GUT MICROFLORA ASSOCIATED CHARACTERISTICS IN CHILDREN WITH CELIAC DISEASE

Bo Tjellström

Stockholm 2009
ABSTRACT

Aim
The over-arching aim of this thesis was to study some metabolic functions of the gut microflora in children with known or screening detected celiac disease (CD) and their first-degree relatives.

Materials
Study I. A number of 36 untreated CD children, 47 after at least 3 months on glutenfree diet (GFD) and 42 healthy controls (HC).
Study II. A number of 76 first-degree relatives to CD children and 93 healthy controls (HC).
Study III. A number of 17 screening detected CD children were included to be compared with the untreated children and controls from study I; with exchange of one child in the untreated group, due to low age.
Study IV. A comparative study regarding correlation between iso-forms of short chain fatty acids (SCFAs) in humans as well as in animals.

Methods
Faecal short chain fatty acids were measured in all four studies. Additionally faecal tryptic activity (FTA) was measured in study II.

Major findings
All groups of CD children demonstrated a similar SCFAs profile, i.e. significantly more total SCFAs and acetic acid and a strong tendency to more iso-butyric and iso-valeric acids compared with HC. The first-degree relatives demonstrated another SCFAs profile, i.e. significantly less total SCFAs and acetic acid and significantly more FTA than HC.

Conclusions and future outlook
Based upon the strong similarities between all groups of CD children we are allowing ourselves hypothesising that CD children have a “celiacogenic” flora compared with healthy controls.
In a similar way it can be said that the first-degree relatives are harbouring a “celiaprotective” microflora.
Our findings open up for challenging new diagnostic, therapeutic and prognostic possibilities.

Key words
Celiac disease, children, faeces, microflora associated characteristics, short chain fatty acids, branched-chain fatty acids, iso-butyric acid, iso-valeric acid, relatives, faecal microflora, faecal tryptic activity, screening
LIST OF PUBLICATIONS

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals;


To

………………………………………………

with best regards from the author

………………………………………………

To my family and all our friends
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. INTRODUCTION</td>
<td>7</td>
</tr>
<tr>
<td>Introduction to the disease</td>
<td>7</td>
</tr>
<tr>
<td>Historical perspectives</td>
<td>7</td>
</tr>
<tr>
<td>Epidemiology</td>
<td>8</td>
</tr>
<tr>
<td>Pathogenesis</td>
<td>11</td>
</tr>
<tr>
<td>Genetic factors</td>
<td>12</td>
</tr>
<tr>
<td>Gluten</td>
<td>12</td>
</tr>
<tr>
<td>Immunological mechanisms</td>
<td>13</td>
</tr>
<tr>
<td>2. DIAGNOSIS</td>
<td>15</td>
</tr>
<tr>
<td>The small bowel enteropathy</td>
<td>15</td>
</tr>
<tr>
<td>Small bowel biopsy</td>
<td>18</td>
</tr>
<tr>
<td>Serological markers</td>
<td>18</td>
</tr>
<tr>
<td>Biochemical tests</td>
<td>18</td>
</tr>
<tr>
<td>Symptoms, signs and complications</td>
<td>19</td>
</tr>
<tr>
<td>3. GUT MICROFLORA</td>
<td>20</td>
</tr>
<tr>
<td>Relevant microflora associated characteristics – MACs</td>
<td>21</td>
</tr>
<tr>
<td>Production of short chain fatty acids – SCFAs</td>
<td>21</td>
</tr>
<tr>
<td>Faecal tryptic activity – FTA</td>
<td>22</td>
</tr>
<tr>
<td>Celiac disease and the gastrointestinal microbiota</td>
<td>24</td>
</tr>
<tr>
<td>4. TREATMENT</td>
<td>24</td>
</tr>
<tr>
<td>5. AIMS OF THE THESIS</td>
<td>25</td>
</tr>
<tr>
<td>6. MATERIAL AND METHODS</td>
<td>25</td>
</tr>
<tr>
<td>Subjects</td>
<td>25</td>
</tr>
<tr>
<td>Paper I-IV</td>
<td>25</td>
</tr>
<tr>
<td>Ethical considerations</td>
<td>26</td>
</tr>
<tr>
<td>Determination of microflora associated characteristics – MACs</td>
<td>27</td>
</tr>
<tr>
<td>Short chain fatty acids – SCFAs</td>
<td>27</td>
</tr>
<tr>
<td>Faecal tryptic activity – FTA</td>
<td>27</td>
</tr>
<tr>
<td>HLA analysis</td>
<td>27</td>
</tr>
<tr>
<td>Statistical methods</td>
<td>28</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGA</td>
<td>Anti-Gliadin Antibodies</td>
</tr>
<tr>
<td>AGUS rats</td>
<td>An albino rat strain of long-Evans origin, which was reared under Germfree (GF) conditions at Dept of Germfree Res. Stockholm, Sweden, since 1956. Conventional strains were repeatedly established by transfer of GF animals to conventional surroundings.</td>
</tr>
<tr>
<td>CD</td>
<td>Celiac Disease</td>
</tr>
<tr>
<td>DGP</td>
<td>Deamidated Gliadin Antibodies</td>
</tr>
<tr>
<td>EMA</td>
<td>Endomysium Antibodies</td>
</tr>
<tr>
<td>FTA</td>
<td>Faecal Tryptic Activity</td>
</tr>
<tr>
<td>GFD</td>
<td>Gluten-Free Diet</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy Controls</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>IEL</td>
<td>Intragpithelial Lymphocytes</td>
</tr>
<tr>
<td>MAC</td>
<td>Microflora Associated Characteristics</td>
</tr>
<tr>
<td>ME</td>
<td>Milieu Exterieur</td>
</tr>
<tr>
<td>MI</td>
<td>Milieu Interieur</td>
</tr>
<tr>
<td>MT</td>
<td>Milieu Totale</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>PCR-SSP</td>
<td>Polymerase Chain Reaction – Sequence Specific Primers</td>
</tr>
<tr>
<td>SCFAs</td>
<td>Short Chain Fatty Acids</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>tTG</td>
<td>Tissue Transglutaminase</td>
</tr>
<tr>
<td>TGA</td>
<td>Transglutaminase Antibodies</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Introduction to the disease

Celiac disease (CD) or gluten-induced enteropathy is next to allergy the most common chronic disorder in children. At the same time CD is a food intolerance and an autoimmune disorder. CD is caused by a complex interplay between many genes and environment. In genetically predisposed individuals dietary ingestion of gluten causes a chronic inflammation in the proximal small bowel mucosa, leading to loss of the normal intestinal barrier function and integrity. There are histopathologic changes in the mucosa starting with an increased number of intraepithelial T lymphocytes (IEL) proceeding to villous atrophy. The morphological changes of the mucosa result in impaired function with decreased absorption of nutrients and a range from no or few clinical symptoms of disease to extensive malabsorption. On treatment with gluten-free diet (GFD) the mucosa usually heals and the patient becomes symptom free. Untreated or insufficiently treated CD is associated with numerous complications. (For reviews see Kagnoff, 2007; MacDonald, 2005).

In patients with CD, the function of the gut flora and its potential connection with or influence on the disease can be studied from a pathogenic, diagnostic or therapeutic point of view. In the present studies we assessed biochemical methods in an attempt to evaluate the function of the intestinal bacterial flora in children with CD, first degree relatives of children with CD and children with screening detected CD. We also investigated the correlation between two iso-acids, i-butyric and i-valeric acid, in faeces from some different mammalian species including humans.

Historical perspectives

Some 10,000 years ago the cultivation of wheat and other cereals began, when the gathering and hunting man gradually became the agricultural man. This led to exposure to alimentary products, cereals with increasing gluten content, not likely presented before to humans in their 2-3 million year old history (Greco, 1997; Guandalini, 2008). The Greek physician Areataeus the Cappadocian is believed to be the author of the first description of CD. In the first century AD he described a form of “coeliac diathesis” (Walker-Smith, 1988). Interestingly, Areataeus was the first to describe not only CD but also diabetes mellitus, two conditions we now know are associated with the same HLA-types. Many centuries later, in 1887, the British paediatrician Sir
Samuel Gee gave a lecture “On the coeliac affection” at the Hospital for Sick Children at Great Ormond Street in London (Gee, 1888). This lecture was published in 1888 and is considered to be the first publication on CD in modern times (Walker-Smith, 1988).

The next milestone in the history of CD is 1950, when the Dutch paediatrician WK Dicke reported his epoch-making discovery that gluten is the substance that causes the harm of CD. His findings were published in Dutch in 1950 and 1953 in English (Dicke WK, 1950; Van de Kamer et al., 1953; Pena, 1991; Van Berge-Henegouwen & Mulder, 1993). A few years later the oral small bowel biopsy technique made it possible to demonstrate the celiac enteropathy in live patients. Indeed, the development of small bowel biopsy is of fundamental importance and represents the birth of paediatric gastroenterology as a subspeciality within paediatrics (Walker-Smith, 2003) and the formation of the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN), today called the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN). In 1970 ESPGAN published criteria for the diagnosis of CD, which have been to the guidance of clinicians and scientists both within paediatrics and adult gastroenterology (Meeuwisse, 1970). CD has been regarded as an autoimmune disease since Dieterich and her group in 1997 discovered that the enzyme tissue transglutaminase is the autoantigen (Dieterich et al., 1997).

Epidemiology

Formerly looked upon as a rare and in most cases transient childhood disease, CD is now recognized as one of the most common chronic diseases in children and adults in Europe and the US. The prevalence of the disease is generally believed to be 1% (Fasano A et al., 2003; Dubé et al., 2005). However, many children with CD go undiagnosed (Ravikumara et al., 2007). This is illustrated by a recent Swedish multicenter screening study, that revealed CD in 3% of 12-year-old schoolchildren, of whom one third of the cases was diagnosed before the screening (Myléus et al., 2009). Interestingly, a Swedish screening study, that has attracted little attention, found a prevalence of CD of 1.9% in the adult general population (Borch et al., 2001).

The prevalence of CD is increasing in the Swedish child population (Olsson et al., 2008) as well as in Finnish adults (Lohi et al., 2007). Moreover, in Finland the rising trend in CD moves parallel with that in type 1 diabetes. These changes cannot be explained by a higher alertness of
clinically working doctors but must reflect a real rise in the prevalence of the diseases, due to factors not yet known.

The epidemiology of childhood CD in Sweden has attracted special interest due to dramatic incidence changes over the past decades (Fig. 1; modified from Ivarsson, 2000; 2002). In 1989 the Swedish Working Group for Childhood Coeliac Disease was formed by the Section for Gastroenterology, Hepatology and Nutrition within the Swedish Paediatric Association. The prime task was to study the incidence of the disease. A national Swedish Childhood Coeliac Disease Register was started. New detected cases of CD in children younger than 15 years of age are reported to the register by local paediatricians. This unique register gives invaluable information on the incidence of CD in children and forms the basis for epidemiologic research (Ivarsson, 2005; Olsson et al., 2008; Olsson et al., 2009). The incidence changes have been abrupt and almost “epidemic” (Fig. 1) with an onset from year 1983 when the time for introduction of cereals was changed. Accordingly, when the regimen was changed again in 1995, the number of cases per year returned to “normal”. Incidentally, the decrease occurred before the official recommendation was taken by the paediatricians in Sweden, probably due to an early public awareness of adverse effects of gluten. This was evident in the youngest age-group (0-1.9 years; line marked with stars in Fig. 1; modified from Ivarsson, 2000; 2002). These unexpected, short-term incidence fluctuations in children under 2 years of age, strongly suggest that interventional as well as environmental factors may operate. Preceding the sharp increase in the mid 1980s, the Swedish Paediatric Association had recommended postponing the introduction of gluten to infants from 4 to 6 months of age (Ivarsson et al., 2000). This recommendation was not strictly evidence based, but rather an expectation to reduce the prevalence of CD in young children. Coincidently, the gluten content of widely used infant feeding products was doubled due to reduction of milk content and use of cereals with a higher percentage of gluten (Olsson et al., 2008).
Figure 1. Three curves showing the age (years) at diagnosis of celiac disease (CD). In Sweden, the first change of recommendation for gluten introduction from 4 to 6 months of age, took place in 1983. It was followed by the sharply increased incidence in the youngest group, 0-1.9 years (line marked with stars). The arrow indicates the year for changed recommendations, back to gluten introduction at 4 months of age, in 1995. (Modified from Ivarsson, 2000; 2002).

By the courtesy of A. Ivarsson, Umeå, 2009
Thus, the amount of gluten consumed by Swedish infants and the time of gluten introduction into the diet has changed considerably over the past decades. These factors have been claimed to be responsible for about 50% of the “Swedish epidemic” of CD (Fig. 1; modified from Ivarsson, 2000; 2002). Other pathogenetic factors of importance for childhood CD are length of breast-feeding (Fälth-Magnusson et al., 1996; Peters et al., 2001; Ivarsson et al., 2002; Akobeng et al., 2006) and intercurrent infections (Ivarsson, 2003).

The major finding of the “Swedish epidemic” of CD in young children is that prolonged breast-feeding and cautious introduction of gluten, when the baby is still breast-fed, seems to reduce the risk of developing CD (Ivarsson, 2005). Whether a postponement of gluten introduction until after 1 year of age would further decrease the risk of CD is the object of an ongoing Italian study (Catassi, personal communication). Theoretically, the risk of CD might be less after infancy due to a more mature small intestinal function.

Girls represent some 2/3 of the childhood CD population (Ivarsson et al., 2003). There is no explanation of this difference between the sexes, which interestingly is not noted in screening-detected cases of CD (Myléus et al., 2009).

Pathogenesis

Over the years several pathogenetic explanation models have been proposed. Originally it was hypothesised that CD was a state of enzyme deficiency, i.e. a type of inborn error of metabolism, with shortage of one or more specific enzymes responsible for detoxifying gluten or gliadin. However, no such enzymes could ever be demonstrated. It soon became obvious that this was a too simple explanation. As a result of the enormous progress of immunology, CD is now being looked upon as a mismatch between the patient’s genetic make-up and ingested gluten. This leads to a complicated mucosal immune response with breakage of the gut barrier defence, ultimately resulting in a diffuse and complex clinical picture with more or less obvious symptoms from several organs in the body.
Genetic factors

It is well-known that CD is a familial disease. Thus, the prevalence of CD is approximately 10% among first-degree relatives (i.e. parents, siblings and children) of patients with CD (Högberg et al., 2003). Furthermore, the concordance rate is 75% in monozygotic twins compared with 11% in dizygotic twins (Greco et al., 2002).

It is also well-known that CD is a complex genetic disorder (Wolters & Wijmenga, 2008). There is a strong association to HLA-genes. Thus, more than 90% of European CD patients are DQ2 positive compared to 20 – 30% in the general population (Sollid et al., 2000; Louka et al., 2003). Most DQ2-negative celiacs are DQ8-positive. Absence of DQ2/DQ8 has thus a negative predictive value of more than 95% in a patient with suspected CD (Karell et al., 2003).

The contribution of the HLA genes to the familial risk of CD has been estimated to 40% (Petronzelli et al., 1997; Bevan et al., 1999). Thus, non-HLA linked genes may be a stronger determinant of the CD genetic susceptibility. Several such genes conferring risk of CD have been identified. There are numerous studies in the literature on the analysis of non-HLA genes in CD. However, various and partly contradictory reports have been published. Hopefully, more consistent results will in the future make genetic analysis usable in the clinical practice (Liu E et al., 2005).

Gluten

Gluten is a complex protein component of wheat, rye and barley. It can be extracted from a dough after water-soluble components are washed out. Gluten is traditionally divided into the ethanol-soluble fraction gliadin and the ethanol-insoluble remainder glutenin, both of which can be toxic to celiac intestinal mucosa. The gluten content, and thus the toxicity to celiacs, was low in ancient bread wheats compared to modern, cultivated grains (Molberg et al., 2005; Pizzuti et al., 2006). This may explain why a Pompeian family of 3 persons could consume 4 kg of bread daily (Catassi, personal communication). Today the consumption of wheat is increasing all over the Western World.
Immunological mechanisms

CD is a unique disease being at the same time a food intolerance and a model of autoimmunity. Thus, CD is the result of three factors:

i) a strong association with certain HLA types,

ii) an immune response against the enzyme tissue transglutaminase, and

iii) gliadin as the environmental triggering factor.

This initiates a complex interplay between the innate and adaptive immunity systems leading to a process, which damages the enterocytes and ultimately breaks the intestinal barrier function (MacDonald & Monteleone., 2005, see Fig. 2). According to the current prevailing pathogenetic hypothesis one of the main features of CD is a leakage of the tight junctions between the enterocytes (Fasano, 2008). The abnormal small intestinal mucosal permeability can be studied using various test molecules (Stenhammar et al, 1989). Moreover, a genetic predisposition for a gut barrier defect has been reported in CD and ulcerative colitis (Wapenaar et al., 2008). In addition to a genetically determined altered permeability, several factors can have a similar effect, e.g. pro-inflammatory cytokines (Bruewer et al., 2003) and intestinal infections (Berkes et al., 2003; MacDonald & Monteleone., 2005, see Fig. 2). For more details the reader is referred to recent reviews on the intestinal barrier regulation (Liu Z et al., 2005; Turner, 2006; Fasano, 2008; Meddings, 2008).

Although much remains to be explained in the pathogenesis of CD, it is now widely recognized that fragments of gliadin pass the intestinal epithelial cell layer, primarily through leaky intercellular tight junctions but also transcellularly, and reach the lamina propria (Kagnoff, 2007). Gluten peptides may also be collected from the gut lumen by subepithelial dendritic cell processes penetrating between the enterocytes. Interestingly, mice depleted in dendritic cells rapidly develop devastating autoimmunity (Ohnmacht et al., 2009).

Gluten consists of peptides with a high content of proline and glutamine, so called prolamins. They are substrate for deamidation by tissue transglutaminase, which makes them negatively charged with a high affinity for DQ2 and DQ8 molecules. After deamidation by tissue transglutaminase the gliadin peptides bind to HLA DQ2 or DQ8 receptors on antigen presenting cells and are presented to CD4+ T-cells, which orchestrate the immune response. Pro-inflammatory cytokines of Th1-type and chemokines are produced leading to enteropathy starting with infiltration of IEL and successively damage to the enterocytes. In addition, NO is produced through induction of inducible nitric oxide synthase (iNOS) (Holmgren Peterson et al., 1998; Daniels et al., 2005) and B-cells produce antibodies to various antigens, including AGA, EMA and TGA.
Figure 2. Intestinal mucosa (MacDonald TT et al., Science 2005).

Printed by permission of Science.
In conclusion, CD has a multifactorial pathogenesis with an impaired intestinal barrier function leading to a miscommunication between the innate and adaptive immune systems starting early in life (Forsberg, 2006). Growing evidence suggests that the defect gut barrier in CD is a cause rather than a consequence of the disease. Still many questions remain to be answered, e.g.

- why do not all HLA DQ2/DQ8-positive individuals develop CD;
- where does the deamidation of gluten by TG2 take place;
- how are the TG2 autoantibodies formed and what are the role of these antibodies;
- is the initial event taking place in the epithelium or lamina propria?

2. DIAGNOSIS

The small bowel enteropathy

The CD enteropathy was traditionally believed to be continuously distributed in the proximal small bowel (Walker-Smith & Murch, 1999). However, it is well documented that the celiac enteropathy may have a patchy distribution in the upper small bowel (Scott & Losowski, 1976; Bonamico et al., 2004) or even be confined only to the bulbus duodeni (Kappinen et al., 2004; Bonamico et al., 2008), which has important implications regarding how to take the mucosal biopsy. Interestingly, CD may affect not only the proximal small bowel since increased IEL density in the terminal ileum has been reported in celiac patients with duodenal villous atrophy (Dickey & Hughes, 2004). The increased number of IELs is believed to be the initial phase of the celiac enteropathy detectable by light microscopy and mirrors the inflammation of the lamina propria, which is the seat of the mucosal immunological response in CD (Ferguson & Murray, 1971). Thus, it is of interest to count the number of IELs in the mucosal biopsy specimen. Various results have been published. Today counts >20 – 30 IELs per 100 enterocytes are considered to be a marker of mucosal inflammation in children (Grant et al., 2008) and adults (Veress et al., 2004). CD3 immunohistological staining may facilitate the evaluation of the biopsy specimen (Fig. 3).
The small bowel enteropathy in CD is today widely classified according to Marsh (Marsh, 1992), modified by Oberhuber (Oberhuber et al., 1999; Dickson et al., 2006). The light-microscopical mucosal changes describe a dynamic progression: increased number of IEL count (Marsh 1); crypt hyperplasia (Marsh 2); various stages of villous atrophy (Marsh 3 a-c) (Fig. 3).

In addition to the spectrum of small intestinal morphological changes in CD, it is well documented that some patients may lack enteropathy detectable with conventional light microscopy (Sbarbati et al., 2003). In such cases electron microscopy may reveal microvillous lesions (Dickson et al., 2006).
Figure 3.

Normal small intestinal mucosa
Partial villous atrophy

CD3 stain normal mucosa
Total villous atrophy

CD3 staining - Partial villous

By the courtesy of Britta Halvarsson, Malmö, 2009.
**Small bowel biopsy**

Although today questioned as “the gold standard”, the small bowel biopsy is still essential in the diagnosis of CD in children. The oral capsule technique is in many clinics replaced by endoscopic procedure, permitting several mucosal biopsies being taken under ocular inspection. Both European and North American paediatric gastroenterologists recommend demonstration of a small bowel enteropathy for a safe and reliable diagnosis of CD (Fasano et al., 2008).

**Serological markers**

The finding of increased serological celiac markers (anti-gliadin antibodies, AGA; endomysium antibodies, EMA; transglutaminase antibodies, TGA) supports the diagnosis but cannot replace the small bowel biopsy, although some researchers question the need for biopsy in children with very high antibody titers (Barker et al., 2005; Donaldson et al., 2008). According to the Swedish working group for childhood celiac disease a diagnosis of CD can be safely made in a child with:

- symptoms or signs of malabsorption
- increased celiac serology markers
- a small bowel biopsy showing enteropathy consistent with CD
- a positive clinical response to GFD

(Danielsson et al., 1998; Stenhammar et al., 2006).

Today TGA is the most used celiac serology test. However, AGA is probably still the best test in infants and young children (Grodzinsky et al., 1995; Lagerqvist et al., 2008). The recently introduced deamidated gliadin antibody test seems promising but needs further evaluation (Agardh, 2007; Kaukinen et al., 2007; Korponay-Szabó et al., 2008).

**Biochemical tests**

A recent approach has shown that $^{13}$C-xylose and $^{14}$C-xylose breath tests can be used for the diagnosis of celiac disease in adults (Tveito et al., 2008). Tveito et al. present even more promising results in a study on $^{13}$C-sorbitol breath test (Tveito et al., 2009).
Symptoms, signs and complications

The typical symptoms of CD in young children are often obvious and include gastrointestinal symptoms, poor weight gain, thin extremities and a protruding abdomen (Fasano, 2005). Older children may present with recurrent abdominal pain or constipation. In teen-agers the symptoms are often more subtle with anaemia (Mody et al., 2003), late puberty or short stature (Stenhammar et al., 1986). Some cases of childhood CD may present with an acute onset of diarrhoea, dehydration and severely affected general condition, so called celiac crisis (Mones et al., 2007). This severe condition may be induced by certain gastrointestinal virus infections.

From screening studies we know that CD can be more or less asymptomatic. The “ice-berg” has been used as a metaphor for the disease in genetically predisposed individuals: clinically overt cases are above the water line and asymptomatic individuals with silent or latent disease form the submerged part of the iceberg (Logan, 1992; Fasano, 2005). Only screening studies can give a reliable estimate of the whole iceberg (Mulder & Bartelsman, 2005).

Interestingly, symptoms and signs of childhood CD seem to have changed over the past decades with milder or more non-specific symptoms. This was first reported from Finland (Mäki et al., 1988) and later from Sweden (Ludvigsson et al., 2004) and UK (Ravikumara et al., 2006; Rodrigues & Jenkins, 2008). The reason for the changing clinical presentation is basically unknown.

In some cases CD is associated with other autoimmune diseases, including type 1 diabetes, thyroid disease and Addison disease, as well as with chromosomal aberrations, such as Down’s, Turner’s and Williams syndrome. There are some indications that untreated CD might increase the risk of autoimmune complications (Ventura et al., 1999), although contradicted by others (Sategna Guidetti et al., 2001). Long-term complications to CD are reduced bone density (Jatla et al., 2009, Agardh et al., 2009), infertility (Collin et al., 1996; Pellicano et al., 2007) and mood disorders (Dewar et al., 2005; Pynnonen et al., 2002). Previous studies have indicated an over-risk for the development of malignant disease in CD (Keating, 1984). However, recent studies show that this association is weak. (Olén, 2008; Elfström, 2009).

Refractory CD is defined as persistence of small bowel enteropathy in spite of GFD for at least 6 months. The condition can be classified into type I with normal IEL and type II with abnormal
IELs. In type II the aberrant IELs may expand resulting in enteropathy associated T-cell lymphoma. This is a very serious complication with a high mortality (Malamut et al., 2009). The mechanism behind this complication is virtually unknown.

3. GUT MICROFLORA

The flora of the adult human body and all other mammals form an extremely complicated ecosystem, exceeding more than 1500 species and about 1000 species live in the intestinal canal. There are at least $10^{13}$-$10^{14}$ bacteria in the microflora of the colon and maybe 1000 times more bacteria in the gut than there are cells in the body.

Soon after birth, the gastrointestinal (GI) tract is colonised with bacteria and complex populations are established successively. The first bacteria to colonise have plenty of room and nutrition. These bacteria are aerobes or facultative anaerobes (S. aureus, E. coli and others) which consume oxygen present, inviting the more obligate anaerobes, especially Bifidobacteria, to be established and soon become the dominating part of the flora, as long as the baby is breast fed. About 99% of the normal gut flora are anaerobes (Wilson, 2005; Ouwehand, Vaughan., 2006; Benno, 2006).

In all mammals throughout life there is in all compartments of the alimentary tract a complex interplay – often called cross-talks – between the three major actors i.e the host, its microflora and environmental factors as diet, drugs etc. These cross-talks are forming structures and functions in all compartments. Over the years, the microbial influences have been worked out by comparative studies in conventional (CONV=organisms harbouring a conventional microflora) and germfree (GF=organisms devoid any microflora) animals. Applying a slight travesty of the well-known terminology introduced by Claude Bernard, the mammalian organism itself is defined as the Milieu Intérieur (MI), its microflora as Milieu Extérieur (ME), and the two together as Milieu Totale (MT) (Midtvedt, 1989).

In order to study the host parameters related to the colonisation of microbes and the microbial influences on the host, the GAC/MAC-concept have been found useful.
A MAC (Microflora Associated Characteristic) has been defined as the recording of any anatomical structure, physiological, biochemical or immunological function in an organism that has been influenced by the microflora (Midtvedt et al., 1985). When microbes that actually influence the parameter under study are absent, as in germfree animals as well as in newborn individuals, and sometimes in individuals receiving antimicrobial treatment, this particular recording is defined as a Germfree Animal Characteristic, GAC. Consequently a MI represents the sum of all GACs and MT represent a sum of MACs. In table 1 is shown a list of some biochemical MAC/GACs.

There are several factors indicating a role for infections in the pathogenesis of CD (Sollid & Gray, 2004). It is a well-known clinical assumption, that the presentation of CD in children often is preceded by an infection. This might theoretically lead to altered intestinal function (Tlaskalova-Hogenova et al., 2005; Drago et al., 2006) including the intestinal absorption of nutrients (Holm et al., 1992).

**Relevant MACs studied**

*Production of short chain fatty acids (SCFAs)*

In the alimentary tract, SCFAs are produced by microbial fermentative breakdown of endogenous and exogenous compounds, mainly complex carbohydrates, but to a certain extent, also proteins. Faecal SCFAs represent a complex interplay between the type and amounts of consumed dietary compounds, host-related enzymes, the microflora presented in various compartments and its capability to perform other chemical processes as production of other organic acids, various alcohols, methane, hydrogen gas and probably also hydrogen sulfide etc, the anaerobic conditions in the different intestinal compartments as well as absorption of the SCFAs produced.

These complex processes can be simplified as follows. At birth, meconium contains only tiny amounts of acetic acid and this acid represent the prominent SCFAs as long as breast milk is the major source of diet (Midtvedt AC, 1994). The situation reflects the enzymatic capability of the dominant flora. The major fermentative product of bifidobacteria and lactobacilli, i.e. the two dominating groups of microbes in breast-fed babies, is lactic acid, and only to a little extend, acetic acid. When the baby is exposed to food other than breast milk increasing amounts of longer SCFAs than acetic acid can be found. The SCFAs “profile” reflects partly type and amounts of dietary compounds, partly alterations in the composition of the GI microflora. At
two years of age, the SCFAs profile in healthy children are similar to that found in adults (Midtvedt AC, 1994) and the composition of the microflora has changed accordingly. Bifidobacteria constitute a minor part, whereas strict anaerobes, as Bacteroides and Eubacterium species, clostridia and others are dominating. It has to be kept in mind that the basic product of microbial breakdown of complex carbohydrates is acetic acid, yielding an excess of electrons to get rid of. In order to keep fermentation going, the microbes can utilise several ways. In the absence of oxygen, i.e. the physiological status in the intestinal lumen, they can, by anabolic processes, produce longer SCFAs, which can be utilised by the host (Roedinger W EW, Moore A. 1981). Shortly spoken, this is a win/win situation for both parts. The microbes can continue their metabolism and the host get access to energy-rich SCFAs. It has to be mentioned that another way to get rid of excess of electrons is formation of other compounds, especially methane and hydrogen gas. However, none of these microbial products have been investigated in patients with CD and consequently, they will not be discussed any further. SCFAs represent major anions in intestinal content and significant deviations from a normal SCFAs profile may represent considerable alterations in the local microclimate. Possible physiological and patho-physiological consequences of such alterations are poorly understood and often neglected

**Faecal tryptic activity (FTA)**

In response to food intake, inactive trypsinogen is secreted by the pancreas gland, together with trypsin inactivator, into the small intestine, where it is activated to trypsin by enterokinase or by active trypsin already present. FTA is the net sum of (i) pancreatic secretion of trypsinogen and trypsin inactivator, (ii) activation, as already mentioned above (iii) inactivation/degradation of trypsin and/or trypsin inactivator by microbial enzymes and exogenous products (Norin, 1985). Microbes responsible for this inactivation/degradation of trypsin seem to be located within the Bacteroides group (Ramare et al., 1996). It might be mentioned that so far, other mammals than man harbouring a normal intestinal flora, which demonstrate zero level of FTA, whereas GF animals always demonstrate high levels of FTA. (Norin et al., 1985, 1986). The mechanism(s) or reason(s) for this remarkable human feature is (are) to the best of our knowledge, still not elucidated.

The daily pancreatic production of trypsin in human adults has been estimated to 1 to 3 g (Kuknal J et al., 1965). To simplify, assuming a daily pancreatic output of 2 gram and a fluid passage through duodenum of 10 l/day, giving 200 mg/kg of tryptic activity, i.e. close to the
values found in faeces from CD relatives. However, assuming a daily output of 100 g faeces, it can be calculated that only 10% of pancreatic trypsin is excreted in its active form. The remaining is either microbial degraded or inactivated by trypsin inhibitors.

Table 1. Influence of the microflora on certain major anatomical, physiological and biochemical parameters of the intestine.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MAC*</th>
<th>GAC*</th>
<th>Microbes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anatomical/physiological</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal wall</td>
<td>Thicker</td>
<td>Thinner</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cell kinetics</td>
<td>Fast</td>
<td>Slower</td>
<td>Unknown</td>
</tr>
<tr>
<td>Migration motor</td>
<td>Normal</td>
<td>Fewer</td>
<td>Unknown</td>
</tr>
<tr>
<td>Size of the caecum</td>
<td>Normal</td>
<td>Enlarged</td>
<td>Partly unknown</td>
</tr>
<tr>
<td>Oxygen tension</td>
<td>Low</td>
<td>As high as in tissues</td>
<td>Several species</td>
</tr>
<tr>
<td>Redox potential (mV)</td>
<td>Low (&lt; -100)</td>
<td>High (&gt;0)</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Biochemical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile acid metabolism</td>
<td>Deconjugation</td>
<td>No deconjugation</td>
<td>Several species</td>
</tr>
<tr>
<td></td>
<td>Dehydrogenation</td>
<td>No dehydrogenation</td>
<td>Many species</td>
</tr>
<tr>
<td></td>
<td>Dehydroxylation</td>
<td>No dehydroxylation</td>
<td>Few species</td>
</tr>
<tr>
<td>Bilirubin metabolism</td>
<td>Deconjugation</td>
<td>Little deconjugation</td>
<td>Many species</td>
</tr>
<tr>
<td></td>
<td>Formation of urobilin</td>
<td>No formation of urobilin</td>
<td>One species</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Formation of coprostanol</td>
<td>No formation of coprostanol</td>
<td>Few species</td>
</tr>
<tr>
<td>Intestinal formed gases</td>
<td>Carbon dioxide</td>
<td>Some carbon dioxide</td>
<td>Many species</td>
</tr>
<tr>
<td></td>
<td>Hydrogen</td>
<td>No hydrogen</td>
<td>Some species</td>
</tr>
<tr>
<td></td>
<td>Methane</td>
<td>No methane</td>
<td>Few species</td>
</tr>
<tr>
<td>Mucin metabolism</td>
<td>Degradation</td>
<td>No degradation</td>
<td>Many species</td>
</tr>
<tr>
<td>Tryptic activity</td>
<td>Little or none</td>
<td>High activity</td>
<td>Few species</td>
</tr>
<tr>
<td>Beta-aspartylglycine</td>
<td>None</td>
<td>Present</td>
<td>Little known</td>
</tr>
<tr>
<td>Formation of SCFAs</td>
<td>Large amounts</td>
<td>Far less**</td>
<td>Many species</td>
</tr>
</tbody>
</table>

* these Microflora Associated Characteristic (MAC) and Germfree Animal Characteristic (GAC), values are adapted from T Midtvedt (1999).

** mainly acetic acid from the diet
Celiac disease and the GI microbiota

As stated earlier, CD might be diagnosed following enteric infections. However, causal relationship remains to be established (Welander., 2009). Some few years ago, a Swedish group showed that rod-shaped bacteria were attached to the small intestinal epithelium in around 1/3 of CD children, but not to the epithelium of healthy controls (Forsberg et al., 2004). This unexpected and thought-provoking finding was commented upon in an Editorial, stating that the study raised more questions than it answered, and that “it will be essential verifying that bacteria may play a role in celiac disease (Sollid & Gray, 2004). To the best of our knowledge, Forsberg et al. have, so far not characterised the rod-shaped microbes down to species level, thus making it difficult for other groups to verify their findings. However, the mere fact that alteration in the intestinal microbiota might be found in celiac patients have prompted many new investigations, as demonstrated in a recent CD symposium in Amsterdam, April 2008. One group from Australia presented data “thought to be the first known study assessing the commensal bacterial counts in patients with CD”. They showed significant differences in the amount of 7 microbial groups, in CD patients and healthy controls (Harnett, 2009). The results of another study, indicate that “virulence features of the enteric microbiota are linked to celiac disease”. Altogether in the Amsterdam congress, there were several posters about CD and the composition of the faecal flora and some posters about the finding of bacterial antibodies but no studies on the function of the gut flora were presented.

4. TREATMENT
The only effective treatment of CD is GFD, i.e. a diet free of wheat, rye and barley. Oats were traditionally excluded from the GFD. Recent studies indicate that patients with CD tolerate pure oats (Högberg et al., 2004). However, a few celiacs may have avenin-reactive mucosal T-cells and thus be intolerant to oats (Arentz-Hansen et al., 2004). It is generally believed that the GFD must be strict. A recent systematic review of the literature suggests that a daily gluten intake of <10 mg is unlikely to cause intestinal mucosal abnormalities (Akobeng et al., 2008). The GFD is recommended for both symptomatic and asymptomatic coeliac individuals (Polanco, 2008) for optimal health and minimal risk of complications (Haines et al., 2008, Pynnönen et al., 2005). It is obvious, that adherence to a strict GFD must be difficult, not only for children and teen-agers with CD but also for adults. This is well-documented in the literature (Hallert et al., 1998; Hörnell, 2008; Wagner et al., 2008).
5. AIMS OF THE THESIS

The overall goal of this thesis was to study the metabolic function of gut microflora by means of analysing the faecal SCFA pattern using the MAC concept.

The specific aims were to investigate:

*study I:* Children with untreated CD and children with CD on GFD to see if there are differences between the groups, indicating a deviant gut microflora function.

*study II:* Healthy first-degree relatives of children with CD to see if there are differences between the groups indicating a deviant gut microflora function. In addition we analysed the faecal tryptic activity (FTA) in this study.

*study III:* Screening detected children with CD to see if there are differences between the groups indicating a deviant gut microflora function.

*study IV:* The correlation between faecal iso-butyric and iso-valeric acids in different species to see if these acids reflect a deviant gut flora function.

6. MATERIAL AND METHODS

**Subjects**

*Paper I*

Faecal samples from thirty-six children (12 boys/24 girls; median age 4.7 years, range 0.7 – 9.9 years) with CD were studied at presentation when still on a normal gluten-containing diet. They all had symptoms and signs indicative for CD, positive celiac serology markers and small bowel biopsy showing severe enteropathy, consistent with CD. Forty-seven celiac children (21 boys/26 girls; median age 4.2 years, range 1.1 – 10.3 years) were studied when they had been on GFD for at least 3 months.

For comparison, faecal samples from 42 healthy children (23 boys/19 girls; median age 3.0 years, range 0.3 – 5.8 years) were investigated.

*Paper II*

Faecal samples from seventy-six first degree relatives (26 fathers, 28 mothers, 9 brothers, 12 sisters, 1 son) (median age 42 years, range 12 – 76 years) to 34 index children (11 boys/23 girls;
median age 3.1 years at diagnosis; 30 HLA DQ2-positive, 3 HLA DQ-negative, 1 no HLA-
typing available) with CD were studied. All first-degree relatives studied were on a normal
gluten-containing diet, had no symptoms of CD or other chronic disease and had normal serum
IgA, EMA or TGA. In 48 out of the 76 relatives small bowel biopsy had been performed, in a
previous study, showing no light-microscopical changes indicative of CD.
For comparison, faecal samples from 93 healthy controls were studied (Siigur et al., 1994).

**Paper III**
Seventeen children (8 boys/9 girls; median age 12 years, all born in 1993) with small bowel
biopsy verified CD were included in the study. The children had been detected in a screening-
study using anti-human tissue transglutaminase of isotypes IgA and IgG and total serum IgA.
All children had increased antibody levels. Faecal sample was not available from one child and
faecal material was too little to permit analysis in one case. Faecal samples from the remaining
15 children were studied.
For comparison we used faecal samples from 36 children (12 boys/24 girls; median age 4.7
years, range 0.7 – 10 years) with symptomatic CD at presentation and 42 healthy children (23
boys/19 girls; median age 3.0 years, range 0.3 – 5.8 years) (*Paper I*).

**Paper IV**
Faecal samples from 42 healthy children (23 children aged 0-3 years and 19 children
aged >3- 6 years (*Paper I*), 93 healthy adult controls (Siigur et al, 1994), rats (48 Wistar rats
and 41 AGUS rats) (Collinder et al., 2000), horses (10 competing Standardbreds, 8 retired
Standardbreds, 25 sport horses) and pigs (16 cohorts, each with 5 – 21 individuals) (Collinder
et al., 2002) were studied.

**Ethical considerations**
The studies in Paper I, II and III were approved by the Research Ethics Committee of Linköping
University, Linköping, Sweden. In addition, the study in Paper III was approved by the
Regional Ethical Review Board at Umeå University, Umeå, Sweden. Informed consent was
obtained from all participating families. The local ethical committees in Stockholm and
Uppsala, Sweden, approved study IV.
Determination of microflora associated characteristics (MACs)

Short chain fatty acids - SCFAs
The faecal samples were frozen within 20 minutes of passage and stored at -20°C pending analysis.

The faecal material was homogenised after addition of distilled water containing 3 mmol/l of 2-ethylbutyric acid (acting as an internal standard) and H2SO4 (0.5 mmol/l). Two millilitres of the homogenate was vacuum-distilled, according to the method used by Zijlstra et al.1977, modified by Höverstad et al.1984. The distillate was analysed with gas liquid chromatography using flame ionisation detection and quantified using internal standardisation. The following SCFAs were analysed: acetic acid; propionic acid; i-butyric acid; n-butyric acid; i-valeric acid; n-valeric acid; i-caproic acid; and n-caproic acid. The results were expressed in mmol/kg wet weight. The relative distribution of the fatty acids was calculated as a percentage of the total SCFA concentration.

Faecal tryptic activity - FTA
Faecal samples were homogenised with saline (1:2) and centrifuged at 5000 rpm for 30 min. Aliquots of 0.1 ml of the supernatant was added to 2.9 ml Tris buffer, pH 8.2, containing 4.4 g/l calcium chloride (Norin E., 1986). After that, 0.6 ml 0.0003 M BAPNA (N-benzoyl-DL-arginine-4-nitroanilide hydrochloride) was added. The reaction was performed at room temperature and was stopped after 10 minutes by adding 0.6 ml 5 M acetic acid. Bovine pancreas trypsin type III diluted in 2 mM hydrochloric acid was used for construction of the standard curve. All samples and standards were analysed spectrophotometrically in parallel with blanks at 405 nm. After correction for blank values, FTA was calculated and expressed as mg trypsic activity/kg faeces.

HLA analysis
Most index children and family members of Study II were genotyped at HLA-DQ for the A1*0501 and B1*0201 alleles by polymerase chain reaction-sequence specific primers (PCR-SSP) (Olerup et al., 1993).
Statistical methods

**Paper I**
The statistical evaluation was performed by Student’s t-test. A $p$ value < 0.05 was considered significant. Correlation was tested using the Spearman rank correlation coefficient $r^2$. A coefficient $r^2 > 0.70$ was considered as a strong and > 0.90 as a very strong correlation.

**Paper II**
Relationships between continuous variables were assessed by the Student’s t-test. A $p$-value < 0.05 was considered significant. Discriminate analysis was performed using SPSS (SPSS Inc, Chicago, IL; v. 14.0 for Windows).

**Paper III**
Statistical analysis was performed using Student’s t-test with Bonferroni correction. SCFA results with skewed distribution were tested by the Wilcoxon rank sum test. A $p$-value < 0.05 was considered significant.

**Paper IV**
The Mann-Whitney U test for unpaired observations was used to compare the total amount of SCFAs in different groups within species. The correlation between the iso-butyric and iso-valeric acids was tested using the Spearman rank correlation coefficient $r^2$.

7. RESULTS SUMMARY

The main findings of **Paper I-III** are presented in Table 2. For further details please see the individual **Papers I-IV**.

**Paper I**
There was a significant difference between untreated CD children and healthy controls (HC) as well as between treated CD children and HC with significantly higher level of acetic, $i$-butyric, $i$-valeric acid and total SCFAs. The propionic and $n$-valeric acids were significantly higher in
children with CD on GFD compared to HC. There was a strong correlation between \(i\)-butyric and \(i\)-valeric acids in all study groups.

**Paper II**

Significantly lower levels of acetic acid and total SCFAs as well as a significantly increased level of \(i\)-butyric acid and FTA were found in relatives compared to HC.

**Paper III**

The children with screening-detected CD had a significant increase in the amount of SCFAs with regard to acetic acid, \(i\)-caproic acid and the total amount of SCFAs compared to HC.

**Paper IV**

High differences in the total output of SCFAs were observed within and between species investigated. Despite these differences, a remarkable correlation between the \(i\)-butyric and \(i\)-valeric acids was found.

<table>
<thead>
<tr>
<th></th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SCFAs</strong></td>
<td>Untreated CD</td>
<td>Treated CD</td>
<td>Healthy controls</td>
</tr>
<tr>
<td>Acetic</td>
<td>50.6***</td>
<td>49.3***</td>
<td>25.4</td>
</tr>
<tr>
<td>Propionic</td>
<td>13.9</td>
<td>14.1*</td>
<td>11.6</td>
</tr>
<tr>
<td>(i)-butyric</td>
<td>2.3**</td>
<td>2.3***</td>
<td>1.6</td>
</tr>
<tr>
<td>Butyric</td>
<td>15.4</td>
<td>15.7</td>
<td>14.9</td>
</tr>
<tr>
<td>(i)-valeric</td>
<td>3.0**</td>
<td>2.8**</td>
<td>2.1</td>
</tr>
<tr>
<td>Valeric</td>
<td>1.8</td>
<td>1.8**</td>
<td>1.4</td>
</tr>
<tr>
<td>(i)-caproic</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Caproic</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>87.4***</td>
<td>86.1***</td>
<td>57.1</td>
</tr>
<tr>
<td><strong>No.</strong></td>
<td>36</td>
<td>47</td>
<td>42</td>
</tr>
<tr>
<td><strong>Age years</strong></td>
<td>0.7-9.9</td>
<td>1.1-10.3</td>
<td>0.3-5.8</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>12m/24f</td>
<td>21m/26f</td>
<td>23m/19f</td>
</tr>
</tbody>
</table>

Significant difference vs. healthy controls: * \(p < 0.05\); ** \(p < 0.01\); *** \(p < 0.001\)

Table 2. Summary of the results regarding SCFAs
8. DISCUSSION

"Paper I - III"

Our finding of an altered specific faecal SCFA profile in CD children, both at presentation, during treatment with GFD, as well as when found in a screening programme, has to the best of our knowledge not previously been reported ("Paper I, III"). This finding might be a genuine phenomenon of CD patients, not affected by either the diet, the gut inflammation or the autoimmune status of the patient. Our results are supported by a recent study from Czechia (Kopekný et al., 2008) that shows an increased level of faecal SCFAs in children with CD before the introduction of GFD. It would have been interesting to learn the results of the individual acids in their study. De Angelis at al., reported differences in the faecal microbiota between children with CD and controls (De Angelis et al., 2009). The significant increase of some faecal SCFA in children with CD compared with HC is in accordance with our findings in children with CD irrespective of diet and may well reflect a deviant gut flora in CD patients.

A lower content of lactic acid producing microbes leaves more carbohydrate substrate to the fermentation process in the gut, leading to an increased production of SCFAs, primarily acetic acid, which is a pro-inflammatory substance supporting an inflammatory reaction in the gut. This might lead to a state of chronic inflammation, which maintains the increased permeability over the enteral mucosa, leading to a persistent increased leakage of antigenic material, e.g. bacteria and gluten, para- or transcellularly. In this way the inflammatory cascade is rolling on. Primarily Bifidobacteria and Lactobacilli produce lactic acid. In CD patients, lower levels of these two species were found (Sanz et al., 2009; De Angelis et al., 2009) in untreated as well as treated patients compared to healthy controls, which might explain our finding of increased levels of acetic acid found in patients with untreated as well as treated CD. Together with the finding of striking differences in some MACs in first-degree relatives of children with CD, our results may well reflect a deviant gut microflora in CD patients and their non-celiac relatives. It can be hypothesised that individuals with CD have a “celiacogenic” microflora and the relatives a “celiacoprotective” flora.

If that is the case it opens up for new therapeutic measures. A manipulation of the intestinal microflora might, theoretically, affect gut mucosal permeability, since it has been shown that colonisation with certain bacteria can change intestinal permeability in rats and humans (Garcia-
Lafuente et al., 2001; Madsen et al., 2001). Another mode of action of a possible “celiacoprotective” microflora might be the formation of a functional barrier against pathogens.

Interesting is the finding of significantly lower levels of acetic acid in first degree relatives of CD children, indicative of them having a celiacoprotective intestinal microflora with a low production of acetic acid, maybe preventing the gluten to exert its toxic effects. In CD patients the GFD might, from the ME, take away the incitement to an initial inflammatory reaction, leading to a decreased inflammatory process in the MI, which leads to a more balanced situation in the MT. Is there a celiacogenic flora in CD-patients?

**Paper IV**

*Study IV* showed high differences in the total output of SCFAs within and between species. Despite these differences, a remarkable correlation between the iso-butyric and the iso-valeric acids was found. The fact that the correlation is strong, irrespective of species, age, diet and living conditions, points to an endogenous common protein source actually reaching the hindgut. We hypothesise that this source is intestinal sloughed cells.

**General discussion**

A prevalence of CD of 1% was found in a screening-study of children in UK, which is comparable to the incidence in adults (Bingley et al., 2004). This suggests that CD starts early in life, since the prevalence of the disease is not greater in adults than in children. The gut microflora is, with regard to function as well as composition, permanently established in children already from the age of 2 years (Midtvedt, 1994; Adlerberth & Wold, 2009). It may be that the presence, or absence, of certain bacteria in the upper small bowel is a prerequisite for developing CD in genetically predisposed individuals. Thus, in small intestinal biopsies performed on children with CD during the “Swedish epidemic” (Fig. 1), rod-shaped bacteria were demonstrated in the intestinal epithelium. (Forsberg et al., 2004; Ou, 2009). The bacteria were found in 37% of children with CD at presentation and during gluten challenge. On gluten free diet 19% still had these rod-shaped bacteria in the small intestinal epithelium. Only 2% of the controls showed the same finding. Post-epidemically, in the years 2004-2007, small intestinal biopsies showed no major gut microbial differences between CD patients and controls.
(Ou, 2009). These findings suggest that certain bacteria indeed could have a role in the “Swedish epidemic” of CD.

At the recent 13th International CD Symposium held 6-8 April 2009 in Amsterdam, Sanz et al. (2009) found reduced numbers of faecal and duodenal Bifidobacteria in both active and dietary treated childhood CD. However, Pusa et al. (2009) did not observe any major difference in the diversity of the small bowel mucosal microbiota between adults with CD and healthy controls. Thus, somewhat conflicting results regarding the impact of gut microflora in CD were presented at the symposium.

In addition to our finding of a deviant SCFA pattern in celiac children there are some indications of a potential role for the gut microbiota in CD. Thus, Collado et al. (2009) recently reported that duodenal and faecal microbiota is unbalanced in children with untreated CD and only partially restored on treatment with GFD. Moreover, Sanz et al. (2007) reported a significantly higher diversity of the gut microflora in children with CD compared with healthy controls. Their findings encourage further classification of the microflora in CD patients aiming at a better understanding of the role of the flora. Moreover, a high prevalence of small intestinal bacterial overgrowth has been shown in adult CD patients with persisting gastrointestinal symptoms in spite of treatment with GFD (Tursi et al., 2003).

CD and Crohn’s disease are prototypic disorders of chronic intestinal inflammation (James, 2005). It is generally believed that the characteristics of the intestinal inflammatory response depend on the cytokines involved. In Crohn’s disease there is an abnormal CD4+ Th1 response to gut bacteria. There is growing evidence suggesting that the situation in CD might be similar with a corresponding reaction towards gluten.

A seasonal variation in the presentation of CD has been observed. Children born in the summer have increased risk for CD (Ivarsson et al., 2003; Lewy et al., 2009). One hypothetical explanation is that children born in the summer are weaned and introduced to gluten in the winter time, when the risk of infections is increased. In addition dietary factors of seasonal character might be of pathogenic importance. Prenatal infections and repeated infectious episodes early in life may also be associated with increased risk of CD (Sandberg-Bennich et al., 2002; Ivarsson, 2005). Recent data by Welander partly contradict these observations, by finding infectious disease, at the time of gluten introduction, not to be a major risk factor for
CD, in children diagnosed at about 1 year of age. The gluten introduction was made parallel with breast feeding (Welander, 2009). Another cause of seasonal variation might be vitamin D deficiency due to lack of sunshine in wintertime. Vitamin D affects epithelial functions and a deficiency might theoretically be a negative factor.

A study from Finland comparing eastern Finland with Russian Karelia revealed a five-fold increased prevalence of CD on the Finnish side of the border, although gluten consumption and HLA-DQ distribution are similar (Kondrashova et al., 2008). However, the mechanisms behind this is virtually unknown.

Twenty-five years ago it was suggested that prior infection by adenovirus 12 could play a role in the pathogenesis of CD (Kagnoff et al., 1984). However, conflicting results were reported from other researchers (Mahon et al., 1991). Moreover, a high frequency of rotavirus infections has been proposed to increase the risk of CD in genetically predisposed children (Stene et al., 2006). If this is confirmed in other studies, it may open the possibility for disease prevention by rotavirus vaccination, since rotavirus is a very common cause of gastroenteritis in infants and young children.

All since the original ESPGAN criteria were published in 1970 (Meuwisse, 1970), the diagnosis of CD, both in children and adults, has been based on the demonstration of a small bowel enteropathy. We are presently moving away from a morphologically based diagnosis. Increased value is being conferred to different serological tests and genetic markers. In the future this will probably limit the need for small bowel biopsy and even make it possible to construct a “genetic risk profile” by combining HLA and non-HLA genes. This will have implications for establishing the diagnosis of CD, but even more for making a reliable prognosis. Interesting is a recently published paper by Tveito et al. (2009), showing a novel one-hour $^{13}$C-sorbitol breath test ($^{13}$C-SBT). The $^{13}$C-sorbitol breath test was positively correlated to levels of serum IgA tissue-transglutaminase antibodies (TGA) and age in the investigated adult celiac group. The degree of histologic damage was shown to correlate positively with age and TGA. Thus, in the study, it was interpreted that the histological severity of CD, indirectly, might correlate to the results of $^{13}$C-SBT-tests.

In this context it could be of interest to correlate the faecal SCFA profiles with results of serological markers and $^{13}$C-SBT-tests, thereby taking a new step on the road towards a non-invasive diagnosis of CD.
9. SUMMARY AND CONCLUSION

Celiac disease (CD) is now recognised as a common, life-long disease in children and adults with a substantial impact on public health. Fortunately, intense research all over the Western World is devoted to the disease. Consequently, piece by piece is added to the exciting celiac jigsaw puzzle. Clearly, the fascinating role of the intestinal microflora is being increasingly unravelled. Hopefully, the findings of this thesis, if confirmed in larger studies, may contribute to a better understanding of the disease process and perhaps even offer future strategies for prevention, diagnosis and therapy of CD. Notwithstanding progress in therapy, the prevention of the disease must be the ultimate goal of the research. Celiac disease has been described as “tricky to find, hard to treat, impossible to cure” (Lohiniemi, 2001). “On the contrary, thanks to previous and present research, CD is possible to find, in almost all cases treatable and, in the near future, perhaps even preventable”, according to Stenhammar (2009).
10. ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and deep appreciation to all children and adults, who has participated in this study by leaving, maybe, the most important of all samples – the faecal sample! Many thanks to all the participants and to the parents of the children for letting us handle that invaluable material in our research.

In short terms I will thank everyone that have helped and supported me as a PhD-student:

- My three supervisors that I have been fortunate to have, assistant professor Elisabeth Norin and professor Tore Midtvedt, at the Dept Microbiology, Tumor and Cellbiology (MTC), Karolinska Institutet (KI) for all exciting functional discussions on microbial ecology and assistant professor Lars Stenhammar, Dept of Paediatric Clinic, Norrköping Hospital, Linköping University – for all your enthusiastice participation and always alert reasoning in clinic as well as science. To all three of you, thank you for all support, respect, inspiration and brilliant supervising tutorship of best brand.

- All my co-authors in England and Sweden.

- The staff at the welfare clinics in Norrköping and surroundings and at the Paediatric Clinic at Norrköping Hospital for their cooperation.

- The staff at the Clinical Chemical Laboratory at Norrköping Hospital, for storing the samples in a -70 degree C freezer.

- Anna-Karin Persson, MTC, KI for excellent technical assistance, analysing the faecal samples and for good instructions about the methods at the laboratory.

- At the Dept of Medical Microbiology, Linköping University I thank our group: professor Tommy Sundqvist for being a skilled and inspiring tutor and for his excellent statistical help. Professor Karl-Eric Magnusson for leading the crew on the best of ways. I am also grateful to professor Karin Fälth-Magnusson, Kajsa Holmgren, Lotta Högberg, Elisabeth Hollén and Eva Grodzinsky.

- My present and former colleagues at the Paediatric Clinics in Norrköping and Linköping.

- Swedish University of Agricultural Sciences, Mälaren Equine Clinic.

- In bright and living memory, my mother Maj-Lis and father Arthur, for letting me grow up in a loving, warm and secure family together with my sister Britt-Marie(Pia) Wall, in the same close memory, and Christina (Nina) Taube, my little sister that is so great.

- Carin – my wife and our four children Gustaf, Martin, Hannes and Ingeborg-Cecilia (median 21, range 18-33) for all love, patience, respect, for putting up with all inconveniences in connection with my work, their ability to improvise and to contribute.
to the work with my thesis, e.g. great help with the computer and to draw a great picture of duodenal villi on the cover of the thesis (Ingeborg-Cecilia, 2009); my family makes everything worth while.

- To all my friends.

- This study was financially supported by the Swedish Medical Research Council, the King Gustaf Vth 80-year Foundation, the Vårdal Foundation, the Swedish Research Council, the Bengt E Gustafsson Fund, the Clas Groschinsky Foudation, the Samaritan Foundation, the Swedish Celiac Society, FORSS (the Health Research Council in the South-east of Sweden) and FORMAS (the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning).
11. REFERENCES


Gee S. On the coeliac affection. St Bartholomew’s Hospital Reports 1888;24:17-20.


Harnett J. Altered levels of commensal gastrointestinal bacteria found in patients with celiac disease. 13th International Coeliac Disease Symposium, Amsterdam, 2009, Poster 213.


42


Lohiniemi S. Tricky to find, hard to treat, impossible to cure. Lancet 2001;358:s14.


Midtvedt A-C. The establishment and development of some metabolic activities associated with the intestinal microflora in healthy children [Thesis], Karolinska Institute, Stockholm, Sweden; 1994.


Olerup O, Aldener A, Fogdell A. HLA-DQA1 and –DQB1 typing by PCR amplification with sequence specific primers (PCR-SSP) in 2 hours. Tissue Antigens 1993;41:119-34.


Wold AE. The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? Allergy 1998;53(suppl):20-5.
