Dietary antibodies and gluten related seromarkers in children and young adults with cerebral palsy
To the children and youth with cerebral palsy and their families.
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The long and winding road, that leads to the door……

(Lennon/McCartney)

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Dietary antibodies and gluten related seromarkers in children and young adults with cerebral palsy
The long and winding road, that leads to the door......
(Lennon/McCartney)
Abstract


Background&Aims; Cerebral palsy (CP), the most common physical disorder in children that affect motor function, is associated with a low weight and height. Celiac disease (CD), an autoimmune disorder precipitated by ingestion of gluten, is another common chronic disease in children that has a negative impact on growth. Based on our findings in a small pilot study, antibodies against gluten, dietary antigens and antibodies against transglutaminase 6(TG6) a new possible gluten related neurological marker have been investigated in an extended group of children with CP. The main aim of this thesis was to find out if the children with elevated gluten related antibodies have enteropathy consistent with CD and if they have antibodies to other dietary antigens as well. We further wanted to study if elevated levels of antibodies were associated to their weight, subtypes of CP and also to investigate if there were an association between the brain damage seen in CP and antibodies against TG6.

Methods; Ninety nine children with CP and matched (study4) controls (study3) were analysed for antibodies against gluten, TG6, egg white, lacto-globulin, casein and wheat. Small bowel biopsies were analysed in the majority of the children with antibody positivity, both by routine procedures and by extended analyse (study 2).

Results; Significantly elevated levels of gluten related seromarkers and antibodies against casein, lacto globulin and egg white were found in the CP-group compared to matched controls. The overall elevated levels of antibodies were more frequent in the tetraplegic (TP) and dyskinetic (DK) CP-subtypes having the most severe neurologic handicap and undernourishment. Routine and extended small bowel biopsies analysis did not indicate an increased prevalence of CD. Elevated antibodies against TG6 were found in the CP-group and significantly in the tetraplegic CP-subgroup.

Conclusion Children with CP do not have increased prevalence of celiac disease but have elevated levels of gluten related seromarkers as well as antibodies against other dietary proteins compared to matched controls. There was a correlation between underweight, CP-subtypes (TP/DK) and occurrence of the tested antibodies suggesting disturbed intestinal permeability related to underweight. Compared to controls TG6 autoantibodies were found in the TP-subtype of CP that could be a result due to the brain damage.

Keywords: Cerebral palsy, children, celiac disease, glutensensitivity, brain, transglutaminase 2 and 6, malnutrition, casein, eggwhite lactoglobulin.

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**List of papers**

This thesis is based on the following original papers.


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Abbreviations

AGA = anti-gliadin antibody  
A = ataxic  
BMI = body mass index  
BSA = bovine serum albumin  
CD = coeliac disease  
CI = confidence interval  
CP = cerebral palsy  
CRM = celiac disease-related marker  
CT = computed tomography  
DGP = deamidated gliadin peptide  
DK = dyskinetic  
DP = diplegic  
ECM = extracellular matrix  
ELISA = enzyme-linked immunosorbent assay  
EMA = endomysial antibody  
EDCD = early developing coeliac disease  
FEIA = fluorescence enzyme-linked immunosorbent assay  
GERD = gastroesophageal reflux disease  
GMFCS = Gross Motor Function Classification System  
GS = gluten sensitivity  
HLA = human leukocyte antigen  
HP = hemiplegic  
IF: immunofluorescence  
IEL = intra-epithelial lymphocyte  
IgA = immunoglobulin A  
IgG = immunoglobulin G  
IQR = interquartile range  
IP = intestinal permeability  
MRI = Magnetic Resonance Imaging  
NSAID = non-steroidal anti-inflammatory drug  
OR = odds ratio  
PVL = periventricular leukomalacia  
PPI = proton pump inhibitor  
SD = standard deviation  
TBS = Tris-buffered saline  
TG = transglutaminase  
tTG / TG2 = tissue transglutaminase 2  
TP = tetraplegic
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Preface

I would like to use this Preface to give the background to my research in this field.

In the mid-1990s I met a patient who at the age of 13 years weighed only 13 kg. The z-score (SD) of this his weight and height was so low, that it was not measurable, an estimated – 6 to -7 SD and the weight and height had not been measured for several years. When I asked the parents if there were difficulties in feeding the child, they said no.

The psychological and human primitive instinct of giving your child food for survival is very strong and overcomes even the greatest barriers. In many cases disabled children do not have the preconditions for eating because the neurological damage affecting their chewing and swallowing mechanisms. The parents blame themselves for being bad parents who are unable to feed their children to ensure a normal growth. At that time when I met this patient the medical care did not have so much to offer a child with cerebral palsy (CP) and it was generally accepted that CP children have a poor growth. Height and weight were measured sporadically and recording of anthropometric data and records were often incomplete, for example owing to difficulties in measuring height in a child with severe contractures.

My co-workers and I started a team to improve the knowledge about the nutritional problems of children with CP and find treatments. We had many discussions and information with different specialists including surgeons and gastroenterologists convincing them that it is important to treat these patients.

It took 2 years from my first meeting with the patient mentioned above until he received surgery, a gastrostomy for feeding and a gastro-oesophageal reflux operation. The reasons why it took so long were the parents’ resistance as well as the lack of tradition of actively treating CP children in health care. After treatment the child had a catch-up in growth and entered puberty. The child became more alert and had fewer infections and a much improved quality of life as also did the parents.

At that time blood analysis was not routinely performed and in fact many habilitation centres claimed to protect the children and provide treatments free from invasive procedures such as taking blood samples. Some centres still adhere to these principles at some places in Sweden.

When we worked on improving the nutritional status of disabled children with poor growth and especially children with CP, we took blood samples for different routine analyses including haemoglobin concentration, plasma albumin, electrolytes and liver enzymes. In addition, we investigated thyroid hormones and coeliac disease (CD) markers; antibodies of immunoglobulin A class (IgA) against gliadin (AGA). Remarkably few of these laboratory test results were
abnormal but an unusually high number of children had elevated levels of IgA-AGA, the only available test for coeliac disease (CD) at the time. These findings resulted in a pilot study¹ and raised the question whether they had CD or whether the increased level of AGA was a marker for another condition. Small bowel biopsies were not performed in most cases since these are difficult to do in a severely disabled child and there was scepticism about the findings among colleagues. (I myself was uncertain as to how to interpret these results).

This scepticism became obvious when I was planning my research studies. Since I am also a biomedical scientist I was fortunate to perform parts of the laboratory work myself under professional guidance from laboratory staff. This has facilitated my research. I performed all the laboratory work in study 3 and part of it in study 1.

Today we know more about different gluten reactions in humans with or without association with other diseases, an area that has attracted considerable interest in the general population as well as in people specializing in this field. The nutritional problems are very complex and there is a big challenge is how to interpret findings and find the best treatments for children with CP. Today we have improved the treatment for CP-patients but there still remain a number of unsolved questions. We still need to learn more about the brain and, the gut interaction and establish whether and how brain damage may affect gut permeability and function. The studies in this thesis can perhaps contribute a small piece in the complex nutritional puzzle.

My main goal when embarking on this thesis has been to highlight these children’s needs for optimal nutrition for increased quality of life and improved health status.
Background

Introduction

My first meeting, in my clinical practise, with a patients with cerebral palsy (CP) having Immunoglobulin A (IgA)-antibodies against gliadin (AGA) was in the mid-1990’s. Since then the knowledge about coeliac disease (CD) a gluten-induced inflammatory disease of the small bowel mucosa and gluten sensitivity (GS) but also about CP has increased significantly. Today we know much more about the nature of CP and can nowadays better identify and interpret symptoms in a child with CP than previously. We also have better treatment both regarding nutrition, gastro-intestinal problems and spasticity. The Swedish national CP-register also known as Cerebral Palsy follow up program (CPUP) established in 2005, which since 2007 has included the whole of Sweden, has contributed to a better –diagnosis of CP. The functional classification of CP based on Gross Motor Function Classification System (GMFCS) as well as other classifications has become widely used, which facilitates research and the assessment of prognosis and quality of life of the children with CP and their families. Still many unanswered questions remain.

During my research studies for this thesis the knowledge of and interest in CD has moved from obscurity into the popular spotlight worldwide.

From diagnosing classic CD in a child with weight loss, diarrhoea and a distended stomach we have now moved to being more aware of different symptoms and diseases connected with CD. The spectrum of disorders related to gluten has emerged and that has made the research in this thesis very challenging. Two papers have recently been published in an attempt to reach a consensus in the classification of different gluten related disorders and CD. In one paper the authors discuss twelve different terms for CD (there are more) and four terms for GS. The terms discussed are based on different serological findings, clinical symptoms and small biopsy findings.

In this thesis the term GS is used when one or more of the markers testing for CD is elevated without evidence of enteropathy.

Cerebral palsy (CP)

CP is one of the most common physical disorder in children that affects motor function to varying degrees, and with one or several accompanying impairments. The CP-group is very heterogeneous and the disability may range from mild to very severe. In Sweden there is a long tradition of research in this field starting with Bengt Hagberg and colleagues in the 1950’s. Research have fo-
Research into the origins and management of CP must remain a high priority, because one of the most severe disabilities in childhood makes heavy demands on health, educational and social services as well as on the families and children themselves. Many unresolved questions still remain including the exact mechanism of the brain damage and the development of the brain injury.

Prevalence
The CP prevalence in Sweden of 2/1000 live births has been relatively stable during the past years and is higher in males than in females; the SCPE in Europe reports an M:F ratio of 1.33:1. There was a rise in CP prevalence during the 1970s and 1980s with a peak in 1983-1986 but since then there has been a decrease in prevalence in preterm and low birth weight (LBW) in children due to the improved neonatal and maternal health care. However Himmelmann et al has shown a recent increase in children born at term and also in the dyskinetic CP.

Definition of cerebral palsy
The definition of “CP” by Hagberg, Mutch et al has been in use for several years. To improve the definition and also to include the accompanying impairment, a new definition was formulated in 2005/2006 based on a workshop in Bethesda, MD, USA, underlining that CP is not an aetiologic diagnosis but a clinical descriptive term:

Cerebral Palsy (CP) describes a group of disorders of the development of movement and posture, causing activity limitation, that are attributed to non-progressive disturbances that occurred in the developing fetal or infant brain. The motor disorders of cerebral palsy are often accompanied by disturbance of sensation, cognition, communication, and behaviour; by epilepsy, and by secondary musculoskeletal problems.

Classification of cerebral palsy
In Sweden the classification by Hagberg, Mutch et al has been in use for many years and has been referred to as the “Swedish classification (SC)” of CP. Following a consensus decision taken in Europe in 2000, the SCPE developed a new classification that differs from the SC as shown in Table 1.
Table 1. Classification of cerebral palsy (CP) according to Hagberg et al and the “Surveillance of cerebral palsy in Europe (SCPE).

<table>
<thead>
<tr>
<th></th>
<th>Hagberg et. al</th>
<th>SCPE</th>
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<tbody>
<tr>
<td>Spastic</td>
<td>Hemiplegia</td>
<td>Unilateral CP</td>
</tr>
<tr>
<td></td>
<td>Tetraplegia</td>
<td>Bilateral</td>
</tr>
<tr>
<td></td>
<td>Diplegia</td>
<td>Spastic CP</td>
</tr>
<tr>
<td>Ataxic</td>
<td>Diplegia</td>
<td>Ataxia</td>
</tr>
<tr>
<td></td>
<td>Congenital(Simple)</td>
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<tr>
<td>Dyskinetic</td>
<td>Dystonic</td>
<td>Dystonic</td>
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<td></td>
<td>Choreoathethotic</td>
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**The Gross Motor Function Classification System**

This is a five-level classification system to assess the motor function in children with CP, based on self-initiated movement such as sitting and walking\(^{14}\). This classification system is used in clinical practise. It is a valuable help in assessing the impairment of the children and youth with CP and has been successfully implemented worldwide. It can also be useful in research and teaching and for administrative purposes.

The classification system has been expanded and revised and now includes children below 2 years and up to 18 years of age\(^{15}\). The validity, reliability and stability of the classification have been stable over the years\(^{16-18}\). At GMFCS level I the children and youth have the same motor performance although they have less speed and more difficulties with coordination and balance as do children without disabilities. At level V, children and youth have no independent movement. Furthermore they have difficulties in controlling their head and trunk postures in prone and sitting position.

**Aetiology**

The aetiologies of CP are numerous and multifactorial. Individual risk factors include birth weight, gestational age and country of birth. Some of the main known causes that increases risk of CP are LBW\(^{19}\), congenital malformation\(^{20}\), infection (maternal and neonatal). In many cases of CP no single cause can be identified and there are probably sequences of associated events, so-called “the
pathophysiological pathways” and interactions that are complicated and not yet fully understood, which result in brain damage. Besides the different known and or associated risk factors for CP, such as infection and malformation, hypoxia seems to be one major cause of damage in the immature brain and the outcome for the child depends on the timing of the adverse event (in the foetus)\textsuperscript{21}. For an overview of risk factors and known causes see article by Reddihough\textsuperscript{22}.

The hypoxic mechanism is complex and has attracted much research attention but there still remain unresolved questions. However, it seems clear from many studies\textsuperscript{23-25} that an ischaemic event severe enough to damage the brain causes an overflow of excitatory neurotransmitters, the major one being glutamate. This may lead to cell death by necrosis and or apoptosis and the immature brain is by far the most vulnerable organ. Both hypoxia-ischemia and inflammatory disorders can activate the apoptotic programmes in the neonatal brain\textsuperscript{26}. Hypoxic-ischaemic injury leads to increased permeability on the blood brain barrier (BBB) both in the immature and in the adult brain and this could also have additional negative impact on the brain damage development\textsuperscript{27-29}. Already 1861 the orthopaedic surgeon William John Little proposed a connection between birth asphyxia and poor neurological outcome in the child\textsuperscript{30}. This proposal was questioned in 1893 by the neurologist and father of psychoanalysis Sigismund Freud meaning that the damage leading to CP may begin earlier in life already in-utero\textsuperscript{31}. Birth asphyxia as the leading cause of CP is still widely accepted although later research has given evidence of more complex etiological pathways as discussed above\textsuperscript{27-29}.

Research is ongoing also in genetics a field that is rapidly growing. Moreno de Luca reports six genes associated with CP and emphasized the importance of thorough investigation of CP-subtypes for example of the dystonic subtypes of CP, because of the potential possibilities with treatment (L-DOPA)\textsuperscript{32}.

Much of the research has been focused on preterm born children although more than half of the children with CP are born at term. In a review on risk factors for CP in term born children, Himmelmann et al report an association between infections, central nervous system (CNS), malformation, intra-uterine growth restriction, social deprivation or multiple gestation and CP\textsuperscript{33}.

Since magnetic resonance imaging (MRI) has become more widely used we have learned more about brain injuries yet still much is unknown about the specific cause of the brain injury. In a study by Bax et al, brain abnormalities were seen in 88\% of the investigated children with CP. The brain abnormalities had a good correlation to the clinical findings and the severity of the condition. Brain-MRI makes it possible to determine the timing of a brain event in the immature brain; however, it does not enable us to establish the exact cause of the injury\textsuperscript{21}. Neuroimaging has shown that the brain abnormalities differ according to the subtypes of CP. In premature born children the typical brain damage seen is
periventricular leukomalacia (PVL) and if CP develops it is of the diplegic subtype. In Bax et al’s study this PVL damage was also seen in the tetraplegic subtype where it tended to be more severe. In this group cortical/sub cortical lesions and malformations, were also reported but to a lesser extend. In the dyskinetic subtype of CP the major finding was basal ganglia damage, which has also been reported by others. In the hemiplegic subgroup, PVL and focal infarcts were mainly seen and in fact PVL was seen in all subtypes of CP. More than half of the ataxic group had normal findings and this group had miscellaneous and cortical/subcortical lesions.

Worldwide attempts to prevent brain damages following birth asphyxia and reduce the risk for hypoxic-ischaemic encephalopathy (HIE) by inducing hypothermia are ongoing. Recent data from a meta analysis concludes that both head cooling and total body cooling improves survival and neurodevelopment in newborns with moderate to severe HIE. A report from China furthermore concluded that hypothermia reduces the combined rate of death or neurodevelopment disability both in moderate and severe encephalopathy. In this connection, Wintermark discusses the importance of finding new, alternative treatments in combination with hypothermia to further increase the neuroprotective effect. Studies have researched molecules that could cross the BBB without being inhibited by hypothermia, and can be used to target the injury mechanism, for example Phenobarbital, Topiramate, and erythropoietin. Another therapeutic approach for preventing CP has been suggested by Fields et al; treatment by activating a specific glutamate receptor.

**Comorbidity**

In a large European study including 818 children, Beckung et al report intellectual disabilities in 53%, seizures of the latest year in 21%, and blindness or no useful vision in 7% of the children. Communication problems of varying degrees were found in 42%. Pain was related to CP type, severity of motor function both gross and fine, feeding disabilities and seizures. Hearing impairment was found in 2%.

Twenty percent of individuals with CP have psychosocial and behavioural problems and 9% have an autistic spectrum disorder, according to Pakula et al.

**Growth and feeding problems**

There are surprisingly few studies of difficulties in gaining weight and height in children with CP, although this is a common finding in these children. The metabolic and micronutrient disturbances are poorly studied and understood. Optimal nutrition resulting in adequate growth is an absolute prerequisite to benefit from the help offered by different professionals in habilitation centres. The health and quality of life of both the children and their families improves when
the child with CP has adequate nutrition. The risk of developing CP in babies born at term is linked to restricted growth status at birth. This is not seen in children born preterm\textsuperscript{39}. But what happens to long-term growth in CP children? The general opinion has been that low weight is a natural consequence of the brain damage itself and poor growth has been accepted as a consequence, without any effort to do something about the undernutrition in these children. During the 1990s, weight and height were not measured at all or only very occasionally in children with CP. This has been confirmed in various studies, for example a Norwegian paper by Dahlseng et al who report that weight and height data were not available for children with CP born in 1993–1996. These authors furthermore found that feeding problems and poor growth are still common, particularly in the most severely disabled children. In their study 40\% of children with GMFCS levels IV–V had z-scores for weight below -2SD and even children in this group without feeding problems had low mean z-scores for weight (-0.64)\textsuperscript{40}. Reports from different studies show that poor growth is a big problem in children with CP. Brooks et al in their impressive work measured 25,545 children with CP in California. Based on their findings, they made a weight for age chart for each GMFCS level that could assist health risk detection and early detection of nutritional deficits in children with CP\textsuperscript{41}. In addition to a low energy intake, micronutrient deficiencies, especially a deficiency in vitamin D, have been reported in disabled children\textsuperscript{42}. This has been verified by others reporting that a deficient intake of iron, folate, niacin, vitamin-E and zinc is common in children with CP\textsuperscript{43}. Most researchers argue that micronutrient deficiency is overlooked by some state that the undernutrition in children with CP is mainly insufficient food intake and that these children do not have micronutrient deficiency compared to malnourished children in developing countries\textsuperscript{44}.

Feeding problems in children with CP are common and are correlated to the severity of the handicap\textsuperscript{45}. Poor oral motor control and persistence of primitive reflexes result in dysphagia, vomiting with or without gastro-oesophageal reflux, aspiration, chest infection and behavioural problems. Feeding and eating are not the same as Petersen et al stated\textsuperscript{46} and to feed a child with severe CP takes a long time and is energy demanding for the child. The oral feeding can be improved by optimizing the posture, treating of oral hypersensitivity and thickening of the foods. Feeding problems in children with CP are related to poor health\textsuperscript{38,47}. In two Nordic studies, difficulties in oral feeding were reported in 21-22\% of children with CP, necessitating tube-fed in 7-14 \%\textsuperscript{38,40}. One study reports that 8 \% of children with CP are tube fed\textsuperscript{48} that increases the weight and body fat but also the total body protein (TBP) as shown in a study of 21 tetraplegic children\textsuperscript{49}. In another study tube feeding increased the life expectancy in children with CP by 7 years\textsuperscript{50}. On the other hand, CP and a gastrostomy is a strong risk factor for death\textsuperscript{51} as has also been shown in a from a study by Westbom et al\textsuperscript{52}. However,
this indicates the severity of the handicap and should not hinder active treatments such as gastrostomy in these children.

Gastrointestinal problems
Children with CP have many gastrointestinal problems. Gastrointestinal motility disorders result in various problems such as constipation, oral motor dysfunction, rumination, delayed gastric emptying, and especially gastro-oesophageal reflux disease (GERD)\(^\text{53}\). In studies of children with neurological impairments, GERD has been reported in a range of 14–75%\(^\text{54,55}\). This indicates the difficulties in diagnosing this disease in neurologically impaired children\(^\text{56}\). The main clinical feature is vomiting and or coughing during or after a meal, regurgitation, choking and cyanotic episodes which could lead to refusal to eat, restlessness, irritability and pain that in turn could lead to abnormal movements. Complications of untreated GERD are oesophagitis, apnoea and aspiration with recurrent lung infections. Many of the symptoms can be successfully treated with medication such as proton pump inhibitors (PPIs) or H2 receptor inhibitors\(^\text{57,58}\). In some cases however, this is not sufficient and anti-reflux surgery such as Nissen fundoplication may be necessary\(^\text{53}\). For children in general, this anti-reflux surgery relieves symptoms in 80% with successful outcome in more than 90%\(^\text{59}\). Sullivan et al state that, based on data from 1987-1992, there is a higher risk of morbidity in these children when fundoplication is performed and that the symptoms of GERD will return since the operation in some cases are not permanent\(^\text{60}\). In a recent study from 2011 the authors state that survival of children with CP following fundoplication surgery is related to the presence of gastrostomy and neurological status and that estimates of children’s life expectancy should be taken into account\(^\text{51}\). This indicates the difficulties in making general anti-reflux recommendations in these children.

The question of performing gastrostomy and anti-reflux surgery at the same time is under debate and there are no convincing data of the efficiency of such combined treatment. Most researchers recommend performing these two procedures separately when needed, but more studies are required\(^\text{61}\). In an attempt to compare the effectiveness of anti-reflux surgery and anti-reflux medications in children with neurological impairments and GERD who are undergoing placement of a gastrostomy feeding tube Vernon-Roberts and Sullivan performed a Cochrane Review in 2007 but found no trials that met the inclusions criteria. They found no randomized controlled trials that provided scientific evidence that could advise the clinicians in this question and highlight the need for robust trials on this issue\(^\text{56}\).
Treatments and life expectancy
There is no cure for CP. Nutritional rehabilitation is one of the absolute most important treatment for these children. During the last two decades increased scientific evidence has shown that treating malnutrition in children with CP is beneficial for the child.

Children with CP and a very low weight have more medical conditions and are at increased risk of death stated by Brooks et al in a study from more than 25000 children with CP.

The severity of disability has the main influence of survival. Besides giving physiotherapy, today we reduce spasticity with botulinum toxin and have Baclofen pumps which also improve the child's weight. Since the knowledge about medical problems has improved during the last years we now have better treatment. We have more and newer antiepileptic drugs and we have better knowledge after orthopaedic surgery with a better follow up. Still, individuals with CP have a shorter life span.

The most common direct cause of death in children with CP was respiratory causes and this has been confirmed in a Swedish study. Westbom et al have shown that the estimated survival rate in children with CP at 19 years of age was 60% for the most disabled children (GMFCS level V) compared with 95% but for the total population group with CP.

Coeliac disease (CD)
Coeliac disease is an immune-mediated small bowel disorder precipitated by ingestion of gluten and related prolamin in genetically susceptible individuals, affecting approximately 1% of the general population. The prevalence appears to be increasing and a Swedish study has reported that as many as 3% of children in Sweden are affected. From having previously been considered largely an enteropathy, CD is now regarded as a systemic disorder with many different clinical manifestations. Our knowledge of CD has undergone tremendous revision in recent years and our understanding of the genetic and immunological characteristics of the disease has greatly improved, as have the diagnostic laboratory tests.

Classification of Coeliac Disease
There have been several classifications of CD during recent years. With improved knowledge about this condition and improved diagnostic laboratory tests, the diagnosis has in a way become more complicated for health care professionals since the reaction against gliadin seems to lead to symptoms not only from the gut but from other parts of the body, leading to different associated conditions.
The information available through the Internet and the different diet trends make people seek information about their conditions and, as reported by Sapone et al, many treat themselves by following a gluten-free diet (GFD)\(^2\). Definitions are well defined for classic CD but problems arise when symptoms, signs and test results are not typical. As mentioned previously, there are today at least 13 terms related to CD, including “atypical”, “asymptomatic”, “latent” and “potential CD”. The European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) recommend using the following three terms for CD\(^6^4\).

1. Silent CD: Clinical symptoms and signs specific for CD are missing but positive CD-specific antibodies and compatible human leukocyte antigens (HLAs) are present and the patient has a small bowel biopsy consistent with CD.

2. Latent CD: There is presence of compatible HLAs but absence of enteropathy in a patient who has had a gluten-dependent enteropathy at some point in life. The patient may or may not have symptoms or signs and may or may not have CD-specific antibodies.

3. Potential CD: There is presence of CD-specific antibodies and compatible HLAs but without histological abnormalities from duodenal biopsies. The patient may or may not have CD-specific symptoms or signs and may or may not develop a gluten-dependent enteropathy later.

Studies in this thesis refer to the earlier definitions of CD\(^6^9,7^0\) and small bowel biopsies has been histologically graduated by the Swedish KVAST klassifikation\(^7^1\). Study 2 investigates the possibility that these children have early developing CD based on further evaluation of previous small bowel biopsies and is discussed below.

Early developing coeliac disease.

The Coeliac Disease Study Group in Tampere, Finland has investigated the presence of IgA-deposits co-localized with tissue transglutaminases (TG2) in small bowel mucosa indicating a forthcoming CD that means before the villous atrophy develops. In Study 2 we further analysed the small bowel biopsies previously performed in order to find out whether these children had mucosal changes indicating early developing CD.

Gluten-triggered small bowel mucosal damage in CD develops gradually from mucosal inflammation to elongation of crypts and finally to overt villous atrophy. Minor small bowel mucosal morphological and inflammatory changes such as increased density of intraepithelial lymphocytes (IELs) may indicate early developing CD\(^7^2\). However, increased numbers of CD3+ IELs is an unspecific finding and only a minority of the subjects with such mucosal changes will eventually develop CD. On the other hand, increased density of γ/δ+ IELs is considered more typical of CD in the Western world and identification of these cells is helpful in borderline cases. Recently, the detection of TG2-targeted intestinal autoan-
tibody deposits proved to be a powerful tool in diagnosing early developing CD without villous atrophy, showing a sensitivity and specificity of 93%\textsuperscript{73}.

Immunoglobulin A class antibodies co-localizing with extracellular TG2 can be demonstrated in the small bowel mucosa, and are regarded as an early mucosal sign of CD, even when antibodies against endomysium (EMAs) and/or anti-TG2 antibodies are not detectable in serum\textsuperscript{74} \textsuperscript{75}.

**Genetics**

The genes on chromosome 6 encoding (HLA) have major importance for the intestinal immunological response in CD. Human leukocyte antigens HLA are surface bound glycoproteins on antigen presenting cells (APCs) and display peptides to T-cells in the intestinal mucosa.

Human leukocyte antigen class I is present on all nucleated cells and platelets while HLA class II is present only on the surface of professional APCs that are able to activate naïve T-cells.

In CD, there is a very strong correlation to HLA DQ2 and/or DQ8. Approximately 98\% of individuals with CD have genes for either of these HLA-molecules and more than 90\% carry DQ2. Since these HLA-types are common in the general population (present in about 35-40 \%)\textsuperscript{76} and the fact that only about 1-3\% develop CD indicates that there are other mechanism involved in the pathogenesis. For instance Sollid and Lie suggests that HLA are necessary but not sufficient\textsuperscript{77}. Non HLA genes also plays a role in the CD-development\textsuperscript{78}. Only absence of HLA DQ2 and DQ8 can be used to, with few exceptions, exclude the diagnosis of CD.
Dietary antibodies and gluten-related seromarkers in CP

Immunoglobulin A class antibodies co-localizing with extracellular TG2 can be demonstrated in the small bowel mucosa, and are regarded as an early mucosal sign of CD, even when antibodies against endomysium (EMAs) and/or anti-TG2 antibodies are not detectable in serum.

Genetics

The genes on chromosome 6 encoding (HLA) have major importance for the intestinal immunological response in CD. Human leukocyte antigens HLA are surface bound glycoproteins on antigen presenting cells (APCs) and display peptides to T-cells in the intestinal mucosa. Human leukocyte antigen class I is present on all nucleated cells and platelets while HLA class II is present only on the surface of professional APCs that are able to activate naïve T-cells. In CD, there is a very strong correlation to HLA DQ2 and/or DQ8. Approximately 98% of individuals with CD have genes for either of these HLA molecules and more than 90% carry DQ2. Since these HLA-types are common in the general population (present in about 35-40%) and the fact that only about 1-3% develop CD indicates that there are other mechanisms involved in the pathogenesis. For instance Sollid and Lie suggests that HLA are necessary but not sufficient. Non HLA genes also plays a role in the CD-development. Only absence of HLA DQ2 and DQ8 can be used to, with few exceptions, exclude the diagnosis of CD.

Fig. 1 CD-pathophysiology

Simplified schematic depicting the process of humoral and cell-mediated immune responses and subsequent mucosal injury in coeliac disease. A) Gluten peptides resistant to digestive enzymes cross the epithelial barrier following an increase in intestinal permeability (IP). B) Relevant gluten peptides are deamidated by TG2, creating epitopes with increased immunostimulatory potential. The gluten peptides may also become covalently linked to TG2 or other proteins through the enzymatic activity of TG2. C) Deamidated peptides are presented by antigen presenting cells, such as dendritic cells, macrophages, or B cells to CD4+ T cells. D) Help from gluten-specific T cells leads to B cell clonal expansion and release of anti-gluten antibodies. TG2-specific B cells might also become activated by gluten-specific T cells through intermolecular help. E) Expression of pro-inflammatory cytokines by activated T cells promotes the release of matrix metalloproteinases that cause epithelial cell damage and tissue remodelling. F) The response to gluten also involves the innate immune system, as epithelial cells secrete IL-15 and express nonclassic MHC class I molecules in response to gluten exposure. This in turn activates CD8+ cytotoxic T cells expressing the natural killer receptors, which can target and destroy the stress-induced molecules. TCR= T cell receptor; APC= antigen presenting cell.

With kind permission from Armin Alaedini
Diagnosis

Laboratory test
Immunoglobulin A antibodies against TG2 and endomysium (i.e. EMAs) are markers with high predictive value for CD, especially where high levels are detected. In case of IgA deficiency, antibodies of immunoglobulin G (IgG) class are considered to have high diagnostic accuracy. On the other hand, antibodies against native gliadin are no longer recommended for CD screening because of their low diagnostic value with low specificity. With use of deamidated gliadin as antigen source the diagnostic value of AGA seems to increase and these tests are now recommended as a second-line test if anti-TG2 or EMAs are negative but CD is suspected. Typing for major histocompatibility complex (MHC) class II molecules HLA-DQ2 and DQ8 is useful for, with high probability, excluding CD in asymptomatic individuals with CD-associated conditions.

Histology of small bowel biopsies taken from duodenum should be graded according to the Marsh-Oberhuber classification; a modified model is used in Sweden (the KVAST classification system) (see “Methods”).

Criteria
The new recommended diagnostic criteria for CD are based on the ESPGHAN’s recently published guidelines for CD diagnosis (2012). They recommend that CD diagnoses be grouped in two; group 1 should be based on symptoms, positive serology and histology that is consistent with CD. If the IgA-TG2 titre is more than ten times the upper limit of normal, then the diagnosis could be set without duodenal biopsies by applying a strict protocol with further laboratory tests. Group 2 are asymptomatic children with increased risk for CD (see above) and positive serology and histology. Testing for HLA-DQ2 and DQ8 could then be of importance (see above).

Symptoms
The symptoms of CD in children show wide variation. Still, impaired growth is a very important sign and there is good evidence that failure to thrive and stunted growth may be caused by CD. With this knowledge, it is of great importance to exclude CD in individuals with CP as a cause of weight loss.

The classic symptoms of CD in a child with severe weight loss, diarrhoea, distended abdomen and malabsorption of nutrients leading to malnutrition are not that common anymore. Weight loss is still one of the most common features of CD. Gastrointestinal symptoms such as diarrhoea are not as common as they used to be anymore. Chronic constipation is also one symptom as well as abdominal pain, although difficult to interpret in children and especially those with neurological deficits.
The clinical symptoms can be very non-specific and there we are becoming more aware about the extraintestinal symptoms in CD. Today we have more precise and specific tests for CD why we should consider the diagnosis despite the difficulties of the broad clinical pictures a diagnosis is important because untreated CD has negative health consequences.

Symptoms and conditions with increased risk of CD include the following besides weight loss; iron-deficiency anaemia, osteoporosis, delayed puberty, IgA-deficiency, chronic fatigue, neurological and or genetic syndromes such as Downs, Turners and Williams syndrome. Coeliac Disease is also more frequent seen in autoimmune disorders such as Type 1 diabetes mellitus (T1DM), autoimmune diseases such as dermatitis herpetiformis (DH), a blistering skin disease with granular IgA deposits in the papillary dermis (unusual in children), autoimmune liver disease, thyroiditis and Addison disease. There is also a higher risk for first degree relatives (10%) and up to 20% risk if a pair siblings are affected.

The treatment is life-long GFD. Untreated and undiagnosed patients have a risk of developing all the listed symptoms above. Recently published data shows low or no risk of developing gastrointestinal cancer if untreated.

**Gut permeability**

The gut with a healthy gut mucosa and intestinal epithelium is the main barrier to environmental factors and separates the outer from the inner milieu of the body. The majority of absorbed proteins cross the intestinal barrier through the transcellular pathways where degradation of the proteins into smaller, non-immunogenic peptides occurs. A smaller percentage of the proteins are transported through the paracellular pathway as intact proteins, which results in an antigen-specific immune response. The complex mechanism in this pathway is regulated by the intercellular tight junction (TJ) keeping the mucosal barrier intact. When these barriers are disturbed, autoimmune disease and food allergy may develop. Evidence of the importance of the increased intestinal permeability in the pathogenesis of many autoimmune diseases such as CD and T1DM is accumulating. Several studies report increased IP seen in autistic patients leading to glutensensitivity and suggest that measuring the increased IP may be useful in identifying those patients who could benefit from a GFD. Probably there is not a connection with CD in autistic patients but an increased intestinal permeability. In patients with dermatitis herpetiformis (DH) increased IP has been reported to be a common finding even in those patients without pathological small bowel biopsies.
There are many factors that could increase IP for instance prematurity, exposure to radiation, chemotherapy, toxins\textsuperscript{91}, drugs such as non-steroidal anti inflammatory drugs (NSAID)\textsuperscript{96}, malnutrition\textsuperscript{97} and gliadin itself.

Sapone et al investigated intestinal permeability in two gluten associated conditions; CD and GS. GS was defined as negative IgA-EMA/TG2 autoantibodies, normal mucosa (Marsh 0 -1) and improvement of gastrointestinal symptoms after gluten withdrawal. Other overlapping diseases such as wheat allergy, type 1 diabetes, and helicobacter pylori infection were ruled out. Half of the GS group had elevated levels of IgG/IgA-AGA. Twelve out of 21 were HLA-DQ2 and/or DQ8-positive. Using the lactulose and mannitol test, the authors found that IP was not increased in GS patients compared with the CD group. However, the authors gave no information about the participants’ weight\textsuperscript{98}.

**Gluten sensitivity (GS)/ non-coeliac gluten sensitivity**

Gluten sensitivity is thought to be due to gluten-mediated mechanisms that are different from the immune reactions in CD. However, due to lack of a clear definition and criteria, this field is controversial and confusing. Only a few years ago, researchers stated that gluten is harmful only with regard to CD pathology, i.e. enteropathy. It has, however, become apparent that gluten can have a negative impact on organs other than the gut, which may explain extra-intestinal manifestations.

In 1995 Marsh concluded that the term “GS” referred to

a heightened immunological responsiveness to ingested gluten in genetically susceptible individuals\textsuperscript{99}.

Hadjivassiliou et al have performed a number of studies of adults with ataxia and GS using this definition. They describe GS as a

systemic autoimmune disease with diverse manifestations meaning that CD could be named gluten-sensitive enteropathy\textsuperscript{100}.

This suggests that the effect on the intestines is only one aspect of a range of possible manifestations of GS. Other terms to describe gluten reactions with or without enteropathy that have been more widely used are “gluten related disorders” and “non coeliac gluten intolerance”\textsuperscript{101}. Sapone et al define GS if patients had gastrointestinal and other symptoms triggered by gluten and that these symptoms are alleviated by gluten withdrawal and also that other diseases such as wheat allergy, T1DM, irritable bowel syndrome (IBS) and Helicobacter Pylori infection has been excluded\textsuperscript{98}.

In a study by Volta et al\textsuperscript{101} of 78 GS patients negative for CD and wheat allergy, had a serological pattern dominated by IgG-AGA (56.4%). IgA-AGA was seen in 7.7%. Only one patient was positive for IgG-DGP. All patients in the GS
group were negative for IgA-EMA and IgA-anti-TG2 and wheat allergy. HLA DQ2 and or 8 positivity were found in 46%. The patients had both intestinal (diarrhoea, abdominal pain and bloating) and extraintestinal symptoms (headache, joint/muscle pain, numbness in leg/arms, tiredness, skin rash, anemia and depression) after gluten ingestion and they promptly responded to GFD. After gluten challenge the symptoms worsened again. The small bowel biopsies showed normal mucosa in half of the patients and increased IEL’s (Marsh 1) in 42%.

In summary, it is not until recently that we have begun to understand the term GS and that it differ from CD and IgE-mediated glutenallergy. Gluten sensitivity seems to have another type of immune mediated reaction and the sufferers do not have antibodies to EMA and have lower titres of TG2 antibodies and in some studies mainly of the IgG-type and they are positive for AGA. Testing for anti-AGA are not recommended for diagnosing CD but it is of seems to be of value when testing for GS and also for testing children under the age of 2 years since anti-TG2, IgA-AGA and EMA fluctuates but IgG-AGA is stable. In one study, approximately 50% of sufferers with GS tested positive for HLA DQ2 and 8 compared with almost 100% of individuals having CD.

Troncone and Jabri mean that there is still a long way to go before all forms of GS can be accurately determined.

**Extraintestinal manifestations of CD and or GS**

Much of the research in this field has not distinguished CD and GS; hence, both terms are included. The initial symptoms of both CD and GS can vary greatly and therefore these extra-intestinal symptoms and signs are important to highlight, as individuals with these disorders who remain undiagnosed may suffer negative health consequences. As well as the abovementioned CD-associated conditions, researchers have found cardiovascular associations, such as cardiomypathy, myocarditis/ acute coronary syndrome, end-stage heart failure and childhood stroke. Many studies but not all has reported associations between coeliac and rheumatologic diseases. There are also reports of hepatic and kidney diseases with the connection of CD. Dermatitis herpetiformis is a well known skin disease that is gluten related and is almost always associated with CD-entheropathy in 80-85 % and in the majority they also have increased IP.

**Neurologic and neuropsychiatric aspects**

The understanding of the pathogenesis of CD has improved but the mechanisms behind the neurological manifestations relating to gluten are more complex. Neurological complications in connection with gluten have been reported since the beginning of 1900.
Jackson et al performed a literature review (Pub Med search) and found 162 articles on, or associated with, neurologic and psychiatric complications of CD or GS. Since the distinction between GS and CD has only recently been made and the two have only recently begun to be discussed as two separate entities one can assume that both GS and neurological/psychiatric manifestations have in the past been underdiagnosed. The neurological and psychiatric manifestations are not negligible and could be the prime symptoms of GS or CD. In a paper by Hadjivassiliou et al they stated that

the fundamental differences among CD, DH and glutenataxia(GA) is that both the gut and the skin have the potentials to regenerate, whereas the neural tissue does not.

which underlies the importance of further knowledge and research in this field.

In Table 2(syndromes are not included in the table) some research articles on neurological and psychiatric manifestations associated with CD are listed. Some of these will be discussed in the following section. The studies and case reports listed in Table 2 cover a wide range, both concerning types of studies and regarding the argument whether these manifestations are linked to gluten or not. In some studies only the occurrence of biopsy-verified CD is investigated and specific laboratory tests used, for instance IgA-EMA and IGA-TG2, but not IgG/IgA-AGA which seems to be valuable when GS is investigated. This makes the discussion confusing.
Table 2. Coeliac disease (CD) and some of its neurological and psychiatric manifestations, discussed in the literature. ADHD = attention deficit hyperactivity disorder; CNS = central nervous system; CP = cerebral palsy; MS = multiple sclerosis.

<table>
<thead>
<tr>
<th>Disorders</th>
<th>Literature: CD in children</th>
<th>Literature: CD in adults</th>
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<tr>
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<tr>
<td>Dementia</td>
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Ataxia

Hadjivassiliou et al has during several years studied a group of adult patients with ataxia and GS, a condition later termed “glutenataxia” and one of the best characterized neurologic manifestations related to gluten. Patients with gluten ataxia have a high level of mainly IgG-AGA and IgG-TG2 antibodies and to a lesser extent also IgA-AGA antibodies and IgA-anti-TG2. The authors showed that 41% of subjects with sporadic idiopathic ataxia had anti-gliadin antibodies compared with 12% of the controls\textsuperscript{179}. Enteropathy is not mandatory in patients with GS with neurological symptoms and as been found in one third of these patients\textsuperscript{100}. Patients with gluten ataxia and positive AGA (GA) had the HLA-types of DQ2 (70%), DQ8 (10%) and DQ1 (20%). They were also found to have IgA/IgG antibodies to a new possible gluten-related neurological marker, transglutaminase 6 (TG6), in 56%\textsuperscript{100,123}. Immunoglobulin A deposition on jejunal TG2 in gut tissues was found in gluten ataxic patients and in one case, widespread deposition of IgA colocalized with TG2 around brain vessels was reported\textsuperscript{129}.

Epilepsy

There are many studies on the association between CD and epilepsy both in adults and children. Italian groups have reported occipital calcifications in combination with epilepsy and CD as a distinct syndrome\textsuperscript{180} which has been confirmed by others. Arroyo et al found an association between CD and epilepsy of 32 children, all of whom also had occipital calcifications. In this group, 37.5% became seizure-free when a GFD was added to their anti-epileptic treatment. The authors also state that the diagnosis of CD in these children may improve the evolution of their epilepsy and also improve their cognitive status. They also suggest that the shorter the latency from onset of epilepsy until GFD the better the chance of seizure control\textsuperscript{143}.

There are conflicting data about whether there is a connection between epilepsy and CD\textsuperscript{140}. The difficulties in the evaluation of different reports are that epilepsy is a heterogeneous condition and some of the studies examined aspects of CD, meaning they used biopsies consistent with CD and/or CD-specific seromarkers (EMA and IgA-TG2) and not gluten-related seromarkers such as IgG/IgA-AGA.

For example, a study by Giordano et al reports no increased risk of CD in a cohort of children with epilepsy; however, the authors found that a higher frequency of children with epilepsy was positive for IgG-AGA and IgA-AGA compared with matched controls\textsuperscript{134}. Another study reports no association between CD and epilepsy; however, it does report that IgA-AGA was more prevalent in patients with primary generalized epilepsy (19.6%) compared with the reference population (10.6%)\textsuperscript{139}.
Others have found associations between hippocampal sclerosis in refractory temporal lobe epilepsy and GS (defined as the presence of AGA, EMA and anti-TG2 antibodies and positive HLA-DQ2 and/or DQ8)\textsuperscript{138}.

In a Swedish population-based cohort study of 28,885 individuals with CD, the risk of epilepsy was moderately increased (hazard ratio (HR) 1.43; 95% confidence interval (CI) 1.10–1.86). This was seen for all ages with CD, including children \textsuperscript{137}.

**Autism**

There is a high frequency of gastrointestinal disorder among patients with autism\textsuperscript{94, 181}. Whether there is a link between autism and CD is controversial. Some studies report that there is no link with regard to the classical diagnosis of CD\textsuperscript{94, 176}. Others suggest that autistic patients have increased IP and this leads to GS but then again there are no clear criteria of the term “GS”. Nevertheless it seems clear from many studies that the autistic patients have increased IP\textsuperscript{92-94} and some researches even suggest that this could lead to intact transport over the mucosal membrane of opioid peptides from gluten and casein\textsuperscript{172, 182}. Whether these children benefit from a GFD is still under debate\textsuperscript{92, 181, 183}. 
The transglutaminases

In this thesis, we studied antibodies against TG2 and TG6. The TGs belong to a family of enzymes consisting of nine members including factor XIII A, TG1, TG2, TG3, TG4, TG5, TG6, TG7 and protein 4.2 without enzymatic activity, that catalyse a variety of Ca2+-dependent reactions. The TGs are important for many different biological processes in the body such as blood coagulation (factor XIII A), skin barrier formation (TG1, 2, 3) and neurodegenerative processes (TG1, 2, 6).

Transglutaminase 2 is a ubiquitous enzyme that has many functions. It is the best characterized member of the TG family. It is found in the extracellular matrix (ECM), where it is important for wound healing, angiogenesis and bone remodelling. Transglutaminase 2 can also be found intracellularly in the cytosol and nucleus and has a mitochondrial association important for many different biological processes in the body such as blood coagulation (factor XIII-A), skin barrier formation (TG1, 2, 3) and in neurodegenerative processes (TG1, 2, 6)\textsuperscript{184, 185}.

Transglutaminase 2 is of fundamental importance for the development of CD. It deamidates and cross-links gluten peptides, resulting in effective antigen presentation by high-affinity binding to HLA-DQ2/DQ8 and initiation of the immunological response, leading to production of antibodies against TG2 as well as to gliadin peptides\textsuperscript{186}.

Regarding the nervous system, TG2 seems to be involved in ischaemic as well as excitotoxic brain degeneration. Several studies have been shown increased levels and activity of TG2 in neurodegenerative diseases\textsuperscript{187} and in vitro models of excitotoxicity using cerebellar granular cells and astrocytes has been shown to upregulate TG2\textsuperscript{188}. The importance and mechanisms are unclear but TG2 may play a role in cell death/survival and both protective and destructive functions have been proposed\textsuperscript{185}. However, the finding of TG2 in tissue damage could be a parallel phenomenon without relevance for the pathological process \textsuperscript{189}. Transglutaminase 3 (TG3) is the main autoantigen in DH a blistering skin disease with granular IgA-deposits in the papillary dermis\textsuperscript{120}.

Another enzyme in the transglutaminase family involved in the neuronal process is the newly identified TG6. It is expressed in humans where the enzyme is probably associated carcinoma cell line with neuronal characteristics and with neurogenesis in mouse brain. \textit{In situ} hybridisation of newborn and embryonal mouse brain has shown that TG6 is wide spread in the brain and most commonly in the cerebral cortex, olfactory lobe and cerebellum. TG6 mRNA expression has been found to be prominent in regions undergoing neuronal differentiation\textsuperscript{190}. 
Malnutrition

“Malnutrition” is defined as “bad nutrition”, meaning that the body does not get the right amount of vitamins, minerals and other nutrients needed to maintain healthy tissues and organ function. Malnutrition includes both undernutrition and overnutrition although the term is mostly used for discussing underweight in the literature.

The World Health Organization (WHO) use anthropometric indicators such as stunting, wasting and underweight as do The International Network for the Demographic Evaluation of Populations and Their Health in Developing Countries (INDEPTH) which is a global network of members who conduct longitudinal health and demographic evaluation of populations in low and middle-income countries191.

**Stunting (H/A)**

“Stunting” is the term used to describe the condition of a child who fails to gain sufficient height, given their age. The term is associated with an extremely low height for age (H/A) score. It is often associated with long-term factors such as chronic malnutrition, often protein-energy malnutrition, and frequent illness. Stunting is an indicator of past growth failure.

**Wasting (W/H)**

A child who has failed to achieve sufficient weight for height (W/H) is said to suffer from wasting. This can be a consequence of starvation or severe disease and is often related to current nutritional status. The term “wasting” can also be used to describe chronic conditions or a combination of current and chronic conditions.

**Underweight (W/A)**

When a child’s weight is less than expected, given his or her age, we used the term “underweight”. Underweight implies an extremely low weight for age (W/A) score and reflects body mass relative to age. Since weight in contrast to height fluctuates over time, underweight could reflect current and acute as well as chronic malnutrition.

**Weight and height recordings**

The recommended recording system for H/A, W/H and W/A is z-scores, which are a statistical measure of the distance from the median, expressed as a proportion of the standard deviation (SD). The cut-off risk level to differentiate malnourished children from those who are adequately nourished is -2 z-score, i.e. 2 SDs below the median values of the growth chart.
The values in this thesis are taken from the Swedish growth chart based on 3,650 healthy children born at term, from birth up to 18 years of age\textsuperscript{192}. In Sweden we often use SD when we are talking in general about the weight and height in children.

Optimal and good nutrition is a prerequisite for healthy growth and development in all children. If that fails the poor growth will negatively affect the brain, cognition, behaviour, muscle strength, cardiac function, immunity, healing and repair\textsuperscript{193}.

Malnutrition in children with CP decreases social interaction, motor function and survival\textsuperscript{194}. These children have reduced levels of protein and energy intake\textsuperscript{195-197}.

**Effect of malnutrition on intestinal function**

Malnutrition can affect the mucosa in the gut and thereby cause increased permeability\textsuperscript{198-202}. Macromolecules can then enter the circulation and stimulate the immune system to produce antibodies.

There are several studies from developing countries that show negative effects on the mucosa due to malnutrition. Lunn et al studied children in The Gambia, Africa, and found that growth failure during the first 15 months of life could be explained by mucosal enteropathy. They further found that this damage could remain throughout life, even when the children concerned had achieved almost normal weight by their third month\textsuperscript{97, 203}.

The authors took mucosal biopsies of 100 severely malnourished children and found a villous structure resembling biopsies from children with CD and cow’s milk enteropathy, meaning partial or subpartial villous atrophy, moderate to severe crypt hyperplasia and intra-epithelial lymphocytes and $\gamma\delta$-T cells were at the same level as seen in CD-patients\textsuperscript{97, 203}.

Using the lactulose and mannitol test they evaluated the IP and found increased values, indicating increased IP in the Gambian children in the study. They also discuss the findings of high plasma concentrations of immunoglobulins (IgA, IgG and IgM) which have generally been interpreted as a result of chronic stimulation of the immune system due to highly contaminated environments but increased mucosal permeability may also be of importance\textsuperscript{97, 203}. This phenomenon has previously been reported in a study by Chandra et al showing that elevated immunoglobulin levels and antibodies of IgG- and IgA-class against different dietary proteins was seen in malnourished children\textsuperscript{204}.

Hossain et al confirm that severe underweight has a negative impact on mucosal function. They compared 77 children at a mean age of 13.1 months and with severe underweight recovering from diarrhoea and other illnesses (not specified) with 17 non-malnourished children from the same communities. They found that 84% of the undernourished children had abnormal IP, using the lactulose and mannitol test at baseline. After 3 months of treatment with food supplement and
psychosocial stimulation this figure had decreased to 60%. Among other findings in this study they report that children who improved in IP also gained more weight

The effect of malnutrition in children is not easily studied and the children in the above mentioned studies from developing countries also had other conditions such as H. pylori infection and other infectious diseases causing diarrhoea. However, there are reports from animal studies such as the study by Rodriguez et al showing that protein malnutrition is associated with increased intestinal paracellular permeability in guinea pigs

Reporting on a study in rats, Weaver et al showed that post-natal protein undernutrition had a negative effect on body weight and length and gastrointestinal function while prenatal protein restriction did not have this effect

Malnutrition and cerebral effects
Many individuals with CP are undernourished, with a W/A score ≤-2 SD, meaning they have chronic starvation. There are millions of children with acute and chronic starvation in the world. About 52% of deaths in young children worldwide are attributable to undernutrition. Despite this, surprisingly few studies are done regarding consequences of undernutrition and especially of the brain and cognition. Many of the studies that have been performed are old and there are mixtures of animal and human studies. The human studies are mainly from developing countries. Children hospitalized for severe malnutrition in early childhood in developing countries have been found to have long-term deficits in cognitive development and school achievement up to adolescence (for an overview, see Grantham-McGregor and Baker-Henningham).

This has been confirmed in several studies pointing out that undernutrition early in life (i.e. in the first 1–2 years of life) affects the brain development, intellectual quotient (IQ) and future school achievement.

Undernutrition per se can influence the brain directly by reducing the number of neurons, synapses, branching of the dendrites and myelination, which could result in a small brain size. Under nutrition during the critical development of the human brain from the last trimester until 2 years of age can cause a diffuse cortical involvement for example the right parietal cortex which is involved in visuospatial functions. The hippocampus, which is involved in learning and memory processes, may also be affected by undernutrition. Purkinjecells and the development of the granular cell layer have also been reported in animal studies to be prone to negative effect from under nutrition. The nutrients that are especially important, and their insufficient intake especially deleterious for the brain are; protein, energy, certain fats, iron, zinc, copper, iodine, selenium, vitamin A, choline and foliates. Undernutrition later in life seems to be less harmful for the brain. Report from a study of anorexic adolescent’s state that
grey matter was more affected than white matter and that this alteration was reversible after nutritional recovery.\textsuperscript{208}

In an excellent review of malnutrition and cognition of human and animal studies, Laus et al state that there is overwhelming evidence that undernutrition, especially early in life, both in animals and humans has a significant and long-lasting negative effect on cognition. The social and cultural deprivation also has an influence and must be considered. Not much research has been done in this field and more studies are necessary.\textsuperscript{212} As Karen Olness state

\begin{quote}
  \textit{effects on brain development leading to cognitive impairment is a worldwide epidemic.}\textsuperscript{215}
\end{quote}

\textbf{The tested dietary antigens in the thesis}

A major task of the gastrointestinal system is to digest and absorb nutrients through trans- and inter-cellular mechanisms\textsuperscript{216} but it is also one of the most important part of the immune system, involved in both defence and tolerance. A small part of protein passes undigested through the mucosa into the systemic circulation. Most people have a small amount of antibodies against various food proteins as a normal physiological phenomenon. Hadjivassiliou et al reported that 12\% of 50 healthy blood donors had (IgG and IgA) to gliadin\textsuperscript{217}. Others report 3\% of IgG-antibodies against gliadin in healthy blood donors in Brazil\textsuperscript{218}.

\textbf{Gluten}

Wheat contains 10–15\% protein, approximately 90\% of which consists of gluten. Gluten is an alcohol-soluble prolamine further divided into gliadins and glutenins, both of which can cause CD. Prolamine is a storage protein found in seeds of cereal grains such as wheat (gliadin), barley (hordein), rye (secalin), corn (zein) and sorghum (kafirin), and as a minor protein in oats (avenin). Studies in this thesis have investigated the presence of IgG/IgA antibodies against gliadin (AGA) and IgE antibodies against gluten and wheat.

\textbf{Milk}

The total protein content in milk is approximately 3\% and milk contains all the nine amino acids essential for humans. Two major categories of the milk protein are casein and serum (whey).

\textbf{Casein}

There are several types of casein, each with its own specific amino acid composition, genetic variation and function. The caseins provide a good source of calcium through their high phosphate content. They form micelles which are dispersed in the water phase of milk. Eighty per cent of the proteins in cow’s milk
are caseins, compared with only 20–45% of the proteins in human milk. Casein also contains proline residues. Cheese has a major component of casein.

**Beta-lactoglobulin**
Milk serum (whey) consists of roughly 50% beta-lactoglobulin and 20% alpha-lactalbumin, as well as albumins, lactoferrin, transferrins, immunoglobulins and enzymes and many other small fractions of proteins. The function of beta-lactoglobulin is not fully understood but it is thought to be a carrier of vitamin A. Beta-lactoglobulin is not present in human milk.

**Egg white**
Egg white is the clear liquid in egg. Its function is to protect the yolk and give the embryo additional nutrition. The major component of egg white is water but it contains about 10% of proteins.
Aims of this thesis

The aims of this thesis were to evaluate whether –

- children with CP have increased prevalence of gluten related antibodies compared to controls
- children with CP have increased prevalence of CD
- children with CP have increased prevalence of serum antibodies against dietary antigens other than those related to gluten compared to controls
- patterns of circulating antibodies differ between different subtypes of CP
- there is a correlation between body weight and serum antibodies in children with CP
- there is an association between brain damage, gluten related antibodies and serum antibodies against TG 6 (an enzyme associated with the CNS) compared to controls

Figure 2. The main variables studied in this thesis: the brain (brain injury; CP), the intestines, and underweight.
Aims of this thesis

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• Children with CP have increased prevalence of CD
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• There is a correlation between body weight and serum antibodies in children with CP
• There is an association between brain damage, gluten-related antibodies and serum antibodies against TG6 (an enzyme associated with the CNS) compared to controls

Definitions

In this thesis work, the following definitions were used:

“Coeliac disease” was defined as elevated levels of EMAs or AGA or IgA antibodies against TG2 and histological evidence of CD, according to the Swedish KVAST classification system.51

“Gluten sensitivity” refers to a condition where one or more of the markers testing for CD are elevated without evidence of enteropathy.

All CP subtypes/groups are defined according to the SC of CP (Hagberg, Mutch et al).10,11

Hemiplegia (HP) was defined as spastic involvement of the leg and arm on one side of the body.

Diplegia (DP) was defined as more severe spastic involvement of the legs than the arms that also are involved but to a lesser extent.

Tetraplegia (TP) was defined as spastic involvement of all the limbs with the arms being equally or more affected.

Dyskinesia (DK) is an umbrella term for different conditions and was defined as the presence of involuntary movements that may be athetoid and/or dystonic (muscle tonus change) and/or choreatic. There could also be spasticity involvements and the motor development level remains at an early infant stage. They often have persisting neonatal reflexes.

Ataxia (A) was defined as a disturbance of coordination of voluntary movements due to dyssynergia of the muscles. That results in balance problems and staggering gait with unsteady hands and there could also be a truncal tremor. The term includes both congenital/simple ataxia and ataxic diplegia the latter includes spasticity of the legs in addition to ataxia.
Methods

Subjects
The subjects were children with CP living in the Swedish counties of Örebro and Värmland, with a total number of inhabitants of 547,467 at the time of inclusion. All children with a diagnosis of CP are registered at specialized habilitation centres where they can obtain specific support such as medical care, physiotherapy, and speech, occupational and psychological therapy until they have completed school. In some cases, CP patients are 24 years of age before they are referred to adult habilitation centres.

The diagnoses and CP classification were performed by a paediatric neurologist. The nomenclature of CP and its subgroups was based on Mutch and Hagberg\textsuperscript{10, 11} since the classification according to the Surveillance of cerebral palsy Europe (SCPE)\textsuperscript{6} was not used in medical recording at the time of the studies. There was also a special interest in TP, for which the SCPE classification would not be suitable. The dyskinetic subtypes were not further divided (see Table 1). The ataxic group included simple ataxia and ataxic DP. During the study a functional assessment of each child was made on the basis of the Gross Motor Function Classification System (GMFCS)\textsuperscript{14}. The classification is graded I–V, with V being the most severe scoring.

A total of 275 children and young adults with CP received an information letter about the study. From Örebro County Council 85/147 (58\%) participated and from Värmland County Council 14/128 (11\%) which resulted in 99 children and young adults with CP. The total participation frequency in the study was 99/275 (36\%). See Flow chart. All children and youth did not participate in all parts in the different studies. This is noted with numbers of participants in tables and figures in the different studies.
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Fig. 3

Flow chart of the included and drop out cases in the study.
Table 3. Distribution of GMFCS levels in relation to subtypes of CP. *Information of GMFCS is missing for 10 patients.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
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<tr>
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<td>17</td>
<td>11</td>
<td>14</td>
<td>22</td>
<td>89*</td>
</tr>
</tbody>
</table>

The analyses in this thesis were performed in the same cohort of children and young adults with CP. At the time of enrolment their age ranged from 1.5 to 24 years (median age 11 years). There was no co-morbidity for CD, such as T1DM or DH, at the time of inclusion. Medical data on the CP group were reviewed retrospectively in medical files by the main author (R.S.).

Study 1
Following the recommendation of the referee only children up to 18 years (median age 9 years) were included in the study. Therefore nine children >18 years of age were excluded. Forty-two girls and 48 boys were included, 90 participants in total.

Study 2
Biopsies from 16 patients from Study 1 were available for immunomorphologic analysis. This group of children consisted of six girls (median age 17 years) and ten boys (median age 9 years). The age range in the group was 3–18 years. The analyses in this thesis were performed on the same cohort of children and young adults with CP. At the time of enrolment the age ranged from 1.5 to 24 years (median age 11 years). There was no co-morbidity for CD such as diabetes mellitus or dermatitis herpetiformis at the time of inclusion. Medical data of the CP group were reviewed retrospectively in medical files (RS).

Study 3
Sera from 96 of the previously included 99 children and young adults with CP were available for analysis of TG6 antibodies.
Study 4
Sera from the previously included 99 children and young adults with CP were available for analysis for IgG against beta-lactoglobulin, casein and eggwhite (n=94), for IgG-AGA (n=90), TG2 (n=90), IgA-AGA (n=91), TG2 (n=91) and IgE against wheat and gluten (n=95).

Control groups

Study 3
Thirty-six children (19 boys and 17 girls), aged 2–18 years and divided into age intervals of 3 years, were included in the serological analysis as unmatched controls. These children had previously been tested for allergy but found negative. No further clinical information about the children was available.

Study 4
Ninety-nine children matched with the cases for age and sex, constituted the control group. Blood samples from children below 7 years of age were collected during 2006 and for older children in 2008 in schools and child health centres in a neighbouring county, Dalarna. Exclusion criteria were asthma and/or atopic dermatitis, fever over the last 3 days, vaccination during the last week, and chronic disease. The children and parents answered a short questionnaire about health and medications. We had no data on weight, height or body mass index (BMI) for these children. One child was on thyroid hormone treatment and another one was on NSAID treatment.

Weight Height and BMI
The measures of weight and height were done in the majority of cases by one person on the same digital sit-scale and height in standing position if possible, otherwise in prone position with an adapted custom-made height instrument. Weight z-score also called weight standard deviation (SD) height and BMI z-scores were calculated for all individuals with CP. Z-scores were standardized to the Swedish general population by age and sex and based on 3650 healthy full term born children up to 18 years of age192.

Serological analyses
Human leukocyte antigen
Human leukocyte antigen-DQB1 typing was performed at the unit for Clinical Immunology, University Hospital, Uppsala, Sweden, by polymerase chain reaction (PCR)-sequence-specific oligonucleotide probe (SSOP) using the Luminex flow bead platform (One Lambda Inc, Canoga Park, CA, USA). Ambiguities were resolved with a PCR-sequence-specific primer (SSP) assay (HLA
HLA DQB1 typing was performed at Clinical Immunology, University Hospital, Uppsala Sweden with PCR-SSOP using the Luminex flow bead platform (One Lambda Inc, Canoga Park, CA), ambiguities were resolved with a PCR-SSP assay (Genovision, Vienna, Austria).

*Antibodies against deamidated gliadin peptides, DGP*

An enzyme-linked immunosorbent assay (ELISA) simultaneously detecting IgA- and IgG antibodies against DGP was used (Quanta Lite TM Celiac DGP Screen; INOVA Diagnostics, Inc., San Diego, CA, USA). The analyses were performed according to the manufacturer’s instructions and the recommended cut-off level of 20 AU/mL was applied.

**Study 1**

*Anti-gliadin antibodies (AGA)*

Anti-gliadin antibodies of IgG and IgA class were determined by ELISA as previously described. The cut-off levels used for IgG-AGA (20 U/ml) and IgA-AGA (30 U/ml) were based on investigations of healthy blood donors (n=1,866) and children 1.5–17 years of age (n=181) and set at the 92.6th and 91.3rd percentile, respectively.

*Anti-tissue transglutaminase antibodies*

Immunoglobulin A and IgG antibodies against TG2 were measured by ELISA, using human recombinant TG2 (Biosystems Electrabox, Barcelona, Spain) in accordance with the manufacturer’s instructions. For IgA-TG2, the recommended cut-off level of 8 U/mL was used, and a grey zone value of 6–8 U/mL. The IgG-TG2 value was based on qualitative analysis in accordance with the manufacturer’s instructions. The results for IgG-TG2 is expressed as a ratio, calculated as absorbance of the sample/absorbance of the cut-off control, and were considered positive if the ratio was 1.

*Endomysium antibodies*

Immunoglobulin A and IgG-EMA were assessed by indirect immunofluorescence (IF) microscopy. For detection of IgA-EMA, in-house cryo-sections of monkey oesophagus were used, while for detection of IgG-EMA, we used commercial slides with fixed sections of monkey oesophagus (SciMedX, Denville, NJ, USA). Briefly, sera diluted 1:10 in phosphate-buffered saline (PBS) (pH 7.2–7.4) with 1% bovine serum albumin (BSA) (fraction V from ICN, cat. No. 810034) were applied to antigen-coated (monkey oesophagus) slides and incubated for 30 minutes in a humid chamber at room temperature. After washing with PBS, the sections were covered with α- or γ-chain specific fluorescein isothiocyanate-conjugated rabbit Fab2 anti-human IgA/IgG (Dako, Copenhagen, Denmark) and incubated for another 30 minutes in a humid chamber at room temperature. Finally, the slides were washed with
PBS and mounted using PBS-buffered glycerin on slides for fluorescence microscopy (HBO50 W ultraviolet (UV) microscope; Zeus’s, Germany). The result was considered positive if presence of a reticulin-like pattern was recorded at a dilution of ≥1/10.

Study 3

Transglutaminase 6

Detection of auto-antibodies to the TG6 isozyme by ELISA followed our previously published protocol with minor modifications. Briefly, full-length human TG6 produced in SF9 cells was obtained from Zedira (Darmstadt, Germany). Results with this antigen were comparable to those obtained with recombinant TG6 produced in-house in Escherichia coli123. Transglutaminase 6 was diluted to 2g/ml in 20mM Tris/HCl, 300mM NaCl, pH 7.6, immediately prior to use and applied to high-capacity protein-binding 96-well plates (ImmulonTM 2HB; Thermo Electron, Waltham, MA, USA) overnight at 4°C. All subsequent incubations were conducted at room temperature. Non-specific binding was blocked by incubation with 3% BSA (immunoassay grade, Sigma 05477) in Tris-buffered saline (TBS) (Tris/HCl, pH 7.4, 150mM Nick) for 1hour. Patient sera were diluted 1:100 in 1% BSA in TBS and any protein aggregates present removed by centrifugation at 10,000 x g for 5 minutes prior to being applied to coated plates. All binding steps were carried out for 90 minutes and followed by five rinses with TBS/0.01% Tween 20. Antibody binding was detected by incubation with peroxidase-conjugated affinity-purified anti-human IgA (109-035-011; Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:1,000 in 1% BSA/TBS, or anti-human IgG (Dako, Carpinteria, CA, USA) diluted 1:250 in 1% BSA/TBS. The reaction was finally developed for 2hours using 5mM 5-amino-2-hydroxybenzoic acid/NaOH, pH 6.0, 0.005% H2O2, as a peroxidase substrate solution and stopped by addition of an equal volume of 1 M NaOH to each well. After 15 minutes, the absorbance at 490 nm was measured. All serum samples were analysed in duplicate on wells containing antigen or only BSA, included on the same plate. The BSA-only background was subtracted from values for antigen. Units were calculated from a series of standards (0,1, 3,10,100 U/mL) (Zedira, Darmstadt, Germany) run in parallel. Results are given as the mean of two independent determinations. Values >14U/mL for IgA-anti-TG6 or >34U/mL for IgG-anti-TG6 were considered positive based on the 98th percentile of a blood donor collective.

Detection of autoantibodies to the transglutaminase 6 isozyme by ELISA followed our previously published protocol with minor modifications123. Briefly, full-length human TG6 produced in SF9 cells was obtained from Zedira (Darmstadt, Germany). Results with this antigen were comparable to those obtained
with recombinant TG6 produced in house in E.coli (data not shown). TG6 was diluted to 2g/ml in 20mM Tris/HCl, 300mM NaCl, pH 7.6 immediately prior to use and applied to high-capacity protein binding 96-well plates (ImmulonTM 2HB; Thermo Electron, Waltham, MA, USA) overnight at 4°C. All subsequent incubations were conducted at room temperature. Non-specific binding was blocked by incubation with 3% BSA (immunoassay grade, Sigma 05477) in TBS (Tris/HCl, pH 7.4, 150mM NaCl) for 1h. Patient sera were diluted 1:100 in 1% BSA in TBS and any protein aggregates present removed by centrifugation at 10,000 x g for 5 min prior to being applied to coated plates. All binding steps were carried out for 90 min and followed by five rinses with TBS/0.01% Tween 20. Antibody binding was detected by incubation with peroxidase-conjugated affinity purified anti-human IgA (Jackson ImmunoResearch, West Grove, PA, USA; 109-035-011, diluted 1:1000 in 1% BSA/TBS) or anti-human IgG (Dako, Carpinteria, CA; P0214, USA, diluted 1:250 in 1% BSA/TBS). The reaction was finally developed for 2h using 5mM 5-amino-2-hydroxybenzoic acid/NaOH, pH 6.0, 0.005% H2O2 as a peroxidase substrate solution and stopped by addition of an equal volume of 1 M NaOH to each well. After 15 minutes, the absorbance at 490 nm was measured.

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Study 4
Sera for analyses of dietary antibodies from the CP-group were stored at -20°C until analysed at the Department of Laboratory Medicine at the University Hospital in Örebro.
• Immunoglobulin G antibodies against beta-lactoglobulin, casein and egg-white were analysed using a fluorescence enzyme immunoassay (FEIA) (Phadia 250; Phadia, Uppsala, Sweden). The cut-off level was based on the 97.5th percentile of controls.
• Immunoglobulin G-AGA was assayed using a FEIA (Phadia 250; Phadia, Uppsala, Sweden). The manufacturer’s recommended cut-off of 7 U/mL (weak positivity 7–10 U/mL) was applied.
• Immunoglobulin A-AGA was analysed by FEIA technique (Phadia, Uppsala, Sweden). The manufacturer’s recommended cut-off of 7 U/mL was used.
• Immunoglobulin G-TG2 was assessed using a FEIA (Phadia 250; Phadia, Uppsala, Sweden). The manufacturer’s recommended cut-off of 7 U/mL was used.

• Immunoglobulin A-TG2 was assessed by FEIA technique (Phadia 250; Phadia, Uppsala, Sweden). The manufacturer’s recommended cut-off of 7 U/mL was used.

• Immunoglobulin E antibodies against wheat and gluten were assayed using a FEIA (Phadia 250; Phadia, Uppsala, Sweden). The manufacturer’s recommended cut-off of 0.35 kUA/L was applied.

• All total serum-IgA (s-IgA) concentrations were measured using an ELISA test for the controls; data in CP cases were available from an earlier study. Immunoglobulin A deficiency was defined as IgA concentration <0.07g/L.

Small bowel Biopsies

Study 1
Gastroscopes used were Pentax 2540, 2940 or 1840 (diameter 8.33, 9.67 and 6 mm, respectively). Biopsy specimens were taken from at least two parts of the duodenum and were then immersed in formaldehyde solution. The specimens were examined histologically at the Department of Pathology of the University Hospital in Örebro and were classified as follows, according to the Swedish KVAST document71: (1) normal (compatible with Marsh 0); (2) duodenal intra-epithelial lymphocytes, i.e. mucosa with normal villi and thickness but with >30 intra-epithelial lymphocytes/100 epithelial cells (Marsh 1–2); CD3 staining was done in most cases; (3) partial villous atrophy; the villi are reduced in length but the length is greater than the breadth and there is an increased number of intra-epithelial lymphocytes (Marsh 3a); (4) subtotal villous atrophy; the breadth of the villi is greater than the length (Marsh 3b) or there is total villous atrophy (flat mucosa) with intra-epithelial lymphocytes (Marsh 3c)220,221.

Study 2
Small-bowel mucosal immunomorphology
The biopsies were freshly embedded in optimal cutting temperature (OCT) compound (Tissue-Tec; Miles, Elkhart, IN, USA), snap-frozen in liquid nitrogen and stored at -70°C. Staining was performed on 5m frozen sections. Thereafter, CD3+IELs were stained with the monoclonal antibody Leu-4 (Becton Dickinson, San Jose, CA, USA), γδ+ IELs with T cell receptor (TCR)-γ antibody (Endogen, Woburn, MA, USA) and αβ +IELs with monoclonal antibody-β F1 (Endogen, Woburn, MA, USA). Positive IELs were counted with an x 100 flat-field light microscope objective in the surface epithelium; at least 30 fields of 1.6 mm epithelial length were counted and IEL density was expressed as cells/mm of epithelium, as previously described73,222,223.
The reference values for the number of cells per millimetre of epithelium are based on a study of 987 adults and set at 37 for CD3+, 25 for αβ T-cells and 4.3 for γδ T-cells. Mucosal HLA DR expression was detected with monoclonal HLA DR antibody (Becton Dickinson, San Jose, CA, USA) at a dilution of 1:1,000. Human leukocyte antigen DR expression was considered normal (grade 0) when there was no staining in crypts and only slight expression in the villous epithelium. When HLA DR expression was seen on the crypts or it was strong in the villous epithelium, the DR expression was considered enhanced and the intensity was graded semi-quantitatively as moderate (grade 1) or strong (grade 2).

**Small bowel mucosal TG2-specific IgA deposits**

The details of these procedures have been published elsewhere. Briefly, 5μm unfixed cryosections of the small bowel biopsies from 16 patients were stained for IgA and TG2 using fluorescein isothiocyanate-labelled rabbit anti-human IgA (Dako, Glostrup, Denmark) and examined by IF microscopy. The IgA deposits were graded semi-quantitatively from 0 to 3 according to their intensity along the basement membrane in the villous crypt area. For double labelling, sections were also stained for TG2 expression using monoclonal mouse antibodies against TG2 (CUB 7402; Neo Markers Inc., Fremont, CA, USA), followed by rhodamine-conjugated anti-mouse immunoglobulin antibodies (Dako, Glostrup, Denmark). Immunofluorescence microscopic examinations were done by one investigator without prior knowledge of disease history or laboratory findings.
Statistical methods
Data were analysed using the Statistical Package for the Social Sciences (SPSS), version 15 and 17 (SPSS Inc., Chicago, IL, USA). Age is shown as median (range) and other continuous variables as median (interquartile range (IQR)) or means (SD) if symmetrically distributed. Differences between groups were evaluated with Pearson’s chi-2 test in cross-tabulations and, where appropriate, Fisher’s exact test. An independent t-test were used for BMI (z-scores), height (z-scores) and weight (z-scores was used for BMI (SD), height (SD) and weight (SD), using 2-tailed significance. Conditional (matched) logistic regression was used to evaluate the association of different antibodies between children and youth with CP and matched controls. The measure of association is odds ratio (OR), with 95% CIs.

Ethical considerations
All studies in this thesis have been approved by the Regional Ethical Review Board in Uppsala and Örebro. Parents have given written informed consent.
Results
An overview of background data of the study population and the results of tested antibodies in the different studies is shown in table 4-6. Several laboratory tests have been performed during this work and the number of test results and included participants in different studies varies.

HLA DQ2 and or 8.
Ninety-three cases were tested for HLA-DQ2 and 8 with 41 that was positive (44%).

Antibodies against DGP
Forty of forty-three children that had positive serology for any of the gluten related markers earlier tested (n=99) and 7/40(18%) tested positive.
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<table>
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<tr>
<th>Category</th>
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Table 5. The frequency of positive antibodies and biopsies in the different studies. AGA = anti-gliadin antibody; CD = coeliac disease; EDCD = early developing coeliac disease; HLA-DQ2 and/or DQ8 = human leukocyte antigen-DQ2/DQ8; IEL = intraepithelial lymphocyte; IgA = immunoglobulin A; IgG = immunoglobulin G; TG2 = transglutaminase 2; TG6 = transglutaminase 6. BMI SD can only be calculated up to 18 years of age.

<table>
<thead>
<tr>
<th>Studies</th>
<th>IgG-AGA n (%)</th>
<th>IgA-AGA n (%)</th>
<th>IgG-TG2 n (%)</th>
<th>IgA-TG2 n (%)</th>
<th>Total antibody positivity of the tested gluten related markers.</th>
<th>IgG against dietary proteins n (%)</th>
<th>IgG and IgA TG6 n (%)</th>
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<td>25/85 (28)</td>
<td>6 (7)</td>
<td>39/90 resulting in: 1 CD + 2 IEL’s</td>
<td></td>
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</tr>
<tr>
<td>Study 2 n=16</td>
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<td></td>
<td></td>
<td></td>
<td>16/27 resulting in: 1 EDCD+2 IELS’s</td>
<td></td>
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<tr>
<td>Study 3 n=96</td>
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<td></td>
<td>12/96 (13)</td>
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<tr>
<td>Study 4 n=(90-94)</td>
<td>55/90 (61)</td>
<td>20/91 (22)</td>
<td>14/90 (16)</td>
<td>1/91 (1)</td>
<td>57/91(63)</td>
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<td>2/36 (6)</td>
</tr>
<tr>
<td>Controls, Study 4 n= 99</td>
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<td>3/99 (3)</td>
<td>3/99 (3)</td>
<td>2/99 (2)</td>
<td>23/99 (23)</td>
<td>9/99 (9)</td>
<td></td>
<td></td>
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</tbody>
</table>
Table 5. The frequency of positive antibodies and biopsies in the different studies. AGA = anti-gliadin antibody; CD = coeliac disease; EDCD = early developing coeliac disease; HLA-DQ2 and/or DQ8 = human leukocyte antigen-DQ2/DQ8; IEL = intraepithelial lymphocyte; IgA = immunoglobulin A; IgG = immunoglobulin G; TG2 = transglutaminase 2; TG6 = transglutaminase 6. BMI SD can only be calculated up to 18 years of age.

<table>
<thead>
<tr>
<th>Category</th>
<th>Positive IgG-AGA n (%)</th>
<th>Positive IgG-TG2 n (%)</th>
<th>Positive IgA-AGA n (%)</th>
<th>Positive IgG against dietary proteins n (%)</th>
<th>Positive IgG/IgA TG6 n (%)</th>
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<tr>
<td>Age (birth-year)mean</td>
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<td>12</td>
<td>11</td>
<td>11</td>
<td>14</td>
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<tr>
<td>Prematurity (n=39)</td>
<td>20/35 (57)</td>
<td>5/35 (14)</td>
<td>8/37 (22)</td>
<td>28/38 (74)</td>
<td>4/37 (11)</td>
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<tr>
<td>Autism (n=11)</td>
<td>8/11 (73)</td>
<td>2/11 (18)</td>
<td>5/11 (45)</td>
<td>6/11 (54)</td>
<td>2/11 (18)</td>
</tr>
<tr>
<td>Mental retardation (n=50)</td>
<td>25/44 (57)</td>
<td>6/44 (14)</td>
<td>9/45 (20)</td>
<td>16/50 (32)</td>
<td>6/48 (12)</td>
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<tr>
<td>Epilepsy (n=30)</td>
<td>22/29 (76)</td>
<td>7/29 (24)</td>
<td>9/29 (31)</td>
<td>14/30 (47)</td>
<td>4/29 (14)</td>
</tr>
<tr>
<td>Controls, Study 4 n= 99</td>
<td>22/99 (22)</td>
<td>3/99 (3)</td>
<td>3/99 (3)</td>
<td>9/99 (9)</td>
<td>2/36 (6)</td>
</tr>
</tbody>
</table>

Table 6. Positivity for any of the tested antibodies from study 4, (IgA-TG2 not presented) in correlation to other diagnoses in combination with CP. When comparing these data in relation to elevated antibodies we found an association between elevated IgG-AGA (antigliadin antibodies) and epilepsy (p=0.03), between autism and elevated IgA-AGA (p=0.048) and between IgG antibodies against casein and epilepsy (p=0.02) and prematurity (p=0.045).
Study 1

Thirty-nine (43%) of the 90 included children had elevated serum levels of one or more of the antibodies tested (Fig. 4). Out of these, four tested low-positive. Neither IgG nor IgA-EMAs could be detected in any of the subjects. None of the children had IgA deficiency (<0.07 g/L). Children with elevated antibodies had lower body weight (p=0.049), height (p=0.041) and BMI (p=0.014). In 25/39 children with elevated serum levels of one or more of the antibodies tested, small bowel biopsy was performed during the study. Another two patients had previously undergone small bowel biopsy because of clinical suspicion of CD. One of these biopsies was performed before the study and confirmed CD, but this child had not since been on a strict GFD. The other child had previously undergone biopsy, with a normal histological result. No major difference in the distribution of subtypes and functional grades of CP between children who did or children who did not undergo a duodenal biopsy was seen, with possible exception of the small number of children in the drop-out group for biopsy (n=12). Intraepithelial lymphocytes were found in two of the 25 children biopsied during the study.

![Figure 4. Distribution of antibody positivity in subgroups of children and youths with cerebral palsy (CP) Study1, n=90](image-url)
Study 2
The small bowel mucosa from one child (with positive serum IgG-AGA) showed elevated numbers of α/β and γ/δ T- lymphocytes and positive staining for CD3 and DR3, as well as co-localized TG2 and IgA. The mucosa from another child (with positive serum IgG-AGA and IgG-TG2) showed slightly elevated numbers of α/β IELs and positive DR-staining. A third patient (with positive s- IgG-AGA and IgG- TG2) had elevated numbers of IELs and positive DR-staining. The biopsies from the twelve remaining children showed normal numbers of IELs and no IgA-deposits were found. However, another seven of these biopsies showed positive DR staining i.e. in total 10/15 indicating mucosal inflammation.

Figure 5. Small bowel mucosal findings of a child with cerebral palsy. Densities of mucosal CD3+ (A, 58 cell/mm, arrow) and γ/δ + (B, 21 cell/mm, arrow) intraepithelial lymphocytes were increased but villous structure was normal. Subepithelial coeliac-type small bowel mucosal immunoglobulin (Ig)A deposits (C, green, arrow) were also detected. Yellow colour in composite picture (D, arrow) indicates co-localization of coeliac-type IgA deposits and transglutaminase 2.
Anti-deamidated gliadin peptide (Anti-DGP) antibodies
Sera from 4/16 children were positive for anti-DGP, one of whom had signs of early developing CD and another of whom had positive DRstaining.

Human leukocyte antibodies (HLA)
Ten out of 16 children (63%) were positive for HLA-DQ2 and/or HLA-DQ8.

Study 3
Sera from 96 patients with CP and 36 controls (children from the same geographical area) were available for analysis of auto-antibodies against TG6. We found elevated levels of anti-TG6 anti-bodies (IgG and/or IgA) in 12/96 subjects (13%) and in 2/36 controls (6%), however, the difference not reaching statistical significance (p=0.35) (Fig. 6). However, a positive test for TG6 auto-antibodies was significantly more frequent in the tetraplegic subgroup of CP, 6/17 (35%) compared with the control group, 2/36 (6%)(p=0.01). We also found statistical significance when the positivity in the tetraplegic subgroup was compared with the outcome in the other CP subgroups (p=0.006). Immunoglobulin A anti-TG6 antibodies were found in 7/96 cases (7%) compared with 1/36 (3%) in the control group (p=0.45). Immunoglobulin G anti-TG6 antibodies were found in 6/96 cases (6%) compared with 1/36 (3%) in the control group (p=0.67). One child had elevated levels of both IgA and IgG to TG6. Transglutaminase 6 expression is associated with neurogenesis in CNS development and in the mature brain in mice, and in neurons in regions associated with motor function including the cerebral cortex and cerebellum. The brain injury in tetraplegic children includes the cerebral cortex and PVL.
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Figure 6. Analysis of serum for auto-antibodies against transglutaminase 6 (TG6) by enzyme-linked immunosorbent assay (ELISA). The relative concentration of antibodies in children (n=96) with cerebral palsy (CP) and controls (n=36) is given in arbitrary units. Bold lines represent the mean titre for the group and the dotted lines the threshold for a positive test.

Study 4
Immunoglobulin G anti-gliadin antibodies and IgG tissue transglutaminase
Fifty-five out of 90 (61%) individuals in the CP group were positive for, i.e. had elevated levels of, IgG AGA compared with 22 out of 99 (22%) in the control group, yielding a statistically significant association (OR 5.6; 95% CI 2.6–11.9). Significantly more cases with CP had elevated levels of IgG against TG2, 14/90 (16%) compared with 3/99 (3%) of controls (OR 7.0; 95% CI 1.6–30.8).

Immunoglobulin A anti-gliadin antibodies and IgA tissue transglutaminase
Levels of IgA AGA were elevated in 20/91 (28 %) in the CP group and 3/99 (3%) in the control group (OR19.0; 95% CI 2.5–142). Only one case in the CP group and two in the control group had elevated levels of IgA TG2 antibodies.

Immunoglobulin G antibodies to beta-lactoglobulin, casein and egg white
When we compared the CP group with matched controls, we found significant associations for IgG-antibodies against beta-lactoglobulin (OR 17.0; 95% CI 2.3–128), casein (OR 11.0; 95% CI 2.6–46.8) and egg white (OR 7.0; 95% CI 1.6–30.8) in the CP-group.

The presence of antibodies against beta-lactoglobulin, casein and egg white was associated with a positive AGA test in the CP group.
There was low occurrence of IgE antibodies against wheat or gluten, with no difference between cases and controls. Positive antibody tests were most frequently found in the tetraplegic and dyskinetic CP subtypes which also had the most pronounced underweight. The correlation between weight only and antibody positivity shows statistically significant lower weight in CP cases with positive antibodies (p=0.03).

Figure 7. Boxplot of weight z-scores by CP subjects with at least one positive dietary antibody compared to CP subjects with all negative dietary antibodies. In the group with negative antibodies the mean weight z-score was -0.77; in the group that tested positive for one or more antibodies the mean weight z-score was -1.74, yielding a difference of 0.97 standard deviation (SD) (95% confidence interval (CI) -1.8 to -0.1), p=0.030.
Discussion

Methodological considerations

This is a report of clinical research performed in a group of CP patients at a time when there was great progress in administration and registration of the CP diagnosis. About 2,000 laboratory analyses were done, which explains the different numbers when results for the cases are presented.

The cohort
A cohort is a group of individuals with some specific factor in common. In this thesis it is CP. The participants are children and young adults. The age span is wide, from 1.5 years to 24 years of age. It would have been preferable to have a narrower age span but then there would have been fewer patients. The medical treatment has changed over the years since the oldest children in the cohort were born, so that the youngest child did not have the same health care as the oldest. In paediatric wards, patients are considered to be children up to 18 years of age. After the age of 18, patients are usually referred to an adult clinic. This is not the case in our habilitation centres where children and young adults can have support up to 24 years of age, which explains the wide age span in these studies.

Study design

Study 1
This first study is a cross-sectional study with no controls. The aim was to investigate whether children with CP have elevated levels of the gluten-related markers previously found in the pilot study1. The results were compared with the manufacturers’ or in-house recommended cut-off levels of the different analyses based on normal values. It would have been better to have had matched controls in this first study but I wanted to find out if there really were elevated levels of these markers in this cohort of individuals. In Study 4 we compensated for this and retested all gluten-related markers and compared these with those in age and gender-matched controls. In Study 1, following the referee’s recommendation we reported data on 90/99 patients who were 18 years of age or younger.

Study 2
The second study is a further analysis of the previously taken small bowel biopsies reported in Study 1. Altogether 16/27 biopsies were further analysed. Not all previously taken biopsies could be analysed because we had the opportunity of collaboration when some patients had already been biopsied and since Study 2 required special preparation of the biopsies, we could not use those that had
been biopsied previously. The total number of biopsied children was 30/43. Furthermore, Study 2 was based on Study 1 and due to exclusion of nine patients in Study 1 only 27/30 small bowel biopsies are reported. Of these, 16 were further analysed.

**Study 3**
This is a cross-sectional study with an unmatched control population. The controls were divided into age groups and the age ranges were the same as in the CP group. The male: female ratio was the same in the CP and control groups. Ideally, there should have been at least as many controls as CP cases, and it would have been a strength if they had been matched. Controls came from the same region in mid-Sweden (from a nearby county). The controls had been referred to a clinic on suspicion of allergy but had been found to be negative. Nothing more is known about the controls. Sera from 96/99 individuals were available for analyses.

**Study 4**
This is a cross-sectional study which has the strength of comparing cases with controls, matched for sex and age, from a nearby county. In this study we retested all the markers tested in Study 1, with additional analysis of dietary antibodies and IgE antibodies against wheat and gluten. The majority of the samples from patients were collected in 2004 and from controls between 2006 and 2008.

**Internal validity**

**Selection bias.**
An invitation letter to the study was sent to all registered children and young adults with CP at the habilitation centres. Fifty-eight per cent of cases from Örebro County participated. Only 11% participated from Värmland County. In total, 99 children and young adults with CP participated, a total participation rate of 36%. This a low participation rate. The higher participation from Örebro was probably due to the researcher (R.S.) being personally known. This could also explain the higher number of the individuals with the tetraplegic CP subtype since compared with hemiplegic CP, many children with tetraplegic and dyskinetic CP have more nutritional and feeding problems, which is one of the researcher’s interests and a focus of the present research. However, there was a lower frequency of participation from the dyskinetic subtype (Fig. 8).
Study 1
This is an historical study in which all children with CP who participated were biopsied between 1996 and 2001 at the University Hospital, Örebro, Sweden. Nine children (4 boys and 5 girls) with CP were biopsied previously. The total number of biopsied children was 30/43. Furthermore, Study 2 was based on Study 1 and due to exclusion of nine patients in Study 1 only 27/30 small bowel biopsies are reported. Of these, 16 were further analysed.

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External Validity
“External validity” refers to the validity of generalized (causal) inferences in scientific studies. The distribution of subtypes of CP compared with other studies is presented in Figure 9.

The distribution into different subtypes has varied over the years. The optimal comparison would have been studies of children born at the same time as the participants, meaning from 1980 on-ward. As previously mentioned, there was...
not that much research on CP before 1980. One of the internationally best known researchers in the field, Bengt Hagberg, performed impressive work in this area during this time. However, due to methodological differences, it is difficult to compare his studies with studies in this thesis.

There are fewer children with hemiplegic CP and more with tetraplegic CP, probably for the same reason as discussed above, in the studies of the thesis. According to Himmelmann et al. there was a rise in the 1970s and 1980s of preterm children with CP, most of whom had diplegic CP. This could have had an impact on the present studies which included more children with DP. Since 1980 there has been a decrease in CP in children born preterm and LBW. This is largely due to improved neonatal and maternal health care. However Himmelmann et al. has showed that in the decrease in total CP-prevalence now has ceased and an increase of CP were seen in children born at term and in the dyskinetic subtype.

![Figure 9. Percentage of different types of cerebral palsy (CP) compared with other studies. A = ataxia; DK = dyskinesia; DP = diplegia; HP = hemiplegia; TP = tetraplegia.](image)

Although more children in the tetraplegic subgroup are present in the studies of the thesis, the GMFCS level did not show that big differences compared to other studies (Fig.10). Comparison to different studies is difficult depending on the birth year of the included subjects and due to the improved neonatal and maternal health care as discussed earlier which also differ between countries\textsuperscript{8,9} and over time. GMFCS and especially the classification for older children have not been used in clinical practise until recently.

**Figure 10.** Gross Motor Function Classification System (GMFCS) levels in the present study population compared with other studies.

<table>
<thead>
<tr>
<th>GMFCS classification</th>
<th>Study 1</th>
<th>Ref. 2</th>
<th>Ref. 3</th>
</tr>
</thead>
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<tr>
<td>I</td>
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</table>


**Misclassification.**

The diagnoses of the CP subtypes were made in the habilitation centres or at the Pediatric departments usually by, or after discussions with, neuropaediatricians. The CP- subdiagnosis could be difficult to decide early in a young patient; however, not many very young children participated. Some patients may not have been included because they had not been referred to the habilitation centres, still receiving treatment at the children’s department. We did not use the new classification recommended by the SCPE because we had a special interest in the tetra-
plegic subgroup. The GMFCS classification was performed during or after the study by physiotherapists. The measures of weight and height were done in the majority of cases by one person on the same digital sit-scale and height in standing position if possible, otherwise in prone position with an adapted custom-made height instrument. The biopsies in Study 1 were evaluated by two experienced pathologists.

Findings

In summary, antibody responses to gluten and other dietary antigens are more common in children with CP compared with matched controls. This is seen particularly in children with the most pronounced handicap and underweight. They do not have increased prevalence of the characteristic findings of gluten enteropathy in small bowel biopsies, i.e. CD. The question is whether they have GS. They have the same serological pattern as discussed for GS and gluten-related disorders; they are also HLA-DQ2 and/or DQ8-positive in 44% of cases, which is in line with GS. However, the gastro-intestinal symptoms were not specifically investigated and asked for. The gastro-intestinal symptoms reported in GS are difficult to evaluate in a severely disabled child.

What are the clinical implications of these findings? (see Figure 11).

Underweight

The mean weight z-score for the whole CP group is low compared with normal children, according to the Swedish growth charts. The mean (SD) z-scores in the whole CP group for weight were -1.40 (A). Underweight can increase the IP, as shown in other studies\(^97, 202\). (D). We has shown that underweight is significantly associated with one or more elevated antibodies (B). Underweight that increase IP, and thereby antibody production against dietary antigens could interfere with other transport mechanisms crossing the mucosal barrier that are important for uptake of nutrients, water, vitamins and the weight (D). Underweight has also a negative impact on the brain, as discussed previously\(^212, 213\). (C). Elevated antibodies and gluten can per se influence the intestines (E) causing increased IP (E).

The Brain

It may be speculated whether other conditions could be the reasons for the high levels of mainly IgG-AGA and IgG-anti-TG2 in the CP-group. Studies have shown cross-reactions between AGAs and Purkinjecells, with loss of these cells in the cerebellum (F). Neurologic and psychiatric symptoms are associated with gluten-mediated disorders (F). Transglutaminase 2 is linked not only to the gut but also to the brain. Transglutaminase 2 has been shown to be important in cell adhesion and synaptic stabilization. Rosenspire et al speculate whether a subpopulation of children with autism may have autoimmunity directed against
TG2, associated with the brain but not the gut (F) 171. The investigated CP-group were negative for EMA that also may indicate that the gut are not involved, endomysium is not present in the brain. The discrepancy of positive TG2 antibodies and negative EMA has also been seen in other conditions such as schizophrenia and glutenataxia without enteropathy 123, 228. One study also report about intrathecal synthesis of auto antibodies against TG2 229(F). The reason for elevated level of antibodies against TG6 in especially the tetraplegic CP subtype is not clear and calls for further research (F). The brain damage itself in children with CP leads to dysfunction of the gut and we still do not know much about the “dyskinetic gut” E. Is the higher frequency of antibodies (mainly AGA and TG2 and 6) in these children due to the brain insult that triggers the immune reactivity or could these antibodies due to the low weight and increased IP exacerbate the brain damage F? There was an association between elevated antibodies (IgG-AGA and anti-IgG against casein) and CP-children with epilepsy, and between CP-children having autism and elevated IgA-AGA (F).

Much is known about the clinical implications of antibodies against gluten but less is known about the other dietary antigens except for the discussion of milk and gluten exorphins 230, 231, 232.

Sapone et al have shown that GS in contrast to CD do not have increased intestinal permeability (IP) 98, but the weight were not reported in that study. The Cp-group in this thesis have malnutrition that probably leads to the increased IP and increased levels of mainly IgG antibodies to gluten and the other dietary antigens tested. Depending on which GS definition that is used they could have glutensensitivity. Glutenfree diet (GFD) is essential for individuals with CD but for individuals with GS it is unclear how gluten affects the body in the long run.
Figure 11. Illustration of the results of the studies in this thesis and their possible effects, as discussed under “Findings”. CP can cause underweight A. Underweight can increase the intestinal permeability (IP) D and thereby cause elevated dietary antibodies B. Underweight has a negative impact on the brain C. Neurologic and psychiatric symptoms are associated with gluten-mediated disorders F. The increased IP, and thereby antibody production against dietary antigens could interfere with other transport mechanisms crossing the mucosal barrier that are important for uptake of nutrients, water and vitamins and weight D. Does the brain damage trigger the immune reactivity resulting in a higher frequency of antibodies (AGA, TG2 and 6) F or could this antibodies harm the brain, thereby exacerbate the brain damage F? The brain damage (CP) has a negative impact on the gut E as has the gluten itself. Results in this thesis shows associations between CP-children with epilepsy and autism and elevated antibody levels (gliadin and casein, see text) F.
Conclusions

Study 1
Elevated levels of antibodies to gliadin and/or to tissue TG were found among 39/90 (43%) children with CP. Even when a cut-off corresponding to the 97.5th percentile was used 35/90 (39%) had elevated levels of these antibodies. In 27/39 children with elevated serum levels of one or more of the antibodies tested, small bowel biopsy was performed during the study, showing CD in one child and intra-epithelial lymphocytosis in another two children. The children with elevated levels of gluten-related seromarkers had significantly lower weight, height and BMI and had dyskinetic or tetraplegic CP. The study shows that children with elevated levels of gluten-related seromarkers do not have increased prevalence of CD.

Study 2
Mucosal IgA-deposits co-localised with TG2 were found in the small bowel biopsy from one patient with serum IgA-class anti-TG2 antibodies, HLA DQ2 and gastrointestinal complaints. Another two children had slightly increased numbers of mucosal α/β+ and/or γ/δ+ IELs. Our findings support previous reports stating that routine small bowel histology may not be sufficient to identify CD at an early stage, and that analysis of IgA co-localised with TG2 may prove useful when CD is suspected despite normal villous morphology. The majority of children with CP and elevated levels of CD-related seromarkers do neither have classical nor early developing CD, but rather an increased immune reactivity to gluten.

Study 3
Antibodies against transglutaminase 6 (anti-TG6), a possible new marker associated with gluten-related neurological dysfunction, were found in 12/96 (13%) of patients with CP compared to 2/36 (6%) in controls, but the difference did not reach statistical significance. However, the tetraplegic subgroup of CP had a significantly higher prevalence of anti-TG6 antibodies 6/17 (35%) compared to the other CP-subgroups, p=0.006 and controls 2/36 (6%) p=0.001. The results of positive TG6 antibodies of controls are similar to other data from 148 controls in an American study of schizophrenic patients.

The interpretation of this data may indicate that an early brain insult and associated inflammation may predispose to future development of TG6 autoimmunity. More studies, however, are required to address the interpretation and clinical relevance of these findings.
Study 4
Children and young adults have a higher frequency of elevated levels of all tested dietary antibodies compared to matched controls. That was especially seen in tetraplegic and dyskinetic CP-subtypes who also had a lower weight. These findings may be related to an abnormal intestinal permeability, possibly secondary to malnourishment and underweight, resulting in immunisation against various dietary antigens which calls for more studies to address the clinical relevance of these findings.

The overall antibody response to IgE-wheat was low.
Future perspectives

The present results call for further studies to address the clinical and therapeutic implications of these findings. Currently, there are no data to support a GFD or other dietary restrictions if CD is not diagnosed. However, a trial GFD evaluating behavioral and gastro-intestinal symptoms and seizure frequency before and after the diet, besides measuring weight, height and IgG/IgA antibodies against AGA and TG2, would provide additional data for the discussion on GS.

Since IgA-TG2, EMA and antibodies to DGP are the most specific markers for the enteropathy in association with CD, the other markers, especially IgG/IgA AGA, and IgG-TG2, is not routinely used today. Further studies of these "GS markers" would be needed to evaluate their usefulness when discussing other extra-intestinal manifestations of gluten.

One area for future research is using the lactose and mannitol test to investigate our assumption that underweight could lead to increased IP and consequently to elevated antibody levels, and whether this is true for this group of children.

Also, in this CP cohort, further analyses of the association between the gut and the brain is of great interest. There have been suggestions of molecular mimicry between gliadin and structures in the brain that could cross react. Alaedini et al have found antibody reactivity against synapsin 1, a neuronal protein in patients with CD. Furthermore, cerebrospinal fluid analyses would be interesting in this group of children to investigate the presence of antibodies to both TG2, which is involved in the brain and especially in ischaemic and excitotoxic brain degeneration, and TG6. Schrodl et al has showed intrathecal synthesis of TG2. Then markers to evaluate if the blood brain barrier is breached could be analysed as well as gut associated CD4+ memory T cells.

Gluten consists of about 100 different proteins and another study could be to investigate if the antibody response in this group of children is directed towards different gluten proteins compared to CD patients and controls. If so, that could indicate another anti-gliadin immune response in this CP-group, which has also been found in patients with schizophrenia.

The main purpose of these studies was to find ways of preventing malnutrition in these children which also is the main message, to prevent malnutrition. Undernutrition affects the muscle, brain and immune system. Undernutrition is treatable in general but is very difficult in this group of children despite great efforts made in this area.
In summary

• The children and youth with CP did not have increased prevalence of CD but they had elevated levels of gluten-related seromarkers and antibodies against other dietary proteins.
• The children with TP or DK more often had elevated levels of these antibodies compared with other subtypes of CP.
• There was a correlation between weight and occurrence of antibodies against dietary antigens.
• Antibodies against TG6 were significantly more frequent in the tetraplegic subgroup of CP compared with controls.
In summary

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Summary in Swedish

Bakgrund


Målsättningar med studien, har varit att undersöka
• om barn och ungdomar med CP i ökad omfattning har gluten relaterade antikroppar jämfört med kontroller.
• om barn och ungdomar med CP och förhöjda glutenrelaterade antikroppar har den specifika tarmskadan som ses vid celiaki.
• om barn och ungdomar med CP har högre förekomst av antikroppar mot andra födoämnen än gluten jämfört med friska kontroller.
• om antikroppssvaren har en annorlunda fördelning i de olika subtyperna av CP.
• om det finns något samband mellan vikt och förhöjt antikroppssvar hos barn och ungdomar med CP.
• om det finns något samband mellan hjärnskadan (CP), glutenrelaterade antikroppar och antikroppar mot TG6 (ett enzym associerat till CNS) jämfört med kontroller.

Studie 1

En tvärsnittsstudie gjordes på 90 barn och ungdomar med CP i Örebro och Värmlands län, avseende alla tillgängliga laborativa test för gluten.

Resultat: 39/90 (43 %) barn med CP hade förhöjda antikropps värden på någon av de testade antikropparna. De flesta hade IgG antikroppar vilka anses vara mer ospecifika för själva tarmskadan vid celiaki. Barnen med antikroppsspositivitet hade lägre vikt, längd och BMI. De tillhörde CP-subgruppen tetraplegi och dyskinesi. Deras funktionsnivå var sämre jämfört med de barn som var antikroppsnegativa bedömda utifrån Gross Motor Function Classification Scale (GMFCS). De hade sålunda ett svårare funktionshinder.

27/39 barn genomgick sedanlig tunntarmsbiopsi. Ett barn hade en tunntarmspåverkan som vid celiaki och 2 hade en viss tarmpåverkan; intraepitelial
lymfocytos (IEL’S). Barnen med antikroppsspositivitet hade sålunda inte någon ökad förekomst av celiaki.

**Studie 2**
I samarbete med en finsk forskargrupp gjordes en utvidgad analys av tunntarmstbiopsier från 16/27 barnen som var biopserade i studie 1. Anledningen till denna studie var att denna forskar grupp har visat att man kan ha en tidig tarmpåverkan; Early Developing Celiac trots att man inte har de mer typiska antikroppssvaren som vid celiaki.

Alla barnen testades också avseende risk för celiaki dvs. en genetisk test (HLA DQ2 och 8). Analys av en mer specifik antikropp som tyder på tarmskada (IgG och IgA- DGP) gjordes också på barnen med positiva glutenrelaterade antikroppar.

Resultat: Ytterligare 1 barn bedömdes ha Early Developing Celiac Disease och ytterligare två barn hade en viss tarmpåverkan i form av ökad förekomst av intraepiteliala lymfocyter.

4/16 barn hade antikroppar mot DGP varav 1 hade Early Developing Celiac Disease.10/16 barn (62%) var HLA DQ2 och/eller HLA DQ8 positiva.

**Studie 3**
De två första studierna visade sammanfattningsvis en ökad reaktivitet mot gluten hos barn och ungdomar med CP och framförallt i den subgrupp av barn som hade det mest omfattande funktionshindret. De flesta har inte den typen av tarmskada som man ser vid celiaki.

Gemensamt för barn med CP är att de har en hjärnskada.

Forskare i England har påvisat antikroppar mot enzymet transglutaminas 6 (TG6) som kan vara en neurologisk markör hos en viss grupp patienter med glutenöverkänslighet och ataxi (balanssvårigheter). Enzymet transglutaminas 6 finns i hjärnan och framför allt då hjärnan bildas under fostertiden. Antikroppssvaret mot gluten hos dessa vuxna som ingick i de engelska studierna liknade det antikroppssvar som vi såg hos de studerade barnen med CP. Därför gjordes i samarbete med dessa forskare en studie där vi analyserade förekomsten av antikroppar mot TG6 på samma barn med CP jämfört med friska kontroller.

Resultat: Vi fann en ökad förekomst av antikroppar mot TG6 i CP-gruppen jämfört med kontroller och signifikant hos barnen med CP- tetraplegi.

**Studie 4**
Barn och ungdomar med CP-tetraplegi och dyskinesi har lägst vikt och det är i den gruppen vi har sett det största utfallet av antikroppar mot gluten. I andra studier hos barn med undernäring har forskare påvisat en tarmpåverkan vilket innebär en ökad tarmpermeabilitet (genomsläpplighet) som i sin tur medför pas-
sage av makromolekyler till blodcirkulationen. Detta kan leda till en ökad immunologisk reaktivitet mot olika födoämnen.

I studie 4 ville vi studera om barn och ungdomar med CP också har en ökad immunologisk reaktion mot andra vanliga födoämnen såsom mjölk (kasein och laktoglobulin) och äggvita med en matchad kontrollgrupp samt en förnyad analys av alla glutenrelaterade antikroppar. Vi undersökte också om det fanns IgE-antikroppar mot gluten och vete.

Resultat: Statistiskt signifikant ökad förekomst av antikroppar mot de studerade födoämnen ågs i hela CP-gruppen jämfört med matchade kontroller. I CP-subgruppen tetraplegi och dyskinesi sågs även ett statistiskt signifikant ökat antikroppssvar mot alla de testade födoämnen utom IgG mot äggvita och IgA/IgG - mot Transglutaminas 2. Vid jämförelse av enbart vikt och antikroppssvar av alla testade födoämnen kunde vi visa att ju lägre vikt desto större risk för antikroppar mot födoämnen. Detta var statistiskt signifikant.

Totalt i hela CP-gruppen var 41/93(44 %) HLA DQ2 och/eller HLA DQ8 positiva och 7/40 (18 %) hade antikroppar mot DGP.

**Sammanfattning**


Dessa studier är de första som undersöker sambandet mellan undernäring, hjärnskada, tarmfunktion och dietära antikroppar hos barn och ungdomar med CP. Syftet har varit att öka kunskapen om nutrition i denna grupp barn för att därmed begränsa deras undernäring.
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