised, double-blind trial of oats given to children with newly diagnosed CD. The children were given either a traditional GFD or a GFD supplemented with oats. There was a rapid decrease in antibody levels in both groups already after three months on diet, and at the end of the study period all but a few had normalised their levels. The same children were also studied using urinary nitric oxide (NO) products as markers for intestinal inflammation. Likewise, these values decreased significantly after three months. At the end of the study four

children in the GFD-oats group and one in the standard GFD group still had extremely high concentrations of urinary NO metabolites. Taken together, these studies strengthen the clinical impression that oats can be tolerated by the majority of children with CD, but they also warrant a caution, since there seem to be children that do not tolerate oats in their diet.

The structure and distribution of occludin and claudins 1-5, tight junction proteins known to play a crucial role in maintaining the barrier function, was studied in biopsy specimens from children at different stages of CD. There was an increased expression of occludin in untreated CD, which reflects the characteristics of crypt cell hyperplasia and altered barrier properties seen in active CD. The findings also indicate that gluten intake does not significantly influence the expression and distribution of claudins 1-5 in coeliac children.

SAMMANFATTNING

Celiaki, eller glutenintolerans, är en av våra vanligaste kroniska sjukdomar i barnaåren. Sjukdomen orsakar en kraftig inflammation i tunntarmens slemhinna efter intag av glutenhaltig föda hos personer med ärftlig benägenhet att utveckla celiaki. En frisk tarm är kraftigt veckad för att öka ytan för upptag av näringsämnen. Ytan består dessutom av åtskilliga fingerliknande utskott, s.k. villi, och mellan villi finns kryptorna där celldelning och celldifferentiering sker. Villi och kryptor kantas av epitelceller, enterocyter, vilkas uppgift är att ta upp näring från tarminnehållet samt att utgöra en selektiv barriär mellan den yttre och inre miljön i tarmen. Den typiska tarmskadan vid celiaki karakteriseras av avsaknad av villi och kraftigt förlängda kryptor, och både näringsupptaget och barriärfunktionen är dessutom störda. Den enda behandling som finns att tillgå vid celiaki är en livslång glutenfri diet. De skadliga proteinerna i vetegluten kallas gliadin, och det finns liknande proteiner i råg, korn, och havre. I havre kallas proteinet avenin. Möjligheten att använda havre vid celiaki har diskuterats flitigt, men numera anses det riskfritt för majoriteten av både barn och vuxna att använda havre i den glutenfria dieten.

Målet med den här avhandlingen var att undersöka hur barn med celiaki reagerar på havre i kosten. Detta studerades med avseende på antikroppar mot avenin samt med en metod som mäter halten av kväveoxid- (NO-) produkter i urinen. Ett andra mål var att studera tunntarmens struktur vid olika stadier av celiaki.

I den första studien undersökte vi om celiakibarn har antikroppar i serum mot avenin. Vi fann att så var fallet och att nivåerna var signifikant högre än hos friska kontrollbarn. När barnen sattes på glutenfri kost sjönk antikroppsnivåerna, för att öka igen när gluten återinfördes i kosten. Blodproverna till den här studien togs innan debatten om havre kom igång, vilket gör att vi tror att de

olika dieterna även speglar ett sant intag av havre. Studien visade också att det inte var någon korsreaktion mellan antikroppar mot avenin och gliadin.

Vi använde sedan vår metod för att mäta antikroppar mot avenin i en randomiserad studie där havre gavs till barn med nydiagnostiserad celiaki. Barnen fick antingen en vanlig glutenfri diet eller en med tillsats av specialhavre. Antikroppsnivåerna sjönk markant redan efter tre månader i båda grupperna, och vid studietidens slut, efter ca ett år, hade alla utom ett par patienter återfått normala nivåer. Samma barn studerades även med avseende på NO-produkter i urinen. NO är en kortlivad molekyl som fungerar som budbärare i och mellan celler, och produktionen av den ökar markant vid en inflammation. Tidigare studier har visat att barn med obehandlad celiaki har extremt höga halter av NO-produkter i urinen. I vår studie sjönk även dessa värden signifikant efter tre månader, och det var ingen skillnad mellan grupperna. Efter ett år hade dock fyra barn i havregruppen och ett barn i den grupp som fick vanlig glutenfri kost, fortfarande extremt höga nivåer av NO-produkter.

Dessa båda studier styrker den kliniska uppfattningen att de flesta barn med celiaki kan tåla havre, men de visar också att man bör följa upp de celiakibarn som kompletterar sin glutenfria kost med havre eftersom vissa barn verkar ha kvarstående tecken på inflammation i tarmen.

I tarmbiopsier från barn med olika stadier av celiaki studerades förekomst och lokalisering av occludin och claudiner, proteiner som är viktiga för att upprätthålla barriärfunktionen i tarmen. Vi fann ett ökat uttryck av occludin vid obehandlad celiaki, vilket vi tror speglar den ökade celldelning och de förändrade barriäregenskaper som man ser vid aktiv celiaki. Resultaten tyder även på att uttrycket av claudin 1-5 inte tycks påverkas av kosten hos barn med celiaki.

CONTENTS

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I **Hollén E**, Högberg L, Stenhammar L, Fälth-Magnusson K, and Magnusson K-E. Antibodies to oat prolamines (avenins) in children with coeliac disease.
Scand J Gastroenterol 2003; 38:742-746

II **Hollén E**, Holmgren Peterson K, Sundqvist T, Grodzinsky E, Högberg L, Laurin P, Stenhammar L, Fälth-Magnusson K, and Magnusson K-E. Coeliac children on a gluten-free diet with or without oats display equal anti-avenin antibody titres.
Scand J Gastroenterol 2006; 41:42-47

III **Hollén E**, Forslund T, Högberg L, Laurin P, Stenhammar L, Fälth-Magnusson K, Magnusson K-E, and Sundqvist T. Urinary nitric oxide during one year of gluten-free diet with or without oats in children with coeliac disease.
Scand J Gastroenterol 2006; 41:1272-1278

IV **Hollén E**, Fälth-Magnusson K, Sundqvist T, Magnusson K-E, and Holmgren Peterson K. Increased expression of tight junction associated occludin but not of claudins in untreated coeliac disease in children.
Manuscript (2006).

ABBREVIATIONS

AGA - anti-gliadin antibodies
APC - antigen-presenting cell
AQP - aquaporin
BSA - bovine serum albumin
CD - coeliac disease
EDTA - ethylenediaminetetraacetic acid
ELISA - enzyme-linked immuno-sorbent assay
EMA - anti-endomysium antibodies
ESPG(H)AN - European Society of Pediatric Gastroenterology, (Hepathology) and Nutrition
GFD - gluten-free diet
HLA - human leukocyte antigen
IEL - intraepithelial lymphocytes
IFN - interferon
IL - interleukin
KLH - keyhole limpet hemocyanin
MHC - major histocompatibility complex
NK - natural killer
NO - nitric oxide
NOS - nitric oxide syntase
PBS - phosphate-buffered saline
PEG - polyethylene glycol
PFA - paraformaldehyde
RT - room temperature
SEM - scanning electron microscopy
TCR - T-cell receptor
TER - transepithelial resistance
TGA - anti-tissue transglutaminase antibodies
TJ - tight junction
TNF - tumor necrosis factor
tTG - tissue transglutaminase
WGA - wheat germ agglutinin

INTRODUCTION

Coeliac disease (CD) is a permanent food intolerance, which causes a severe inflammation of the small intestine in genetically predisposed individuals, after ingestion of gluten-containing cereals. It is one of our most common chronic diseases and it is most often emerging during childhood. In small children the main symptoms are reduction in weight, or absence of weight gain, diarrhoea (or constipation), swollen abdomen and gastrointestinal pains. In adults the symptoms are more diffuse, but fatigue, depression and malnutrition are common. The treatment is a lifelong adherence to a gluten-free diet, i.e. wheat, rye, and barley are excluded from the diet.

The use of oats in CD has been a matter of discussion. Oats is considered to be less harmful for CD patients than the other cereals, due to its lower content of the prolamin fraction. It is also taxonomically more distantly related to wheat than are rye and barley. The addition of oats to the gluten-free diet would have several benefits for CD patients. It would make the food more palatable and it would increase both the fibre content and the nutritional value.

The cells lining the small intestine have a dual functional role. Firstly, they compose the main area for absorption of nutrients and, secondly, they build up a selective barrier between the inner and outer environment of the body. In CD both these roles are dysfunctional.

This thesis will focus on the use of oats for children with CD, especially on the humoral response to oats in an otherwise gluten-free diet, and also on nitric oxide (NO) as a marker for the mucosal inflammation. It will also discuss the altered mucosal integrity and ultrastructure seen in active CD.

BACKGROUND

HISTORY

The name coeliac disease dates back to the first or second century AD, when the famous Greek physician Aretaeus the Cappadocian reported a description of the disease (Walker-Smith 1988). Aretaeus wrote: "If the diarrhoea does not proceed from a slight cause of only one or two days duration and if, in addition, the patient's general symptoms be debilitated by atrophy of the body the coeliac disease of a chronic nature is formed." He wrote that the illness was associated with a swollen belly and therefore used the term coeliac disease, derived from the word coelom, meaning the body cavity. Aretaeus thought that the disorder only affected adults (Adams 1856).

In 1888 Dr. Samuel Gee published a paper called "On the Coeliac Affection" (Gee 1888). This was the first description of the disorder in modern times and it begins with a short clinical account: "There is a kind of chronic indigestion which is met with in persons of all ages, yet is especially apt to affect children between one and five years old. Signs of the disease are yielded by the faeces; being loose, not formed, but not watery; more bulky than the food taken would seem to account for; pale in colour, as if devoid of bile; yeasty, frothy, an appearance probably due to fermentation; stinking, stench often very great, the food having undergone putrefaction rather than concoction." Gee gave a description of the disease that was quite similar to that of Aretaeus, and he also concluded that "But if the patient can be cured at all, it must be by means of the diet." However, his diet recommendations, although rather detailed, did not say anything about avoiding cereals.

It was not until the 1950s that the direct cause of CD was discovered. During World War II there was a shortage of cereals and bread in the

Netherlands and the Dutch paediatrician Dicke noted that the occurrence of CD declined during this period. When cereals were again provided, the patients with CD relapsed. Using a faecal fat absorption test, Dicke and his colleagues showed that a component of wheat flour was responsible for the harmful effects seen in CD (Dicke 1953). This "wheat factor" was also found in rye and in oats. The Dutch study, which was first published in thesis form in 1950 (Dicke 1950), was later confirmed by an English group (Anderson 1952), assessing also the influence of wheat on the clinical condition in children with CD. During this time it was shown that the toxicity of wheat was in the gluten fraction (Anderson 1952, Van de Kamer 1953).

Since the discovery of gluten as the factor having deleterious effects in CD patients, CD is treated with a life-long adherence to a strict gluten-free diet, i.e. a diet without wheat, rye, and barley. The use of oats in CD will be discussed later on.

CLINICAL FEATURES IN CD

Symptoms

As we learnt from Gee and Aretaeus there are several symptoms and signs of CD and they can be divided into intestinal features and symptoms caused by malnutrition (Feighery 1999). The most distinct symptoms occur when CD is presenting during early childhood, i.e. in infants less than two years of age. Diarrhoea, or constipation, vomiting, failure to thrive, muscle wasting, unhappy behaviour, and enlarged abdomen are some of the typical symptoms at that age (Schmitz 1992, Catassi 1997a, Visakorpi 1997). The signs of malabsorption often include iron-deficiency anaemia, hypoalbuminemia, and vitamin deficiencies (Fasano 2001). A small number of infants, with early onset of symptoms, may present in

a shocklike state termed “coeliac crisis”, with massive watery diarrhoea, which leads to dehydration and severe hypoproteinemia and edema (Fasano 2001).

Older children and adolescents more often present with vague or atypical symptoms, such as recurrent abdominal pain, failure to grow normally, anaemia, and delayed puberty (Catassi 1997a, Ciclitira 2005). In fact, iron-deficiency anaemia can be the only presenting sign of CD in older children and adults, especially when the anaemia is refractory to oral iron supplementation (Carroccio 1998). Short stature can also be the only sign in an otherwise symptom-free CD in some older children and adolescents (Verkasalo 1978, Groll 1980, Cacciari 1983, Stenhammar 1986). Some patients in this group may present with the more typical form of CD, but these features usually become less frequent with increasing age.

In adults the symptoms can be even more diffuse. While diarrhoea is the most common intestinal symptom of adult CD, patients may also present with metabolic symptoms, e.g. anaemia, neurological symptoms, e.g. epilepsy, or psychological disturbances such as anxiety and depression (Howdle 1992, Corazza 1995, Ciclitira 2005).

It now seems that there is a change in the age of onset of CD, towards increasing age at diagnosis and milder and more atypical symptoms (Ivarsson 2000, Ludvigsson 2004, Rampertab 2006).

The coeliac lesion

The classical histopathologic features of CD are villous atrophy, crypt epithelial cell hyperplasia, and increased numbers of intraepithelial lymphocytes (IEL) and lamina propria mononuclear cells (Trier 1991, Lionetti 2002). In the normal small intestine the surface is heavily folded to increase the area for uptake of nutrients. The folded mucosa is composed of numerous finger-like projections,

villi, into the lumen. Between the villi are the crypts where the cell proliferation takes place. Both the villi and the crypts are covered by a single-cell layer of epithelial cells, which form a selective fence between the outer and the inner environment of the body. The individual epithelial cells have numerous small protrusions, microvilli, on their apical side. In normal tissue the villi are at least twice as long as the depth of the crypts (Fig. 1).

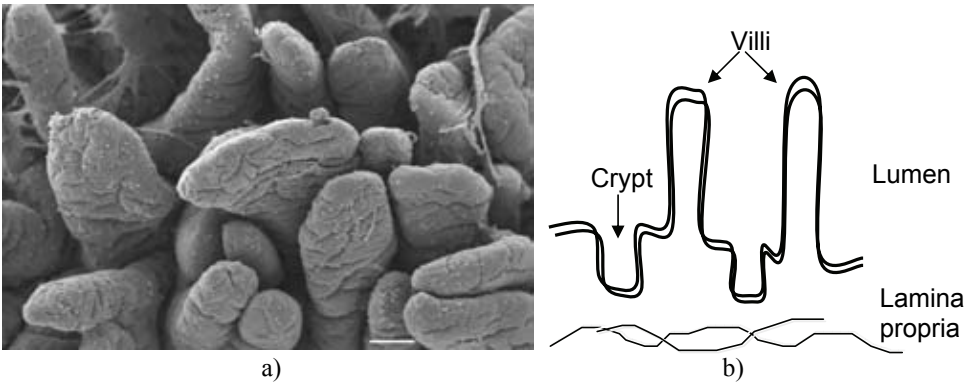
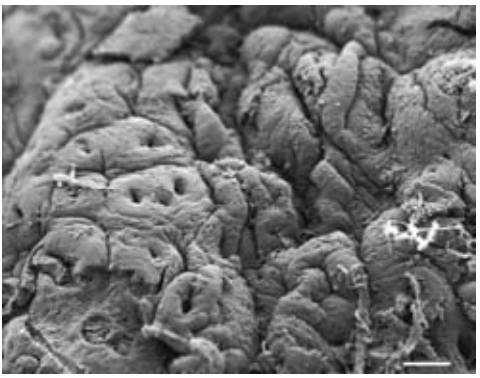
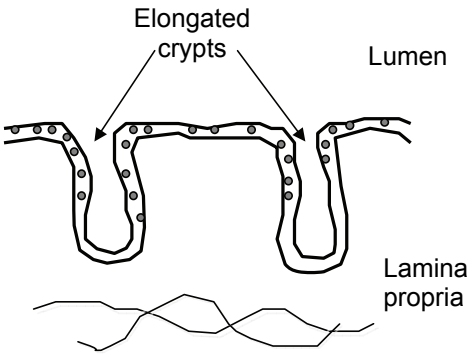


Figure 1. The normal small intestinal mucosa. a) Scanning electron micrograph of normal mucosa showing finger-like villi. Bar 100 μM. b) Schematic drawing of the mucosa showing the normal proportions between crypts and villi.

In CD the classical mucosal lesion shows a flat mucosa with absent villi, villous atrophy, and heavily elongated crypts, crypt hyperplasia (Fig. 2). The intestinal absorptive area is thereby decreased leading to malabsorption, the severity of which depends on the extent of proximal small intestinal involvement. When the patient is put on a gluten-free diet, there is a complete resolution of the lesions, although the density of IELs often does not fully normalise (Ferguson 1977).





a) b)

Figure 2. The flat destructive mucosa seen in coeliac disease. a) Scanning electron micrograph of the flat mucosa with absent villi and visible crypt openings. Bar 100 μM. b) Schematic drawing showing the elongated crypts and the infiltration of intraepithelial lymphocytes.

However, the typical mucosal lesion described above represents one end of a spectrum of pathological changes seen in CD. Different classifications have been used to describe the mucosal damage in CD. In 1975, Alexander (Alexander 1975) described the mucosal changes seen in Dermatitis herpetiformis, a skin disease often regarded as a skin variant of CD. He introduced four grades of the morphologic deterioration of the mucosa as it appeared under the dissecting microscope, but his classification did not account for the infiltrative cells in the epithelia and lamina propria. Alexander Grade I represents the normal mucosa with finger-like, and occasionally leaf-like villi, and Grade IV represents the flat mucosa with no villi, called sub-total atrophy.

In 1992, there was a new classification of the coeliac spectrum, when Marsh (Marsh 1992) described a sequence of progression of the lesion. This classification is divided into five types and it also accounts for the cell infiltration (Table 1).

Table 1. Histological classification of small intestinal biopsy specimens according to Marsh

Type		Histomorphology
0	Preinfiltrative	Normal
1	Infiltrative	Normal villous architecture; normal crypt depth; IEL increase
2	Hyperplastic	Normal villous architecture; crypt hyperplasia; IEL increase
3	Destructive	Villous atrophy; crypt hyperplasia; IEL increase
	a*	mild villous flattening
	b*	marked villous flattening
	c*	total villous flattening (flat mucosa)
4	Hypoplastic**	Villous atrophy; normal crypt depth; normal IEL counts

IEL = intraepithelial lymphocyte

* A division in subgroups has been added for the Type 3 lesion, depending on the degree of villous atrophy (Oberhuber 1999)

** The hypoplastic lesion is an irreversible stage that is very rare today

Infiltration of IELs is a hallmark of the epithelium in CD. The high IEL count does not always correlate with the degree of mucosal lesion, since it can also be seen in the type I infiltrative lesion together with normal villous architecture. There are three main populations of IELs in the intestinal mucosa, T-cell receptor (TCR)αβ+ $CD8^+$ $CD4^-$, TCRαβ+ $CD8^-$ $CD4^+$, and TCRγδ+ $CD8^-$ $CD4^-$, and both the TCRαβ+ $CD8^+$ and the TCRγδ+ cells are increased in the untreated coeliac mucosa (Sollid 2000, Lionetti 2002). When the patient receives a gluten-free diet, only the TCRαβ+ $CD8^+$ IELs return to normal, whereas the TCRγδ+ IELs appear to remain at an elevated level (Kutlu 1993). Both TCRαβ+ $CD8^+$ and TCRγδ+ IELs recognise MICA and MICB antigens, which are stress-induced major histocompatibility complex (MHC) molecules mainly expressed by intestinal epithelial cells (Groh 1996, 1998). It has been shown that gliadin can induce the production of interleukin (IL) -15 in lamina propria mononuclear cells of CD patients (Maiuri 2000, 2003), and that IL-15 promotes the expression of

MIC molecules in the intestine of CD patients (Hue 2004). The MIC molecules interact with the natural killer (NK) lineage receptors of the NKG2D family, expressed at the surface of human TCRαβ+ CD8+ -cells, TCRγδ+ -cells, and most NK cells. This interaction could turn these T-cells into NK-like "lymphokine-activated killers", an action which is suggested to play a role in the IEL-mediated destruction of intestinal epithelial cells seen in CD (Hue 2004, Meresse 2004).

Besides the increased loss of surface epithelial cells in CD, there is also an increased proliferation of the epithelial cells in the crypts. The latter has been shown in several studies (Lionetti 2002) and appears to be due to an increased production of the growth stimulating keratinocyte growth factor (Bajaj-Elliott 1998).

Another grading for the classification of the small intestinal mucosa in CD has been developed by a Swedish group of pathologists, the so called KVAST-group of gastrointestinal pathology. This KVAST-grading (Table 2) is now being used in many Swedish pathology laboratories. This classification, however, has not been published and hence cannot be referred to in international journals.

Table 2. Histological classification of small intestinal biopsy specimens according to the KVAST-group of Swedish pathologists

	Villous atrophy	IEL	Cell infiltrates in lamina propria	Mitotic frequency in crypts
Normal mucosa	-	-	-	-
"Borderline" mucosa	-	+	-/(+)	-
Partial villous atrophy	+	+	+	+
Subtotal/total villous atrophy	++	+	+	++

IEL = intraepithelial lymphocytes; - = normal; + = mild change; ++ = marked change

GENETICS

Coeliac disease is a genetic disorder with a clear association with the MHC molecules, also called the human leucocyte-antigen (HLA) system. The functions of MHC class I and II molecules are to act as recognition molecules and to present antigens to effector cells in the immune system. The MCH class I molecules are present on almost every nucleated cell in the body, while the MHC class II is present only on professional antigen presenting cells (APCs), such as dendritic cells, B-cells, macrophages, and intestinal epithelial cells. The primary association in CD is with the MHC class II. The genes encoding for the MHC II molecules are on chromosome 6 (Dieterich 2003) in the loci HLA-DP, -DQ, and -DR. Up to 95% of the CD patients carry the HLA class II haplotype DQ2, a heterodimer of one α and one β chain, which is encoded by the alleles DQA1*0501 and DQB1*0201 (Sollid 1993), either *in cis* as DR3-DQ2 on the same chromosome, or *in trans* as DR5/7-DQ2 on the opposite chromosomes (Sollid 1989).

It has been observed that individuals who are HLA-DQ2 homozygous have at least five-fold higher risk of developing disease than those who are HLA-DQ2 heterozygous (Mearin 1983). A much stronger gluten-specific T-cell response have been demonstrated by APCs homozygous for HLA-DQ2 than heterozygous APCs, possibly due to a much lower abundance of HLA-DQ2 molecules capable of presenting gluten peptides on heterozygous APCs (Vader 2003b) (Fig. 3).

Those CD patients who do not carry the DQ2 molecule carry, with a few exceptions, the HLA-DQ8 molecule.

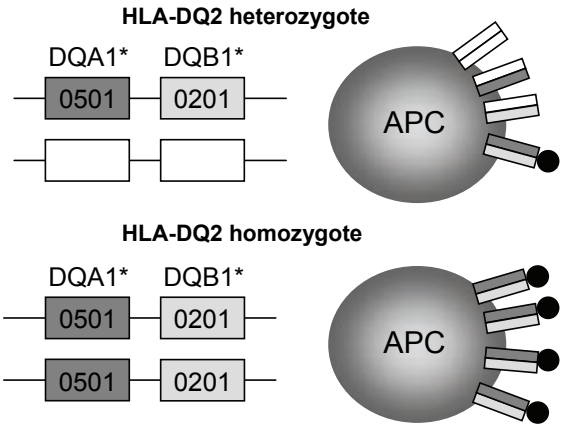


Figure 3. Gluten binding capacity of APCs homozygous or heterozygous for HLA-DQ2. Transdimer formation in HLA-DQ2 heterozygotes results in decreased gluten presentation. Modified from (Stepniak 2006).

However, the association of CD with the HLA-genes does not explain the entire genetic susceptibility. The concordance in HLA identical siblings is around 30%, while in monozygotic twins it is around 70% (Sollid 1993, Houlston 1996, Greco 2002). The risk of CD in first-degree relatives varies between 10% and 20% (Houlston 1996), and the HLA-genes has been estimated to contribute for no more than 40% of the familial risk of CD (Bevan 1999). Furthermore, the HLA-DQ2 and DQ8 haplotypes are also found in 20-30% of the general population. These findings implicate that non-HLA genes might be stronger determinants for CD susceptibility. There are, amongst others, suggestions for regions on chromosome 2, 5 and 19 (Branski 2006).

DIAGNOSIS

Serological markers

Patients with CD develop serum antibodies both to gliadin and to some self-antigens, and the assessment of these antibodies is an important tool when CD is suspected. Antibody testing can also be used for screening, e.g. in relatives or a population, and for follow-up of the response to a gluten-free diet. The first antibody to be used in such tests was the anti-gliadin antibody (AGA). The enzyme-linked immunosorbent assay (ELISA) for AGA yielded high specificity for IgA class antibodies, and high sensitivity for the IgG class. However, the specificity and sensitivity for this assay have varied between different studies (Unsworth 1981, Savilahti 1983, Grodzinsky 1990), and IgG AGA is also frequent in other gastrointestinal disorders as well as in healthy subjects, thereby giving many false-positive results (Scott 1992). Today, AGA assays are used most frequently in the paediatric population, due to the high sensitivity in children (Stenhammar 1984, Burgin-Wolff 1989, Grodzinsky 1995).

CD patients also have antibodies against endomysium, a connective tissue of smooth muscle fibres. These anti-endomysium antibodies (EMA) are detected by immunofluorescence using either monkey oesophagus (Chorzelski 1983) or, more often, human umbilical cord (Ladinser 1994, Volta 1995) as antigens. This assay is reported to have both high sensitivity and specificity for CD (Burgin-Wolff 1991, Grodzinsky 1994, Ladinser 1994, Volta 1995), although the analysis is time-consuming and the subjective interpretation of the immunofluorescence might lead to variability in different laboratories. Another drawback with the EMA assay is that children younger than two years often have negative EMA, in spite of having villous atrophy (Burgin-Wolff 1991, Kwiecien 2005).

In 1997, the predominant autoantigen recognised by EMA was identified (Dieterich 1997). It turned out to be tissue transglutaminase (tTG), an enzyme expressed both intra- and extracellulary in many different tissues and organs in the body. In the extracellular environment, tTG has been shown to play a role in extracellular matrix assembly, cell adhesion and wound healing. The calcium-dependent transglutaminase activity of tTG is to catalyse selective cross-linking or deamidation of protein-bound glutamine residues (Sollid 2002). An ELISA assay for assessment of tTG antibodies in CD was introduced, using guinea pig tTG as antigen, showing that measurement of IgA antibodies to tTG (TGA) was a well-suited tool to detect untreated CD (Dieterich 1998, Sulkanen 1998). Since then, numerous studies have been done measuring the sensitivity and specificity of this assay, and especially when using human recombinant tTG the results have been comparable and even better than EMA assays (Hansson 2000, Sblattero 2000, Leon 2001, Wolters 2002, Wong 2002, Neri 2004). The advantages of TGA as compared to EMA are that it has an objective interpretation, is quantitative, and less expensive.

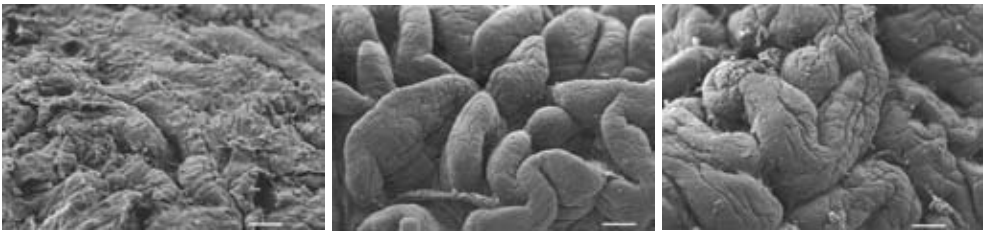
One problem with antibody testing for CD is that IgA deficiency is frequently associated with the disease, with an incidence 10- to 15-fold higher than in the general population (Collin 1992, Cataldo 1998), and both TGA and EMA tests are based on IgA antibodies. The use of IgG-EMA and IgG-TGA can be useful for diagnostic purposes in individuals with IgA deficiency (Cataldo 2000, Basso 2006).

Small bowel biopsy

Even though the serological tests used today display high sensitivity and specificity, the golden standard in diagnosing CD is still the small intestinal biopsy. This can be performed either as an upper endoscopy or a capsule biopsy

under fluorometric control. The most representative samples are taken at or near the ligament of Treitz, at least in children, since more proximal samples may be affected by intestinal infections. Capsule biopsy often yields more material than needed for the diagnostic procedure, and excess material can be used for research purposes.

In 1969, the original criteria for the diagnosis of CD in children were proposed by members of the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) (Meuwisse 1970, McNeish 1979). It was based on the morphological assessment of biopsies from the small intestinal mucosa obtained from three different occasions: 1) an initial biopsy on suspicion of CD, on normal diet, showing absent or almost absent villi, 2) a second biopsy on gluten-free diet, showing normalisation of the mucosa, and 3) a third biopsy showing relapse of the atrophy after reintroduction of gluten (Fig. 4).



a) b) c)

Figure 4. Three repeated small intestinal biopsies in the same child, obtained at different occasions: a) flat mucosa at presentation on normal diet, b) normal mucosa after one year on gluten-free diet, and c) relapse of the atrophy after a period of gluten challenge. Bars 100 μM.

In 1990, the criteria were revised by ESPGAN, as many clinicians questioned the gluten challenge procedure and the follow-up biopsy (Walker-Smith 1990). These revised criteria recommended a follow-up biopsy only in children in whom any doubt regarding the initial diagnosis of CD existed, and in children

aged two years or less at presentation. In these children a gluten challenge was recommended.

Other markers in CD

The procedure of taking small intestinal biopsies can be experienced as traumatic for the child as well as a demanding task for the doctor, hence there is a need for non-invasive diagnostic tools in evaluating the mucosal status in CD. Indeed, the improved assays for serological markers have outruled the importance of follow-up biopsies in many patients according to the revised criteria.

Measurements of the intestinal permeability have been suggested as another non-invasive method. The set up for permeability tests is that a mixture of different test molecules in an aqueous solution is given orally to the patient, and the recovery of the molecules is measured after certain time points, preferably in urine. Test molecules used are di-/mono-saccharides, e.g. lactulose/mannitol (Catassi 1997b) or lactulose/rhamnose (Menzies 1979, Cummins 2001), ^{51}Cr-labelled ethylenediaminetetraacetic acid (EDTA) (Bjarnason 1983), and different sized polyethylene glycols (PEGs) (Fälth-Magnusson 1989, Stenhammar 1989). There are conflicting results on the permeability of these molecules in CD; lactulose/rhamnose and ^{51}Cr-EDTA indicating increased and PEGs indicating reduced permeability during active CD (Bjarnason 1994), possibly due to different routes for permeation. However, whether increased or decreased in active CD, the permeability of all test molecules returns to normal after a period of gluten-free diet, implicating a good usefulness in monitoring children with CD, especially when serial measurements are available in the same child (Stenhammar 1989, Johnston 2001).

Assessment of urinary nitric oxide (NO) metabolites can also provide a cheap and non-invasive tool in the follow-up of CD patients. NO is a short-lived signal molecule the production of which is increased during inflammation. In the body NO is oxidised to the metabolites nitrite and nitrate, which are excreted in the urine. In children with active CD the urinary levels of NO oxidation products are considerably increased (Sundqvist 1998, van Straaten 1999, Koster-Kamphuis 2003, Laurin 2003), and these levels decrease after a period on gluten-free diet (Sundqvist 1998, Spencer 2004). Since the levels of urinary NO metabolites might vary between individuals, the best results are obtained from serial assessments from the same patient. The method requires no more of the patients than an over-night fast, after which morning urine is collected. This is a simple and robust method which can be performed on children at the ordinary, annual check-up visits at the clinic.

GLUTEN

Gluten is the cohesive mass that remains after washing wheat flour dough, and it consists mainly of storage proteins. Due to its cohesiveness, gluten is to be acknowledged for the baking quality of wheat flour. Gluten is divided into two main fractions, the prolamins and the glutenins, based on their solubility in 45-70% alcohol (Shewry 1992) (Fig. 5). The alcohol soluble prolamins are so named because of their high content of the amino acids proline and glutamine. Prolamins of different cereals are called gliadins from wheat, secalins from rye, hordeins from barley, and avenins from oats. The gliadins are further classified in four groups, α-, β-, γ-, and ω-gliadins, depending on their relative electrophoretic mobility (Woychik 1961), and they all have shown toxicity in CD both *in vivo* and *in vitro* (Ciclitira 1984, Howdle 1984). The role of the glutenins in

CD is not clear, however some studies have indicated that these proteins may be involved in the disease process (van de Wal 1999, Vader 2002b, Molberg 2003).

Few studies have been done on intestinal immune responses to rye and barley. However, Vader *et al*. showed that the disease-inducing properties of rye and barley could in part be explained by T-cell cross-reactivity against gliadin-, secalin-, and hordein-derived peptides (Vader 2003a). A recent study also showed that rye and barley caused immune activation on the mucosa of CD patients (Bracken 2006).

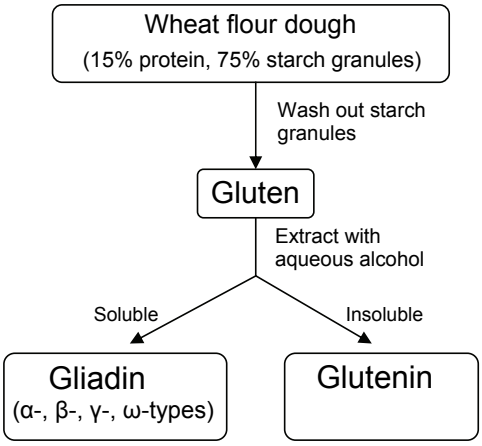


Figure 5. Production of wheat gluten from water-flour dough and fractionation of gluten by solubility into the fractions gliadin and glutenin. Modified from (Kasarda 1996).

OATS IN CD

CD patients were previously advised to exclude oats from their gluten-free diet. This recommendation was based on early feeding experiments. Recently, this recommendation has been changed, as the possible harmful effect of oats has been questioned. Oat is considered to be less harmful than the other cereals,

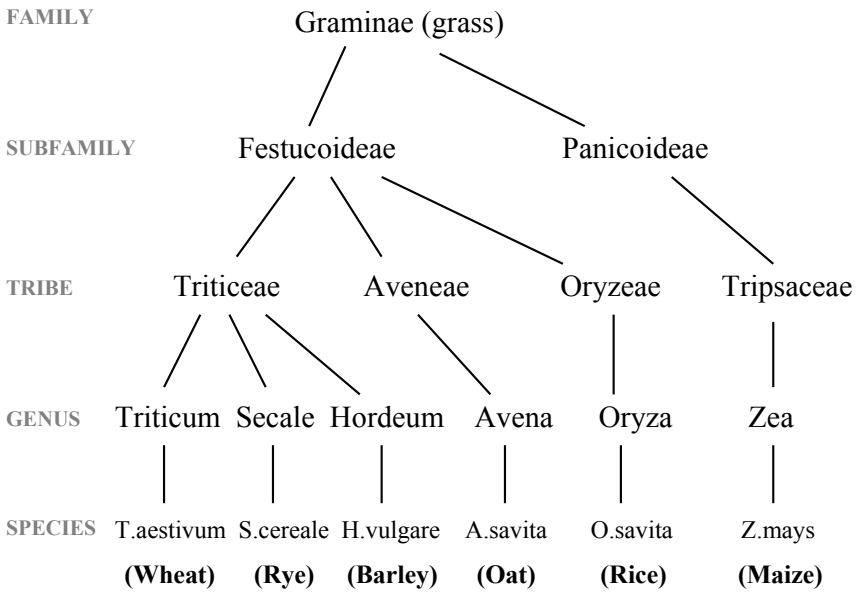


Figure 6. Taxonomic relationships of the major cereals from the Family Graminae (grass), showing that oat falls in another Tribe than wheat, rye and barley (Kasarda 1996).

possibly due to the lower proportion of prolamins. The prolamins of oats, avenins, account for only 5-15% of the total protein content, while in wheat around 40% of the proteins are gliadins (Shewry 1992). Avenin also contains fewer units of the amino acids proline and glutamine. Furthermore, oat is taxonomically more distantly related to wheat than rye and barley (Fig. 6).

Most studies on oats in CD have been performed on adult patients. In a large Finnish study, CD patients were randomised to a standard GFD or a similar diet containing oats (Janatuinen 1995). After 6-12 months, mucosal morphology and the nutritional status of the patients were studied (Janatuinen 1995), as well as gliadin and reticulin antibodies and IELs (Janatuinen 2000). No adverse effects of oats were shown. The patients were re-evaluated after five years, and still no harmful effect of oats was shown (Janatuinen 2002). Other studies have shown good usefulness of oats both in moderate (Srinivasan 1996, 2006) and

even in large amounts (Storsrud 2003a, 2003b). Furthermore, some *in vitro* studies also indicate lack of oats toxicity in CD patients (Picarelli 2001, Kilmartin 2003). One study, where mucosal T-cells were isolated from coeliac patients, showed that these T-cells reacted with the prolamins of wheat, rye, barley, and oats (Kilmartin 2006). However, while treatment with tTG enhanced the response to gliadin, secalin, and hordein, the enzyme caused little or no enhancement of avenin responsiveness. It was suggested that the lower number of proline residues in avenin makes the protein a less suitable substrate for tTG, and that the binding of avenin to HLA-DQ2 is less effective than of the other prolamins.

Fewer studies have been done on children, and until recently, CD children were advised not to include oats in their diet, because of lack of scientific evidence concerning safety. One study reported good usefulness, but it included only ten children and did not have a control group (Hoffenberg 2000). Recently, Högberg *et al*. presented a study including 116 children randomised to a standard GFD or a diet supplemented with oats in a double blind design (Högberg 2004). Serological markers, AGA, EMA, and TGA, were monitored every third month during the one year study period, and small intestinal biopsies were performed at the start and at the end of the study. The study showed that the addition of oats to the gluten-free diet did not prevent clinical or mucosal healing, or humoral immunological down-regulation. It was concluded that oats could be tolerated by the majority of children with CD. In another study, published recently, a 2-year controlled trial and a 7-year follow-up of oats to children with CD were performed (Holm 2006). The study included both CD children in remission, who were either challenged with oats or with gluten, and children with newly diagnosed CD, who were given a GFD containing oats. When the mucosa relapsed in the gluten-challenge group, the patients were put on GFD with oats. Except for two patients that had acute onset of diarrhoea

and/or vomiting after intake of oats, complete recovery from the disease was accomplished in all relapsed and newly detected patients on an oat-containing GFD. Most of the children also preferred to continue with oats after the trial, and they all remained in remission.

There are, however, studies suggesting that at least a small subset of adult CD patients are intolerant to oats (Lundin 2003, Arentz-Hansen 2004). In the first study, one patient developed villous atrophy and dermatitis after challenge with oats. In the second study, two further patients were found that had intestinal inflammation typical of CD after intake of oats. Biopsies from these patients were challenged with oats, and avenin-specific, HLA-DQ2 restricted T-cells were isolated. The peptide epitope specificity was defined to an oats peptide rich in proline and glutamine residues, which was similar but not identical to wheat peptides. The avenin-specific T-cells did not cross-react to gluten.

Although only a few patients with intolerance to oats have been found, it appears that oats is not without toxicity as was previously thought. Oats can be of benefit for CD patients, since it would make the food more palatable and it would increase both the fibre content and the nutritional value. It has also been suggested that oats would improve compliance to a gluten-free diet by making it more diversified. In Finland, where CD patients have used oats since 1997, a study on symptoms and quality of life was performed. It showed that patients with CD eating oats had more intestinal symptoms than those who were on a traditional diet (Peraaho 2004). It should be observed that if CD patients are to eat oats, it must be oats grown and handled in special facilities. Many oat products generally available are contaminated with the other cereals and are therefore unsuitable for individuals with CD (Thompson 2004).

PATHOGENESIS

CD is a complex immunologic disorder with interactions between environmental and genetic factors. It is also regarded as an autoimmune disease. Decades of intense research in this field has resulted in an increased understanding of the underlying immunological mechanisms in CD. Three factors have made CD an important model disorder for studying complex immunological diseases: 1) the environmental factor that triggers the disease is known (gluten), and by this factor the immunological events can be turned on and off, 2) one genetic factor has been identified (the HLA-DQ2/DQ8), which predisposes for the disease, and 3) the access to the affected organ (the small intestine) is simple, which allows detailed studies and isolation of relevant cell populations (Sollid 2002).

T-cell recognition of gluten peptides

The major event when gluten peptides reach the lamina propria is that APCs present the peptides to gluten-specific T-cells in the context of HLA-DQ2 or DQ8 molecules. Challenge of small intestinal biopsies with gluten has been shown to activate CD4+ T-cells in the lamina propria of CD patients but not of controls (Halstensen 1993). When these T-cells were isolated and cultured, it was shown that they only recognised gluten peptides that were presented by DQ2 or DQ8 molecules (Lundin 1993, 1994).

The peptide binding clefts of HLA-DQ2 and DQ8, however, preferably bind peptides with negative charges at certain anchor positions (van de Wal 1996), and gluten molecules contain only few negative charges. The findings that T-cells from CD patients predominantly recognised deamidated gluten peptides (Sjöström 1998), and that this deamidation could be mediated by the enzyme tTG (Molberg 1998, van de Wal 1998, Molberg 2001), shed further lights into this

question. In fact, gluten has been found to be a preferential substrate for tTG (Bruce 1985). The deamidation activity of tTG converts certain glutamine residues to the negatively charged glutamic acid. In gliadin peptides, the T-cell specific epitopes seem to cluster in regions rich in the amino acid proline (Arentz-Hansen 2002). The spacing between proline and targeted glutamine residues plays an important role in the specificity of tTG deamidation (Fleckenstein 2002, Vader 2002a).

The high content of proline residues in gliadin seems to have additional roles in CD pathogenesis. Normally, ingested antigens are processed by digestive enzymes in the gut, but the gliadin peptides that contain the important epitopes are highly resistant to these enzymes, because of their high content of proline (Hausch 2002). When treating α-gliadin with gastric and pancreatic enzymes, Shan *et al*. found a large fragment, a 33-mer peptide, which remained intact despite prolonged exposure to proteases (Shan 2002). The 33-mer fragment contained three known T-cell epitopes, and it was a good substrate for tTG. Furthermore, proline residues help in anchoring the peptide to the HLA-molecule (van de Wal 1996).

Cytokines

The activation of intestinal T-cells is followed by production of cytokines, bioactive polypeptides that are secreted mainly by immune cells in order to modify the behaviour of themselves or other cells. In CD this secretion is clearly dominated by interferon (IFN)-γ (Nilsen 1995, Nilsen 1998, Lahat 1999, Westerholm-Ormio 2002), which is a cytokine typically secreted by T helper (Th)1 lymphocytes involved in cell-mediated inflammatory responses. The expression of both Th1, e.g. IFN-γ and IL-2, and Th2, e.g. IL-4 and IL-10, associated cytokines, as well as macrophage-derived tumor necrosis factor (TNF)-α have been reported in CD (Lahat 1999). A recent study by Forsberg *et al*., showed that

in active CD there was a significant increase in production of IFN-γ and IL-10 by intestinal T-cells, and that the intraepithelial lymphocytes were responsible for the majority of the production (Forsberg 2002). IL-10 is a down-regulatory cytokine, but it can also have proinflammatory effects. It enhances IFN-γ and IL-2 production by cytotoxic T-cells (Santin 2000). Both IFN-γ and TNF-α have been shown to have cytotoxic effects that can damage the target tissue, in this case the intestinal epithelial cells (Westerholm-Ormio 2002).

The gluten-lectin theory

There have been several theories of the pathogenesis of CD over the years. One such theory is the gluten-lectin theory. Lectins are carbohydrate-interacting proteins that can elicit several biological effects, including cell agglutination, cell activation and mitogenesis. According to the gluten-lectin theory, coeliac lesions represent a response to a toxic lectin, putatively wheat germ agglutinin (WGA). In a series of experiments on the effects of WGA on rat intestine and cultured epithelial cells, Sjölander *et al*. showed that the interaction caused an increase in cell proliferation and intracellular calcium concentrations, as well as a reduction of F-actin and morphological changes of microvilli (Sjölander 1984, 1986, 1988a, 1988b). The finding of elevated levels of serum antibodies to WGA gave further support to this theory (Fälth-Magnusson 1995). It was hypothesised that the crypt hyperplasia seen in CD could be due to a mitogenic response induced by WGA, since this lectin can mimic the effect of epidermal growth factor at the cellular level.

Zonulin

Little is known about the mechanism(s) through which gliadin crosses the intestinal epithelial barrier and enters the sub-epithelial compartment. Under physiological circumstances, the intestinal epithelia are almost impermeable to

macromolecules such as gliadin (Fasano 1998). Zonulin, a newly discovered modulator of intestinal permeability (Wang 2000), has been shown to be up-regulated during the acute phase of CD (Fasano 2000). Recent studies on the early effects of gliadin on intestinal epithelial mucosa showed that gliadin activates the zonulin signalling, resulting in rapid reduction of the barrier function and passage of gliadin into the lamina propria (Drago 2006). This process was dependent on the presence of zonulin receptors but was independent on individual genetic predisposition.

Bacteria in CD

It has also been suggested that bacteria might be involved in the pathogenesis of CD. An intestinal infection could cause inflammation and damage to the mucosa, thereby raising the activity of tTG, possibly increase the leakage of gluten peptides across the epithelial barrier, and stress the epithelial cells to express MIC antigens. In a recent study, Forsberg *et al*. showed that bacteria were associated with the intestinal epithelium in children with CD but not in controls (Forsberg 2004). Furthermore, by studying short chain fatty acids in faecal samples from CD children and healthy controls, Tjellström *et al*. found an altered pattern of these fatty acids in both treated and untreated CD, as compared to controls (Tjellström 2005). They suggested that their findings might reflect a deviant gut flora in CD patients, and that this could bring new clues to the pathogenesis of CD.

NITRIC OXIDE

Nitric oxide (NO) is a free radical gas produced by many cell types. It is a signalling molecule in blood vessels, where a continuous production from endothelial cells acts on the underlying smooth muscle to regulate

vasodilatation and blood flow. It also has a role in immune defence, since activated macrophages can produce large amounts of NO which destroys microorganisms and cancerous cells (Änggård 1994). In the gastrointestinal tract NO has a wide variety of physiological and pathological roles. It can be associated with severe cell damage due to free radical oxidative damage (Rachmilewitz 1995), but it can also have a protective role at low concentrations, by improving mucosal integrity (Kubes 1992).

NO is produced from L-arginine by a family of enzymes called nitric oxide synthases (NOS) (Fig. 7). There are three distinct isoforms of NOS identified, which are products of different genes, and have different localisation, regulation, and catalytic properties. Two isoforms are constitutively expressed, nNOS and eNOS, which are mainly found in neuronal tissue and endothelial cells, respectively (Alderton 2001). Both are Ca^{2+}-dependent enzymes and they respond to increased intracellular Ca^{2+}-levels by giving off a short burst of NO. nNOS and eNOS are collectively called constitutive NOS (cNOS), and they regulate several basal body functions at physiological concentrations. The third isoform is called inducible NOS (iNOS) and it is a Ca^{2+}-independent enzyme that can be expressed in a wide variety of cells, including macrophages, neutrophils, and mast cells, and also endothelial cells (Änggård 1994). The main characteristic of iNOS is that it releases large amounts of NO continuously.

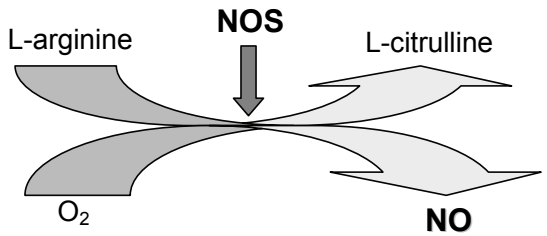


Figure 7. Synthesis of nitric oxide (NO) from L-arginine by the enzyme nitric oxide synthase (NOS).

In the body, NO is oxidised to the metabolites nitrite and nitrate, which are excreted in the urine. Several studies have shown that CD children excrete large concentrations of these NO metabolites in urine (Sundqvist 1998, van Straaten 1999), and that in CD children in remission, challenge with gluten resulted in an increased excretion of NO products (Koster-Kamphuis 2003, Laurin 2003). NO metabolites have also been found to be elevated in plasma from untreated CD patients (Murray 2003, Spencer 2004). This increased NO production is probably due to an increased activation and expression of iNOS in the small intestinal mucosa of CD patients (Holmgren Peterson 1998, Murray 2002, Daniels 2005). The up-regulation of iNOS is assumed to be the result of an inflammatory response to the pro-inflammatory cytokines secreted in CD (van Straaten 1999), and increased urinary excretion of NO metabolites is thus a strong indication of ongoing inflammation in the small intestine.

TIGHT JUNCTIONS

Polarised epithelial cells provide a permeability barrier between two very different environments, and they allow a selective transport across the cellular layer. Adjacent cells are held together by junctional complexes such as gap junctions, desmosomes, adherence junctions, and the tight junction (TJ) (Fig. 8). Gap junctions mediate communication between cells by allowing small molecules to pass from cell to cell. Desmosomes are points of intercellular contact that provide anchoring for intermediate filaments of the cytoskeleton. Adherence junctions function to hold cells together by linkage between cell-adhesion molecules and the actin cytoskeleton (Denker 1998). The TJ complex is the most apical one and the most important for formation of the barrier and also for maintaining the polarity of the epithelial cells (Gumbiner 1996). The complex

consists of many different associated proteins and there are three known transmembrane proteins: occludin which is a single gene product (Furuse 1993), junctional adhesion molecules which are members of an Ig-superfamily (Bazzoni 2003), and claudins (Furuse 1998) which is a multi-gene family of at least 24 members.

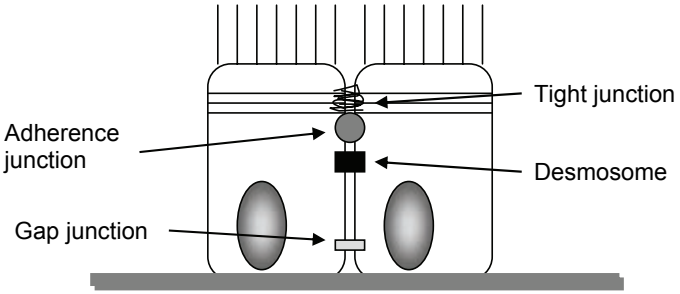


Figure 8. Schematic drawing of two adjacent epithelial cells showing the distribution of the junctional complexes.

Occludin

Occludin is a 60-kDa integral membrane protein, and it was the first component of TJ strands to be identified (Furuse 1993). Occludin has four transmembrane domains and two extracellular loops. The loops are typically rich in tyrosine and glycine residues, and charged residues are mostly located at the cytoplasmic domains. The exact functions of occludin remain unclear and the importance of occludin in the barrier and fence functions of TJ has been debated. A knock-out study where occludin was deleted from embryonic stem cells, showed that TJs could be formed without the presence of occludin (Saitou 1998). Furthermore, due to the lack of charged amino acid residues in the extracellular loops of occludin, it has been questioned whether this protein can create a charge selective barrier (Anderson 2001). The level of expression of occludin in various tissues has, however, been shown to correlate well with the number of TJ strands in the

epithelial cells (Saitou 1997). Findings in a recent study that occludin-deficient mice did not show any defects in the epithelial barrier but a dramatic change in gastric morphology and secretory functions (Schulzke 2005), indicates that occludin is involved in gastric epithelial differentiation.

Claudins

Claudin-1 and claudin-2 were the first members of the claudin family to be discovered (Furuse 1998). These two proteins are also integral membrane proteins with four transmembrane domains, but they do not show any sequence similarity to occludin. There are at least 24 members identified in the claudin family, and they are differently expressed in various tissues (Morita 1999, Rahner 2001, Tsukita 2001). In contrast to occludin, the amino acid composition of the two extracellular loops of claudin varies significantly among different claudins, resulting in a wide range of isoelectric points (Schneeberger 2004), which indicates that claudins are proteins that can determine the range of ion selectivity in different epithelia. Indeed, claudins have been shown to create charge selective channels in tight junctions of cultured epithelial cells (Amasheh 2002, Colegio 2002).

AIMS OF THE THESIS

The aims of the research in this thesis were to investigate the humoral and inflammatory reactions to oats in children with CD. Another aim was to study the intestinal mucosa at different stages of the disease.

The objectives were to:

- Develop a method for analysis of serum antibodies against avenin
- Elucidate if children with CD had antibodies against the oat prolamine avenin
- Evaluate the effect of oats in a gluten-free diet on the levels of anti-avenin antibodies and the concentrations of urinary nitric oxide metabolites, in children with newly diagnosed CD
- Delineate the structure and distribution of occludin and claudins, tight junction proteins known to play crucial roles in maintaining the barrier function, in different stages of childhood CD
- Study the ultrastructure of the intestinal mucosa in CD

METHODS

This is a summary of the methods used in this thesis. For detailed description of the methods the reader is referred to the papers (I-IV).

PATIENTS

Paper I

This was a retrospective study in which we obtained sera from 81 children with suspected CD as the reason for referral. In 47 children the primary investigation discarded the suspicion of CD and they were used as a reference group. The other 34 children were diagnosed as coeliacs. Serum samples from the CD children were drawn at different stages of the diagnostic procedure: at the time of the primary biopsy when on normal diet including gluten (n=16), at the time of a control biopsy after approximately one year on a gluten-free diet (n=18), and at a second control biopsy after a period of gluten challenge (n=7). In seven children serum samples were drawn both on gluten-free diet and after challenge, making the total number of samples 88.

Papers II and III

These studies were parts of a randomized, double-blind trial of oats given to small children with newly diagnosed CD (Högberg 2004). That study included 116 children under investigation for CD from eight Swedish paediatric clinics. Inclusion criteria were: patients less than 18 years of age with a small bowel biopsy showing Type 3 enteropathy according to Marsh-classification, whose families agreed on participating in the study and had a good understanding of the Swedish language. The children were randomised to either a traditional GFD (GFD-std; n=59) or a GFD supplemented with oat products (GFD-oats; n=57). The oats used were grown and handled so as not to become contaminated with

wheat, rye, or barley (Semper AB, Sweden). The study period was approximately one year. Ninety-two patients completed that study.

For the antibody assessments (**paper II**), sera were obtained from 86 of the children originally included in the above study. Out of them 48 were in the GFD-std group and 38 in the GFD-oats group, of which 33 consumed at least 10 g of oats daily as an average throughout the study period. Serum samples were drawn at the time of the primary biopsy, after 3 and 6 months, as well as at the time of the control biopsy after 12 months. From 10 children in the GFD-std group and 7 children in the GFD-oats group, sera were only available from two or three checkpoints. Sera were stored at -20 °C until analyses were performed.

For the NO measurements (**paper III**), morning urine was collected after an overnight fast at 0, 3, 6, and 9 months, and at the time of the control biopsy after approximately one year. Urine samples were not available from all patients at every checkpoint and we chose to evaluate those patients from which urine was collected at least at two checkpoints. That left 87 children to be included in this study, out of which 39 were in the GFD-oats group and 48 in the GFD-std group. Urine samples were stored at -20 °C pending analyses.

Paper IV

In this study we obtained intestinal biopsies from children under investigation for CD in 2003-2006, when excess material from the routine examination was available. Eighty children were included in this study and they were from three different diagnostic groups: children with untreated CD (n=25), children with CD, treated with GFD for about one year (n=36), and children with suspicion of CD as the reason for referral, but with a primary biopsy classified as normal

(n=19). Six CD patients were studied at two occasions, all the others at one occasion each.

Ultra structure of the intestinal mucosa (unpublished)

Scanning electron microscopy (SEM) was performed on 81 small intestinal biopsies from 44 children with coeliac disease. Biopsies were taken at different stages of the disease: primary biopsy on normal diet (n=41), control biopsy on GFD (n=27), and a second control biopsy after gluten challenge (n=13).

PREPARATION OF CRUDE AVENIN (I, II)

Crude avenin was prepared from pure oat meal, guaranteed to be free from contamination of other cereals (Graintec 000514, Semper AB). The oat meal was defatted with water saturated n-butanol and left to separate for 30 min and then the fatty phase was removed. Ethanol was added to a final concentration of 45% in order to dissolve the alcohol soluble proteins. After being stirred for 1 h at room temperature (RT) the solution was centrifuged. Proteins in the supernatant were precipitated with two volumes of cold 1.5% NaCl and recovered by centrifugation and then dried. Protein content of the precipitate was assessed using a BSA Protein Assay Kit (Pierce, Rockford, Ill., USA).

ANTIBODY ASSESSMENTS (I, II)

Anti-avenin and anti-gliadin antibodies in serum were assessed using an indirect enzyme linked immunosorbent assay (ELISA). Crude avenin and gliadin were dissolved in 70% ethanol and diluted in phosphate-buffered saline (PBS) to 100 µg/ml and 50 µg/ml, respectively. The antigens were then allowed to adhere to wells of eight-well strips for 2 h at 37°C and then at 4°C for 2.5 h. Wells were

then blocked with human serum albumin for 2 h at 37°C and at 4°C overnight. Sera were diluted 1:25, added in triplicates and incubated for 1 h at 37°C. The primary antibodies used were mouse anti-human IgG and IgA (Skybio, Bedfordshire, England) at 0.5 µg/ml, and alkaline phosphatase conjugated rabbit anti-mouse IgG (Sigma-Aldrich) were used as secondary antibodies, diluted 1:10000. As substrate for the enzyme p-nitro phenyl phosphate (Sigma) was used. The resulting colour was read as optical density on a kinetic microplate reader (Molecular Devices, Sunnyvale, Calif., USA) at 405 nm.

Controls

In order to minimise and measure the day-to-day variation of the assay, reference sera, with high and low reactivity of the antibodies measured, were included in each run. The high-level serum was from a donor with high levels of several different food antibodies but with a normal jejunal mucosa. The low-level sera were from healthy blood donors without reactivity to the antigens in question. In Paper I, reaction to keyhole limpet hemocyanin (KLH; Sigma) was also tested as an additional control and test of background levels. PBS with 0.05% Tween 20 (Fluka), which was used for dilution of antibodies and sera, was used as blank.

In Paper II, all patient sera were evaluated in relation to the high-level sera, which OD values were normalised to the value 1. The low-level reference serum was used for calculation of the day-to-day variation, and values were related to the high-level reference in the same way as the samples. The coefficient of variation (CV) for the inter-assay variation was 13% for IgA, and 14% for IgG. The CV for the intra-assay variation was 6.8%. Cut-off values were determined using values from Paper I, in which a control group of non-coeliacs was included. The cut-off OD values were then calculated relative to

the median for the high-level reference, and were accordingly set at 0.8 and 0.4 for IgA and IgG, respectively.

Cross-reaction test (I)

An absorption test was performed in order to elucidate a possible cross-reaction between antibodies to avenin and gliadin. The object was to see if antibodies to one protein could be absorbed by the other protein, and *vice versa*. The absorption test was performed on sera from coeliac children on normal diet (n=23). The diluted sera were first added to wells coated with avenin or gliadin, or as negative control, to wells coated with KLH. After incubation for 1 h at 37 °C, sera were moved to wells coated with avenin or gliadin and an indirect ELISA was performed as described earlier (Fig. 9).

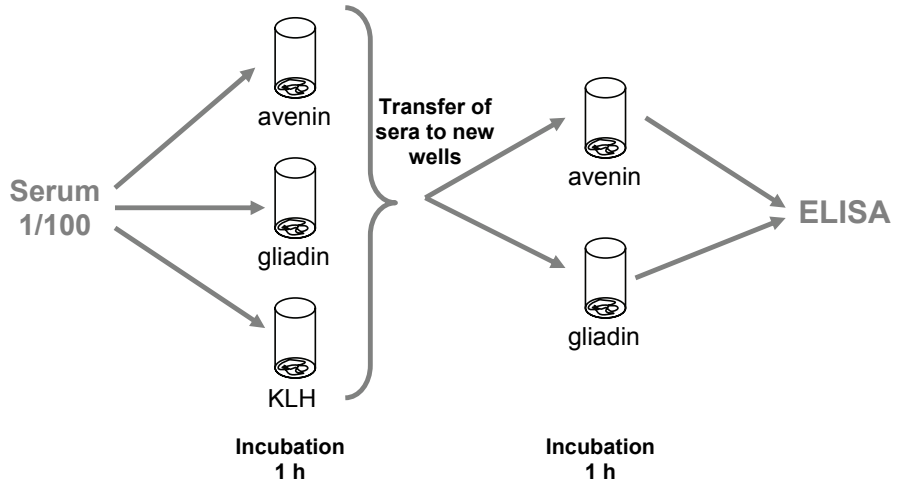


Figure 9. Method for the test of cross-reaction between anti-avenin and anti-gliadin antibodies. KLH = keyhole limpet hemocyanin.

NITRIC OXIDE MEASUREMENTS (II, III)

In the body, NO is oxidised to the metabolites nitrite (NO_2^-) and nitrate (NO_3^-). Therefore, in the urinary samples, we measured the sum of nitrite and nitrate (NO_2^-/NO_3^-) as an indirect indicator of the NO production using a method described previously (Verdon 1995). Urine was diluted in PBS and nitrate was converted to nitrite using nitrate reductase from *Aspergillus*. This was done by mixing 50 µl of the diluted urine with 10 µl NADPH (1 µM) followed by 40 µl containing nitrate reductase (80 U/l; Roche), glucose-6-phosphate (500 µM) and glucose-6-dehydrogenase (160 U/l). The reaction mixture was incubated for 45 min at RT. The mixture was then used for the Griess assay of nitrite by adding 100 µl sulphanilamide (1%, in 5% phosphoric acid) and 100 µl naphtyl ethylenediamine (0.1%). The resultant colour was read with a spectrophotometer (Vmax; Molecular Devices) at 540 nm.

A cut-off value was set at 1406 µM in accordance with a previous study, in which a reference group of non-coeliacs was included (Sundqvist 1998).

IMMUNOFLUORESCENCE (IV)

Fixation

Immediately after dissection, biopsy specimens were placed either in formaldehyde for fixation or in cold PBS and transported to the lab. Upon arrival to the lab, within 30 min from the dissection, biopsies transported in PBS were subjected to 2.5% paraformaldehyde (PFA; Fluka). When put in formaldehyde or PFA, the biopsy specimens were fixed for 24 h at 4°C and subsequently placed in Krebs Ringers solution, without glucose, supplemented with 50 mM NaN_3, and stored at 4°C.

Labelling

The biopsy specimens were embedded in O.C.T Compound Tissue-Tek (Sakura Finetek, Zoeterwoude, NL), frozen and cryosectioned in 15 µm sections and mounted on glass slides. The sections were permeabilised in methanol and incubated with blocking solution containing 1% bovine serum albumin (BSA, Sigma), 100 mM glycine (Fluka) and 10% normal goat serum (DakoCytomation). They then were incubated with mouse anti-occludin, anti-claudin 2, anti-claudin 4, or anti-claudin 5, or rabbit anti-occludin, anti-claudin 1, or anti-claudin 3 (Zymed Laboratories Inc.) As secondary antibodies Alexa 568 conjugated goat anti-mouse or anti-rabbit IgG (Molecular probes) were used. For double staining of filamentous actin (F-actin) and TJ components, cryosections were permeabilised in 0.1% Triton X-100 (Sigma) before blocking and incubation with primary and subsequently secondary antibodies. When 20 minutes remained of the incubation time for the secondary antibody, Alexa 488 conjugated phalloidin (Molecular Probes) was added. All sections were mounted in anti-fading reagent (ProLong Gold, Molecular Probes) and stored at 4°C until examined. Before the examination, each biopsy was coded to ensure blinding and an unbiased assessment of the fluorescence. As negative controls, sections treated in similar conditions but without the primary antibodies were used.

Microscopy

The blinded biopsy sections were studied in a standard fluorescence microscope (Zeiss Axioskop) using a 63x Plan-apochromat oil immersion objective with a numerical aperture of 1.4. Presence and localisation of the different proteins were documented with a digital camera (Canon PowerShot G3), handled by the software ZoomBrowser EX 4.0 (Canon Utilities). The fluorescence for each protein was classified as negligible, diffuse/intermediate, strong, or very strong.

For calculation of frequencies in the three study groups, the strong and very strong fluorescence were regarded as positive for each protein. Documentation of typical labelling of the different antibodies, as well as assessment of the double stained sections, was done using a BIO-RAD Radiance 2100 confocal laser scanning microscopy system (BIO-RAD and Carl Zeiss) based on a Nikon Eclipse TE 2000-U microscope (Nikon) equipped with a 60x high numerical aperture (1.4) oil immersion objective. An argon laser at 514 nm was used for excitation, and for the emission a 570 long pass filter was used. For the double stained sections an argon laser at 514 nm was used for excitation of the Alexa 568 probes, together with a LP560 dichroic mirror and HQ600/50 emission filter. For excitation of the Alexa 488 probe the 488 nm line from the argon laser was used, with the emission filter HQ515/30. LaserSharp 2000 Software (BIO-RAD) was used for handling of the microscope and for collection of data.

SCANNING ELECTRON MICROSCOPY

Biopsy specimens were fixed in 2% glutaraldehyde (Agar Scientific Limited) diluted in 0.15 M sodium cacodylate buffer (VWR Prolabo) for 1 h at RT. After washing in sodium cacodylate buffer the specimens were postfixed for 1.5 h in 1% osmium tetraoxide (Agar Scientific) diluted in 0.15 M sodium cacodylate buffer. The biopsies were dehydrated stepwise in 50-100% ethanol, dried from liquid carbon dioxide by the critical-point drying method, and sputter-coated with 20 nm platinum. Digital micrographs were obtained from a JSM 840 scanning electron microscope (JEOL) operated at 10 kV and with a tilt angle of 42°.

ETHICS

The studies in this thesis were performed with the approval of the Human Research Ethics Committee at the Faculty of Health Sciences, Linköping University, Sweden.

RESULTS & DISCUSSION

ANTIBODIES TO AVENIN IN COELIAC CHILDREN (I)

When this study was initiated, the addition of oats to the GFD was considered safe for adult CD patients (Janatuinen 1995, Srinivasan 1996, Janatuinen 2000, Janatuinen 2002), but there was little information on the safety of oats for children with CD. One study reported good usefulness (Hoffenberg 2000), but it was performed on only 10 children and without a control group. We therefore performed this retrospective study in which we developed an ELISA for assessment of serum antibodies to the oats prolamin avenin in children with verified CD and reference children. The study was intended to serve as a background to an intervention trial on the possibility of adding oats to the diet of coeliac children.

We found that children with CD had serum antibodies against avenin, of both IgG and IgA type, and that the levels were significantly higher than for reference children. The anti-avenin antibodies also followed changes in the diet, i.e. the levels were attenuated after a gluten-free period and after the gluten challenge the antibodies increased, but not to the same levels as at presentation (Fig. 10). The sera used in this study were drawn prior to the debate on the use of oats for CD patients, and hence, the changes in the diet presumably also included changes in intake of oats.

In active CD the intestinal mucosa is greatly damaged which leads to altered uptake and processing of food antigens. This in turn could cause a humoral reactivity to antigens other than gliadin. One study showed that CD children at presentation had antibodies against β-lactoglobulin and ovalbumin at higher levels than reference children (Fälth-Magnusson 1994). The levels declined after gluten withdrawal without any change of the diet regarding intake of milk

and egg. Our anti-avenin ELISA was able to clearly distinguish between the coeliac children and the reference group, and we then speculated if this was a true humoral response to oats. There are, indeed, reports on both T-cell recognition of (Lundin 2002) and serum reactivity against (Rocher 1992, Alfonso 1998) avenin.

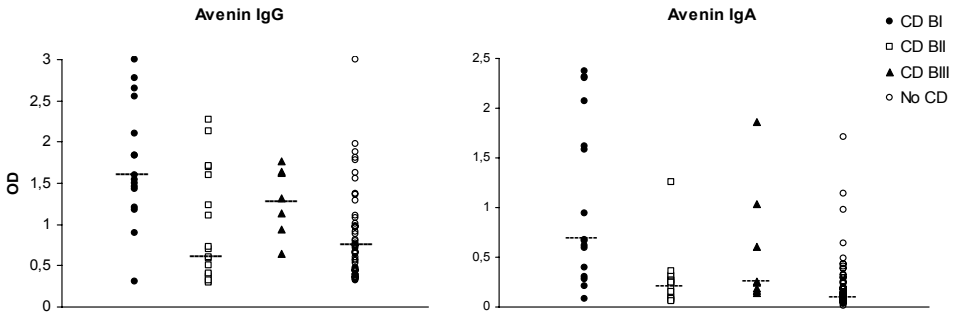


Figure 10. Optical density (OD) values for anti-avenin antibodies of IgG and IgA type, respectively, in children with CD and reference children. Dotted lines represent the median values in each group. CD BI, first biopsy at presentation, n=16; CD BII, control biopsy after one year on gluten-free diet, n=18; CD BIII, second control biopsy after gluten challenge, n=7; No CD, reference children, n=47.

CROSS-REACTIVITY BETWEEN AVENIN AND GLIADIN (I)

In this study the antibodies to avenin correlated well with antibodies to gliadin. It has been suggested that there is a cross-reaction between these antibodies, due not only to sequence similarities between the antigens but also to common structural conformations with β-turn motifs (Alfonso 1998, Ribes-Koninckx 2000). In order to elucidate this proposed cross-reactivity we performed an absorption test using both avenin and gliadin as antigens. Furthermore, we also used keyhole limpet hemocyanin (KLH), a protein to which there should be no reactivity, as a negative control.

We found no evidence of cross-reaction between the antibodies to gliadin and avenin. The absorption of anti-gliadin antibodies by avenin was the same as by KLH, whereas the absorption of gliadin was about two-fold higher. This was also true for the anti-avenin antibodies, which gliadin and KLH absorbed equally (Fig. 11), indicating that both the anti-gliadin and the anti-avenin antibodies were specific.

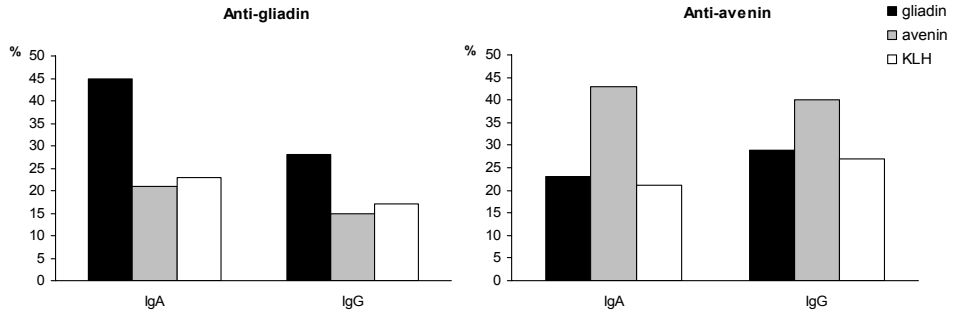


Figure 11. Median values of the absorption of anti-gliadin and anti-avenin antibodies, respectively, by the proteins gliadin, avenin, and keyhole limpet hemocyanin (KLH) in sera from children with CD (n=23). The antibodies were only absorbed by their true antigens, since the other antigen and KLH absorbed equally.

ASSESSMENTS OF A GLUTEN-FREE DIET WITH OR WITHOUT OATS (II, III)

These studies were parts of a randomised, double-blind trial of oats in the GFD of newly diagnosed coeliac children (Högberg 2004). The conclusion of that study was that the addition of oats in the GFD does not prevent clinical and mucosal healing, and that oats can be tolerated by the majority of children with CD.

In the present investigation we further studied the reactions to oats in CD-children randomised to either a traditional GFD or a GFD supplemented with oats products. In Paper II we studied the humoral immune reaction to oats, by performing serial assessments of serum antibodies to avenin, using the anti-

avenin ELISA developed in Paper I. In Paper III we evaluated the effect of oats in a GFD by means of urinary nitric oxide oxidation products as markers of mucosal inflammation.

Anti-avenin antibodies

Serum antibodies against avenin, of both IgG and IgA type, were measured at four occasions during the 12-month study period; at 0, 3, 6, and 12 months. There were no significant differences in antibody titres between the two study groups at any of the four checkpoints. Already after 3 months had the titres of both IgG and IgA antibodies decreased significantly in both groups (Fig.12). At the end of the study, after one year on diet, only three children were still positive for IgA anti-avenin. Out of them two were in the GFD-oats group and one was in the GFD-std group. The IgG titres, however significantly decreased, did not decline as fast as the IgA titres and at the end of the study only 38% were negative for IgG anti-avenin.

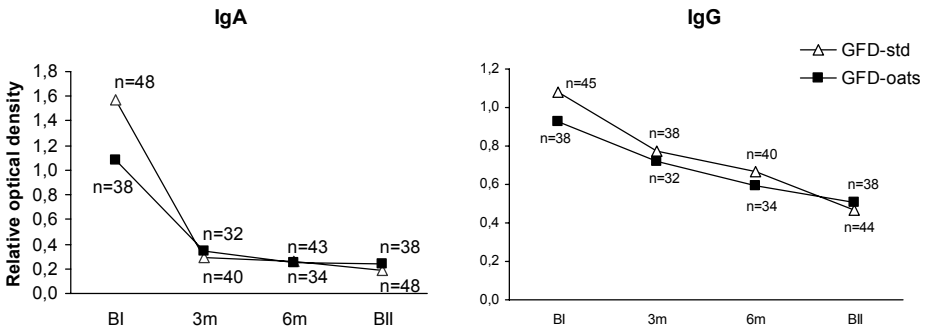


Figure 12. Median values of anti-avenin antibodies at the different checkpoints. Optical density values are given in relation to a high antibody level reference serum. Both IgA and IgG antibodies declined significantly after 3 months. No significant differences were found between the study groups.

It seems that avenin alone is not able to initiate a humoral immune response, and that the reaction requires gluten challenge. Hence, as soon as gluten is withdrawn, the production of anti-avenin antibodies abates.

Nitric oxide measurements

The urinary nitrite/nitrate concentrations were measured every third month during a 12-month period, and on no occasion were there any significant differences between the two study groups. Irrespectively of whether the diet included oats or not, the diet regimens resulted in a marked reduction in urinary NO products already after 3 months (Fig.13). At the 6-month control 8% in the GFD-oats group and 16% in the GFD-std group had concentrations above the cut-off value, and these frequencies increased to 26% and 19% in the two groups, respectively, at the 12-month control (Fig. 14). This increase in frequencies was significant in the GFD-oats group.

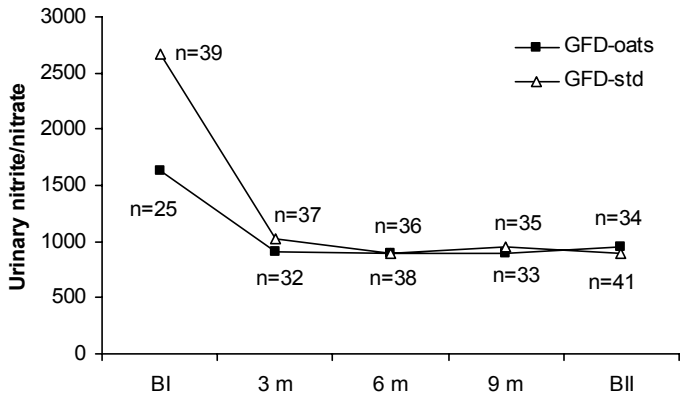


Figure 13. Urinary nitrite/nitrate concentrations (μM) in children with coeliac disease on GFD with or without oats. Values are expressed as median concentrations. The levels decreased significantly after 3 months, and at no occasion were there any differences between the study groups.

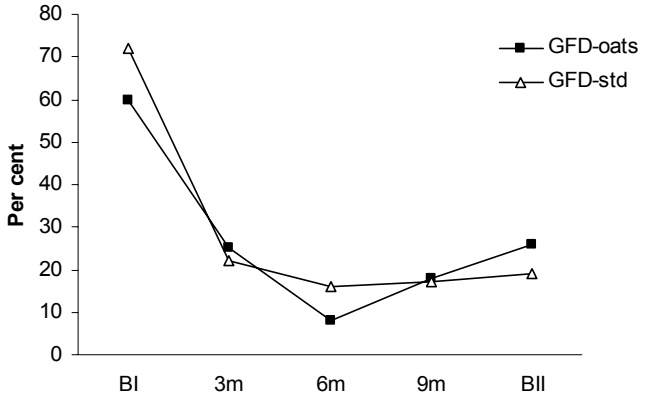


Figure 14. Frequencies of individuals with urinary nitrite/nitrate concentrations higher than the cut-off value, i.e. >1406 μM, at the various checkpoints. In the GFD-oats group there was a significant increase from 6m to BII ($p=0.043$, chi^2-test).

It has previously been shown that iNOS is up-regulated in the small intestine during active CD (Holmgren Peterson 1998, Murray 2002, Daniels 2005), as the result of an inflammatory response to secreted pro-inflammatory cytokines (van Straaten 1999). Interestingly, in the present investigation, there was a subgroup of children that had still high values at the end of the study period, indicating an ongoing inflammation in the intestine. In the GFD-oats group, 4 children had extremely high values, i.e. two times the cut-off value, as compared to only one in the GFD-std group with these high values (Fig. 15).

Of the 116 children included in the original study, 22 declined to participate further and they therefore withdrew from the study (Högberg 2004). Among the children in the GFD-oats group 6 out of 15 withdrew because of adverse symptoms, while in the GFD-std group 2 out of 7 patients withdrew because of symptoms. It would, of course, have been interesting to be able to evaluate both the anti-avenin antibodies and the NO levels in these individuals

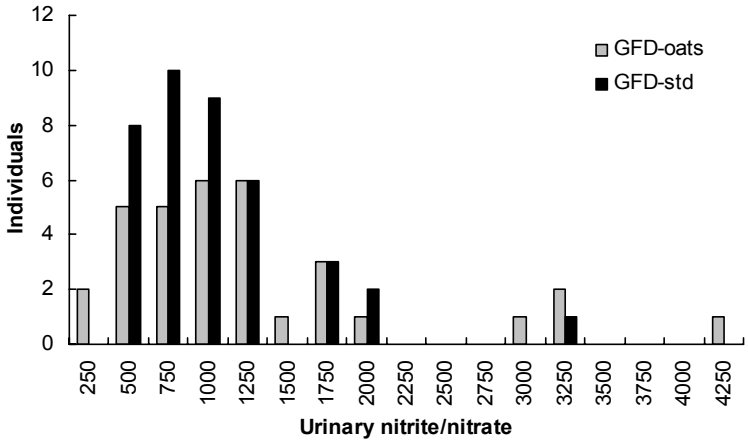


Figure 15. The number of children in each study group with specific concentrations of urinary nitrite/nitrate (μM), showing a subgroup of five patients having concentrations more than two times the cut-off value at the end of the 12-month study period. Four of these individuals were in the GFD-oats group. The cut-off value was set at 1406 μM

that withdrew from the study, and we believe that this could have given further information about the usefulness of these methods in the assessments of reactions to oats. Several studies (Hoffenberg 2000, Arentz-Hansen 2004, Högberg 2004) have indicated a need for follow-up studies on the use of oats in the gluten-free diet for both children and adults with CD. We believe that our method for measurement of nitrite/nitrate concentrations in urine could provide a cheap and non-invasive tool in the follow-up of these patients.

The studies on oats in CD presented in this thesis suggest that oats can be tolerated by the majority of children with CD, but that there is a subgroup of children that do not, and hence, there should be a long-term follow-up on the children having their diet supplemented with oats.

OCCLUDIN AND CLAUDINS IN COELIAC DISEASE (IV)

In order to elucidate the expression and the localisation of tight junction proteins in CD, we performed immunofluorescence labelling of occludin and claudins 1-5 in biopsies from children with CD, both at presentation and after one year on GFD, and reference children. The fluorescence for each protein was classified as negligible, diffuse/intermediate, strong, or very strong. For calculation of frequencies in the three study groups, the strong and very strong fluorescence were regarded as positive for each protein.

We found a significant difference in the frequencies of positive staining for occludin between untreated CD and the other groups. In untreated CD 80% showed strong, or very strong, staining, while in the treated CD and the reference group only 44% and 42%, respectively, showed positive staining for occludin. For the different claudins, however, there were no significant differences in frequencies of positive staining between the groups, although the frequencies were slightly lower in untreated CD for claudins 2, 3, and 4. There were only negligible staining of claudins 1 and 5 in all the subjects studied. Frequencies of positive staining for occludin and claudins 2-4 are shown in Figure 16.

The staining pattern of the biopsy specimens positive for occludin was distinct, strong bands at the apical level of the lateral membrane, with no difference along the crypt-to-villous axis. In those specimens regarded as negative there were more diffuse, granular staining in the region of the lateral membrane.

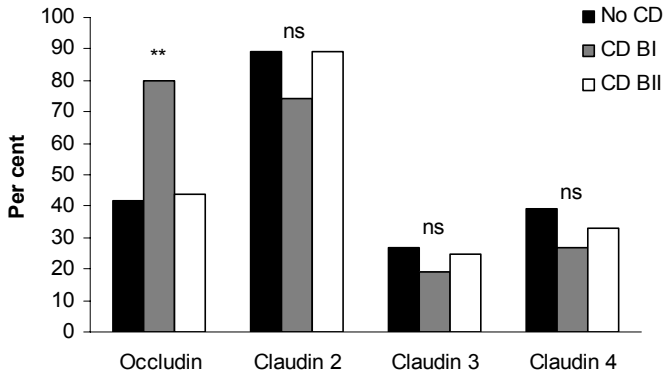


Figure 16. Percentage of biopsy specimens that stained positive for the tight junction proteins occludin and claudins 2, 3, and 4, in the three study groups. There was a significant difference in occludin staining between untreated CD and the other groups, while the differences for the claudins were not significant.

Claudin 2 was the most abundant of the claudins assessed in the present study, and it had a staining pattern completely different from occludin. Along the villi there was an intense granular staining in the apical parts of the cytoplasm, while in the crypts the staining was weaker with a granular pattern at the lateral membrane, where sharp bands could also be seen. Claudins 3 and 4, when present, were also expressed at the lateral membrane and the fluorescence was diffuse and granular.

The higher level of expression of occludin in children with active CD in the present study might be explained by either an up-regulation of this junctional protein in order to maintain the polarity of the enterocytes, or that it is a characteristic for the phenotype associated with the crypt cell hyperplasia typical for the coeliac lesion. A study on the expression of occludin and claudin 1 in HT 29 and MDCK-1 cells showed that upon stimulation with phorbol ester the two cell lines responded differently. The HT 29 cells, with low

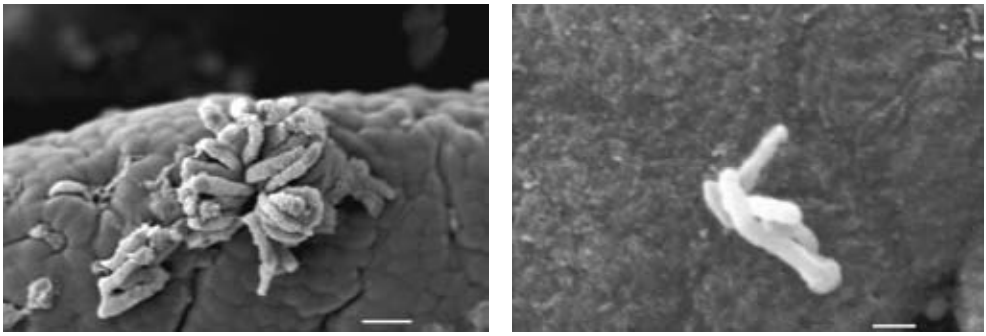
transepithelial resistance (TER), responded with a higher resistance as well as with an increased expression of occludin and claudin 1. The MDCK-1 cells, with high TER, responded in the opposite way (Sjö 2003). It seems that regulation of the barrier and the distribution of the TJ proteins involve different mechanisms depending on the basal characteristics of the cells.

Studies on the intestinal permeability in CD have revealed conflicting results (Menzies 1979, Bjarnason 1983, Fälth-Magnusson 1989, Stenhammar 1989, Sander 2005), possibly due to the use of different test molecules with not only varying sizes, but also different net charges of the molecules. In occludin, there are no charged amino acids in the extra cellular loops, indicating that this protein creates uncharged barriers (Anderson 2001), while the charged claudins create charge selective pores between the epithelial cells (Amasheh 2002, Colegio 2002). This could explain the divergent permeability characteristics for molecules such as small PEGs and ^{51}Cr-EDTA, both of equal size around 300 Da, but with different charges. The urine excretion rate of the uncharged PEG molecules are almost 20 times higher than the excretion of the charged ^{51}Cr-EDTA in normal controls (Bjarnason 1994), while in untreated CD there is a decrease of PEG recovery and an increase of ^{51}Cr-EDTA recovery. Hence, the uncharged PEG molecules would possibly be hindered by the uncharged fence made up by occludin, and the permeation of the charged ^{51}Cr-EDTA molecules would be additionally and more strongly dependent on the charge selective claudins. In the PEG studies (Fälth-Magnusson 1989, Stenhammar 1989), the breakpoint for PEG size, i.e. the molecular weight at which 50% of the intestinal filtering has occurred, was lower in untreated CD, indicating that the functional pore was tighter in those patients. This is in accordance with the present investigation, since a narrower pore would have more strands and thereby more TJ proteins, e.g. occludin.

ULTRA STRUCTURE OF THE INTESTINAL MUCOSA IN CD (UNPUBLISHED)

This study was initiated in order to elucidate the possible association of bacteria to the intestinal mucosa of patients with CD. It has been shown that a variety of bacteria adhere to the small intestinal mucosa (Hörstedt 1989), and that in biopsies from children with active CD, bacteria were present to a significantly higher degree than in treated CD and healthy controls (Forsberg 2004). We therefore performed scanning electron microscopy on 81 biopsies from 44 children with CD at different stages of the disease.

Interestingly, we found bacteria in only two of the biopsies studied. These were colonies of rod-shaped bacteria (Fig.17). We can offer no definite explanation of these results, which deviates from the previous studies, since we believe that the methods used were quite the same.

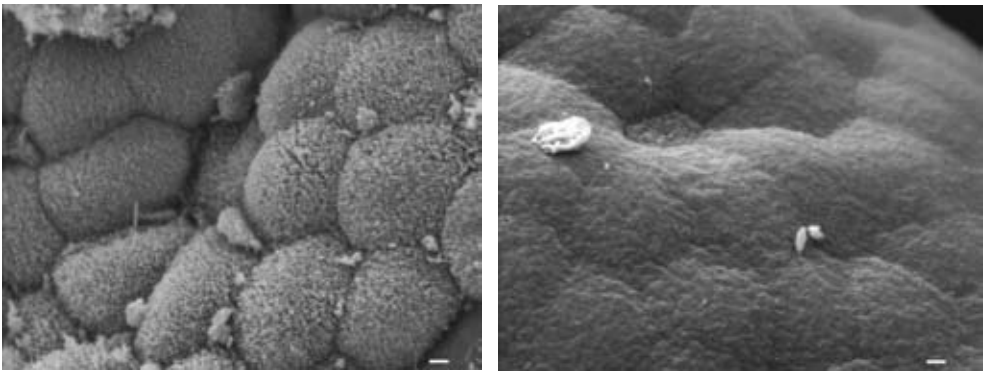


a) b)

Figure 17. Scanning electron microscopy (SEM) pictures of small intestinal biopsies from a) a child with untreated CD, and b) a child with treated CD, showing colonies of rod-shaped bacteria. Bar in a) 10 µM, and in b) 1 µM.

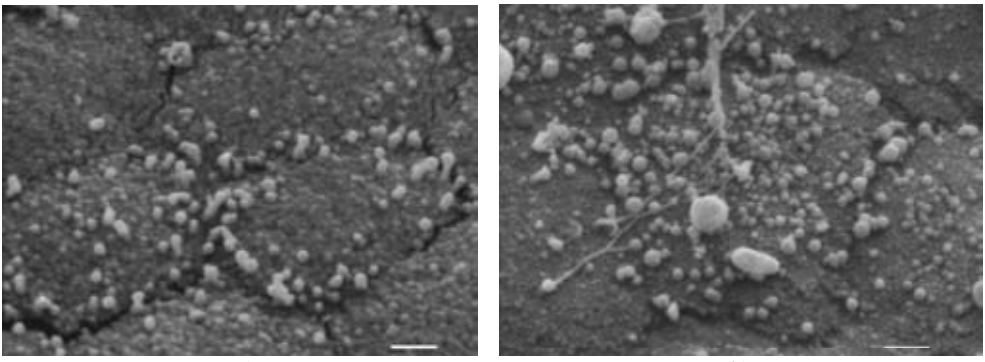
However, when studying the biopsy specimens at a higher magnification, i.e. 4000-10000 x, we found another striking difference between untreated CD and the other groups. The microvilli, which could hardly be seen in treated CD due

to regular shape and an intact mucus layer, appeared in untreated CD swollen, irregular in shape, and heavily elongated (Fig. 18 and 19).



a) b)

Figure 18. Scanning electron micrographs of small intestinal biopsies from a) a child with untreated CD, showing visible microvilli, and b) a child with treated CD, with no visible microvilli. Bars 1 μM.



a) b)

Figure 19. Scanning electron micrographs, at higher magnification, of small intestinal biopsies from two children with untreated CD. In a) the apical microvilli are irregular and some are heavily elongated, and in b) microvilli appear swollen. Bars 1 μM.

Aquaporins (AQP) are channels specific for water and other small non-ionic molecules (Smith 1991) and they are expressed in epithelia and endothelia of organs in which water absorption or excretion is active. A large volume of water

enters everyday through the epithelium of the small and large intestine. Recent findings have demonstrated the presence of several types of AQP members in the small intestine (Hatakeyama 2001, Ishibashi 2002, Parvin 2002, Okada 2003), which implies that the water transporting activity is principally transcellular. It has been suggested that AQPs play a crucial role in cell migration (Loitto 2002, Loitto 2004, Verkman 2005), possibly by causing membrane protrusive activity by directed water flux. Localised membrane protrusions are crucial to numerous cell and tissue functions including cell migration, phagocytosis, formation of cell-cell junctions, and polarisation of the intestinal cell layer. Since water fluxes appear to underlie active alterations in cell shape, our observations imply a severely dysfunctional water transporting activity in the epithelial cell lining in children with CD. Therefore, we will continue this study by investigating how the expression of aquaporin water channels is affected at various stages of CD and how they distribute in tissue in dysfunctional as well as in healthy control mucosa.

CONCLUSIONS

Children with coeliac disease have antibodies against oat proteins at significantly higher levels than reference children. No cross-reactivity between the prolamines of wheat and oats could be demonstrated.

Both anti-avenin antibodies and urinary nitric oxide metabolites declined in coeliac children on a gluten-free diet, whether or not the diet was supplemented with wheat-free oats. However, some children did not normalise their values, indicating that although the majority of children with CD tolerate oats in their diet, there are children who do not. This calls for long-term follow-up studies on CD children having oats in their gluten-free diet.

There was an increased expression of the tight junction protein occludin in untreated CD, which reflects the characteristics of crypt cell hyperplasia and altered barrier properties seen in active CD. The findings also indicate that gluten intake does not significantly influence the expression and distribution of claudins 1-5 in coeliac children. Further studies will be done in order to address whether the findings reflect true differences in occludin expression or if the protein is differently translocated during the acute phase of CD.

Negligible bacteria were found associated with the intestinal epithelium. However, great structural changes were found with enlarged and irregular shaped microvilli in active CD. Further studies will be done, elucidating the role of aquaporin water channels in these ultra structural changes.

ACKNOWLEDGEMENTS

Jag vill rikta ett stort, varmt TACK till alla er som varit inblandade på ett eller annat sätt under den här resans gång! Jag säger som Lennon-McCartney:”I get by with a little help from my friends”. De som jag framför allt vill tacka är:

Karl-Erik Magnusson, huvudhandledare, för att du gav mig möjlighet att syssla med forskning, för att du stöttat mig och trott på mig, och för dina aldrig sinande kunskaper om stort och smått.

Kajsa Holmgren Peterson, bihandledare, rumskamrat, god vän, och vetenskaplig mentor (kärt barn har många namn!), för att du alltid har haft tid att diskutera forskning och även andra livsviktiga saker, för råd, stöd, uppmuntran och omtanke. Utan dig hade detta varit mycket svårare att genomföra, och inte lika roligt!

Karin Fälth-Magnusson, bihandledare, för din glada entusiasm och intellektuella skärpa, och för att du involverade mig i celiakiforskningen.

Tommy Sundqvist, bihandledare, för att du alltid engagerar dig, och för dina otroliga kunskaper om bl a statistik, som mer än en gång satt myror i huvudet på mig.

Lars Stenhammar, Lotta Högberg, Pia Laurin och Ewa Grodzinsky, för medförfattarskap och fruktsamma diskussioner vid våra celiakimöten.

Sköterskorna på barndagvården, US, speciellt *Ann-Louise Karlsson*, för att ni hjälpt mig med handhavandet av biopsierna.

Bengt-Arne Fredriksson, EM-avdelningen, för hjälp med ”sputtring” och allt annat som rör elektronmikroskopiering.

Alla medarbetare på Med mikro för att ni är så hjälpsamma och skapar en sån trevlig stämning. Speciellt vill jag framhålla: *Olle Stendahl*, för att jag har fått utnyttja avdelningens resurser, *Anita Sjö*, för att du kan alla metoder, för att du alltid har tid att svara på frågor och för trevliga diskussioner (utflykter) i vår lilla epitelgrupp, *Charlotte Immerstrand*, för att du är en bra förebild och för att du stöttat och uppmuntrat mig, *Christina Samuelsson*, för att du fixar allt åt oss, för att du håller på våra traditioner och gör det mysigt och trevligt på avdelningen

och för att du är så omtänksam, *Vesa Loitto*, labkamrat och f d rumskamrat, för att du alltid hjälper till och är så ”obehagligt uppriktig”, *Katarina Tejle*, för hjälp med ELISA och cryostat, *Kristina Orselius* för trevligt ”stallsnack” och för hjälp med studentlabbar, *Maggan Lindroth* och *Farideh Tafazoli*, för att ni hjälpte mig igång med elektronmikroskopet, och *Tony Forslund*, för medförfattarskap och för att du är en ”fixare” på avdelningen.

Släktingar och vänner, för att ni bryr er om mig och för att ni stått ut med att bli försakade under alla dessa år.
Jag vill speciellt tacka:

Familjerna *Söderbäck* och *Törnborg*, för att ni är så urtrevliga och får mig att tänka på annat än forskningen; tack också för härliga utflykter till fjälls och till sjöss.

Stallgänget, med *Anki* i spetsen; med er har jag alltid kunnat koppla av och ladda batterierna. Jag saknar det!

Ellert, för att du är en bra svärfar, och för att du hjälpte mig med korrekturläsandet. Jag tänker även på dig, *Sonja*.

Hasse, för att du är världens snällaste och för att du skämmer bort din lillasyster.

Mamma och *pappa*, för att ni stöttat och peppat mig när det har varit tungt, och för att ni hjälper mig och familjen så mycket. Jag vet faktiskt inte hur jag skulle ha fått ihop det utan er!

Mina underbara barn, *John, Karolina* och *Siri*, för att ni finns!

Lasse, min älskade make och bästa vän, utan dig hade jag inte vågat ta ett enda steg. TACK!

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