Tampere University

MARLEENA REPO

Anaemia and Iron Deficiency in Paediatric Gastrointestinal Diseases

A Focus on Coeliac Disease

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ACADEMIC DISSERTATION
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Tampere University, the Faculty of Medicine and Health Technology
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Finland

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PunaMusta Oy – Yliopistopaino
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To my family, friends and colleagues.

ABSTRACT

Anaemia is a common finding in children, and besides tiredness, pallor, and other well-known acute symptoms, it can cause long-lasting disabilities such as impairment in cognitive development and in the immune system. Therefore, the reason underlying anaemia should always be investigated. Most often anaemia is caused by iron deficiency, which in low-income countries is most often dietary, but in developed countries in particular is also often secondary to a gastrointestinal disease. In fact, international guidelines advise gastrointestinal endoscopy for all children with obscure anaemia to rule out alimentary tract diseases, but the evidence behind this recommendation remains scarce.

In the variety of symptoms and findings caused by coeliac disease, anaemia and iron deficiency are among the most common. Coeliac disease is an autoimmune-mediated disorder where dietary gluten induces inflammation and gradual destruction of the small bowel mucosal structure. The ensuing reduction of the absorption area of iron has traditionally been suggested to be the cause of anaemia in coeliac disease, but since seropositive patients with only mild or even normal villous morphology (potential coeliac disease) have also been shown to present with anaemia, other factors seem to play a part. Changes in levels of iron regulatory hormone hepcidin or in expressions of duodenal iron transporter proteins have been proposed as possible explanations.

In the present dissertation, the diagnostic yield of gastrointestinal endoscopies in anaemic children was investigated in Study **I**, while Studies **II-IV** examined anaemia and iron deficiency in children with coeliac disease from different perspectives.

In Study **I**, the diagnostic outcomes were evaluated in 864 children who underwent endoscopies in the Paediatric Department of Tampere University Hospital during the years 2007-2014. Besides possibly obtained diagnoses, demographic and clinical characteristics as well as laboratory and histology results were collected from the patient records and compared between children with and without anaemia. Additionally, the associations between different findings and diagnoses set were evaluated. Anaemia was found to increase the probability of being given a diagnosis, especially when presented with bloody stools, growth failure, diarrhoea, positive coeliac antibodies, high faecal calprotectin, high erythrocyte

sedimentation rate or hypoalbuminemia. However, anaemia in patients without additional findings seldom improved the diagnostic yield of endoscopies. Thus, in these children a short period of observation with possible iron supplementation should be considered before proceeding to invasive investigations.

The impact of anaemia on the clinical and histopathological presentation of coeliac disease in children was investigated in Study **II**. Again, comprehensive clinical data was collected systemically from the medical records of a total of 455 children and compared between children with and without anaemia at coeliac disease diagnosis. The results showed anaemia to be associated with more severe clinical, serological and histological disease. Additionally, the haemoglobin levels did not fully recover after one year of follow-up on a well-maintained gluten-free diet.

In Study **III**, the prevalence of anaemia and iron deficiency were evaluated in potential coeliac disease and in coeliac disease with different levels of mucosal atrophy at diagnosis. Iron parameters, including hepcidin, were measured in altogether 125 children. The prevalence of anaemia and iron deficiency were shown to increase along with the severity of mucosal damage. Additionally, abnormal iron parameters were more common in potential coeliac patients than in healthy controls, suggesting altered iron metabolism in the early stage of the disease.

In Study **IV**, the aim was to evaluate the role of iron transporter proteins in the pathogenesis of anaemia in coeliac disease. This was done by measuring their expression semi-quantitatively in the epithelium of 43 immunohistochemically stained duodenal biopsies from children with coeliac disease and controls. No differences in the expressions between anaemic and non-anaemic patients were seen. However, possibly reflecting the atrophic disease state, the stainings of ferroportin and hephaestin differed between coeliac patients and healthy controls.

To conclude, this dissertation highlights the high frequency of anaemia in gastrointestinal diseases, as well as its importance as an alarm symptom (**I**). In children with coeliac disease anaemia was shown to be associated with more severe presentation and to occur in even the histologically early stages of the disease, supporting early diagnosis and dietary treatment (**I, II**). In future, the aetiology of anaemia in coeliac disease needs further research, as it seems that neither hepcidin nor altered iron transporter expression offers an explanation (**IV**).

TIIVISTELMÄ

Anemia on yleinen löydös lapsilla, ja voi paremmin tunnettujen oireidensa, kuten väsymyksen ja kalpeuden, lisäksi aiheuttaa pitkäaikaisia häiriöitä kognitiivisissa toiminnoissa ja immuunipuolustuksen kehityksessä. Anemian syy tulisikin siksi aina selvittää huolellisesti. Anemian tavallisin aiheuttaja on raudanpuute, joka etenkin kehittyvissä maissa johtuu useimmiten ravitsemuksellisista syistä. Kuitenkin erityisesti kehittyneissä maissa taustalta löytyy usein myös ruoansulatuskanavan sairaus, esimerkiksi keliakia tai tulehduksellinen suolistosairaus. Kansainvälisissä suosituksissa ehdotetaankin ruoansulatuskanavan tähystystutkimusta kaikille aneemisille lapsille suolistosairauksien poissulkemiseksi. Tieteellinen näyttö suosituksen taustalla on kuitenkin vähäistä.

Anemia on yksi keliakiapotilaiden yleisimmistä kliinisistä löydöksistä. Keliakiassa ravinnon gluteeni aiheuttaa ohutsuolen limakalvolla tulehduksen ja asteittain etenevän suolinukkavaurion. Limakalvovaurion aiheuttamaa imeytymispinta-alan pienenemistä on perinteisesti pidetty syynä raudanpuutteelle. Anemiaa on kuitenkin todettu myös keliakia-autovasta-ainepositiivisilla, mutta vielä suolinukaltaan terveillä, eli ns. potentiaaleilla keliaakikoilla. Vaikuttaakin siltä, että keliakiassa anemian taustalla vaikuttaa muitakin mekanismeja kuin limakalvovaurio. Selityksiksi esitettyjä tekijöitä voisivat olla esimerkiksi raudansäätelijähormoni hepsidiinin pitoisuusmuutokset tai rautaa ohutsuolessa ravinnosta elimistöön siirtävien raudansiirtäjäproteiinien ilmenemisen muutokset.

Väitöskirjan osatyössä **I** selvitettiin ruoansulatuskanavan tähystystutkimusten arvoa aneemisten lasten diagnostiikassa. Osatöissä **I-IV** käsiteltiin anemiaa ja raudanpuutetta keliaakikkolapsilla useista eri näkökulmista.

Osatyössä **I** verrattiin Tampereen yliopistollisessa sairaalassa lastentautien yksikössä vuosina 2007–2014 tähystettyjen aneemisten ja ei-aneemisten lasten löydöksiä ja heille tehtyjä diagnooseja. Yhteensä 864 lapsen laajat kliiniset tiedot, tutkimustulokset ja diagnoosit kerättiin retrospektiivisesti potilastiedoista. Anemian todettiin lisäävän diagnoosin todennäköisyyttä etenkin esiintyessään samaan aikaan ripulin, veriulosteiden, kasvuhäiriön, positiivisten keliakiavasta-aineiden, korkean ulosteen kalprotektiinin, korkean laskon tai matalan albumiinin kanssa. Endoskopia johti kuitenkin harvoin diagnoosiin anemian ollessa lapsen ainoa kliininen löydös.

Näin ollen lapsilla, joilla lievä anemia on ainoa poikkeava löydös, voisi harkita lyhyttä seurantaa ja mahdollista rautahoitokokeilua ennen etenemistä kajoaviin tähystystutkimuksiin.

Osatyössä **II** selvitettiin anemian vaikutusta keliakian taudinkuvaan lapsipotilailla. Yhteensä 455 diagnoosivaiheessa olevan keliakiapotilaan kliiniset, histopatologiset, serologiset ja laboratoriolöydökset kerättiin potilastiedoista ja niitä verrattiin aneemisten ja hemoglobiiniarvoiltaan normaalien potilaiden kesken. Tuloksissa anemian nähtiin liittyvän kliinisesti, serologisesti ja histologisesti vakavampaan taudinkuvaan. Lisäksi havaittiin, etteivät hemoglobiiniarvot korjaantuneet kaikilla potilailla vuoden gluteenittoman ruokavalion aikana.

Osatyössä **III** tavoitteena oli selvittää anemian ja raudanpuutteen esiintymistä ns. potentiaaleilla keliaakikoilla verrattuna suolinukkatuholtaan eri vaiheisiin edenneisiin keliakiapotilaisiin ja terveisiin kontrolleihin. Rautaparametrejä mitattiin yhteensä 125 lapselta. Anemian yleisyyden todettiin lisääntyvän yhdessä suolinukkavaurion vaikeusasteen kanssa, mutta myös potentiaaleilla keliaakikoilla oli enemmän anemiaa ja raudanpuutetta verrattuna keliakiavasta-ainenegatiivisiin kontrolleihin.

Raudansiirtäjäproteiinien mahdollista vaikutusta anemian syntyyn keliakiaa sairastavilla lapsilla tutkittiin osatyössä **IV** mittaamalla ko. proteiinien ilmenemistä ohutsuolen immunohistokemiallisesti värjätyissä koepaloissa (n=43). Eroja aneemisten ja ei-aneemisten keliaakikkolasten välillä ei havaittu, mutta keliaakikoiden hephaestiinin ja ferroportiinin ilmeneminen erosi terveistä kontrolleista, viitaten mahdollisesti suolinukkavaurion aikaansaamaan solujen epäkypsyyteen.

Väitöskirjassa todettiin anemian lisäävän riskiä ruoansulatuskanavan sairauteen ja toimivan varoitussignaalina, jonka yhteydessä lisätutkimukset tai vähintään tarkka seuranta ovat paikallaan (**I**). Lisäksi anemian todettiin liittyvän keliakialasten vakavampaan taudinkuvaan, sekä anemian yleisyyden kohonneen myös taudin varhaisvaiheessa, tukien sairauden varhaista diagnosointia ja gluteenittoman ruokavalion aloittamista (**II, III**). Hepsidiinipitoisuuden tai ohutsuolen pinnan raudansiirtäjäproteiinien muutokset eivät selittäneet anemiaa keliaakikkolapsilla (**IV**), joten anemian monitekijäinen etiologia keliakiassa vaatii jatkotutkimuksia.

CONTENTS

ABBREVIATIONS

BMP	bone morphogenic protein
CI	confidence interval
DCYTB	duodenal cytochrome B
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
EmA	endomysium antibody
EPO	erythropoietin
ERFE	erythroferrone
ESPGHAN	European Society for Paediatric Gastroenterology Hepatology and Nutrition
ESR	erythrocyte sedimentation rate
Fe/S	iron-sulphur
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
FPN	ferroportin
GERD	gastroesophageal reflux disease
GFD	gluten-free diet
HAMP	hepcidin gene
HEPH	hephaestin
HFE	human homeostatic iron regulator protein
HIF	hypoxia-inducible factor
HJV	haemojuvelin
HLA	human leucocyte antigen
HMOX	haem oxygenase
IBD	inflammatory bowel disease
IDA	iron deficiency anaemia
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
IgA deposit	TG2-targeted IgA antibodies bound to small bowel mucosa
IL	interleukin

IRE	iron responsible element
IRP	iron regulatory protein
MCV	mean corpuscular volume
mRNA	messenger ribonucleic acid
OGD	oesophagogastroduodenoscopy
OR	odds ratio
P/SVA	partial and subtotal villous atrophy
RBC	red blood cell
Rf	reference value
rS	Spearman's rank
sTFR	soluble transferrin receptor
TF	transferrin
TfR	transferrin receptor
TG2	transglutaminase 2
TG2-ab	TG2-autoantibody
TVA	total villous atrophy
UK	United Kingdom
USA	United States of America
WCE	wireless capsule endoscopy

ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred in the text by the Roman numerals **I-IV**.

Publication I Marleena Repo, Teemu Rajalahti, Pauliina Hiltunen, Antti Sotka, Laura Kivelä, Heini Huhtala, Katri Kaukinen, Katri Lindfors, Kalle Kurppa (2020): Diagnostic findings and long-term prognosis in children with anemia undergoing GI endoscopies. Gastrointestinal Endoscopy. 91:1272-1281.e2.

Publication II Teemu Rajalahti, Marleena Repo, Laura Kivelä, Heini Huhtala, Markku Mäki, Katri Kaukinen, Katri Lindfors, Kalle Kurppa (2017): Anemia in children with celiac disease: association with the clinical, serological and histological findings and response to the gluten-free diet. Journal of Paediatric Gastroenterology and Nutrition. 64:e1-e6.

Publication III Marleena Repo, Katri Lindfors, Markku Mäki, Heini Huhtala, Kaija Laurila, Marja-Leena Lähdeaho, Päivi Saavalainen, Katri Kaukinen, Kalle Kurppa (2017): Anemia and iron deficiency in children with potential celiac disease. Journal of Paediatric Gastroenterology and Nutrition. 64:56-62.

Publication IV Marleena Repo, Markus Hannula, Juha Taavela, Jari Hyttinen, Jorma Isola, Pauliina Hiltunen, Alina Popp, Katri Kaukinen, Kalle Kurppa, Katri Lindfors (2021): Iron transporter protein expressions in children with celiac disease. Nutrients. 13:776.

INTRODUCTION

Anaemia is one of the most common global medical problems, affecting over 30% of the world population (Kassebaum et al. 2015). Anaemia is especially common in children, although the prevalence varies widely by geography and especially between high- and low-income countries. On average, 16.7% of preschool children in Europe and as many as 64.6% in Africa have been shown to present with it (McLean et al. 2009). The most common form of anaemia is iron deficiency, which in developing countries is usually caused by inadequate nutrition and deficits of essential micronutrients. Instead, in developed countries anaemia may be either dietary or secondary to a gastrointestinal or haematological disease or other chronic condition (Camaschella 2019; Kassebaum et al. 2015). Anaemia and iron deficiency in children have been shown to impair psychomotor and cognitive development and to lead to impaired functioning of the immune system (Ekiz et al. 2005, Lozoff et al. 2007; Lozoff et al. 2013; Shafir et al. 2008). Besides its role in oxygen transport, iron plays a crucial part in many biological functions such as energy production, deoxyribonucleic acid (DNA) synthesis and cell proliferation (Evstatiev and Gasche 2012).

Anaemia is not a diagnosis as such but a clinical sign, the underlying cause of which should always be investigated. Gastrointestinal diseases are a common cause of anaemia as they damage the alimentary tract causing both bleeding and decreased absorption of iron as they are often inflammatory in nature. The international guidelines in fact suggest that these diseases should always be ruled out particularly in adolescents with unexplained anaemia (Thomson et al. 2017). However, the evidence behind this recommendation remains scarce. A reliable examination of the intestine requires invasive methods, particularly oesophagogastroduodenoscopy (OGD) and colonoscopy. These investigations in children are usually performed under general anaesthesia, which is labour-intensive and may even impair cognitive development (Hu et al. 2017). Thus, the optimal targeting of these invasive procedures would be of particular importance, but currently the decision whether to conduct an endoscopy relies more on paediatricians' experience than scientific

evidence (Franciosi et al. 2010; Sheiko et al. 2013; Thomson and Sharma 2017; Wang et al. 2017).

One of the most common gastrointestinal pathologies behind anaemia in children is coeliac disease, a life-long gluten-driven disorder characterized by small-bowel mucosal damage and heterogeneous clinical picture (Kivelä et al. 2015; Winter and Halsey 2014). Screening studies have revealed the prevalence of the disease to be as high as 1-2% (Mäki et al. 2003; Singh et al. 2018; Webb et al. 2015). Anaemia is one of the most common extra-intestinal symptoms of untreated coeliac disease, with a prevalence among patients varying from 12 % up to 84 % (Halfdanarson et al. 2007; Husby et al. 2012; Kreutz et al. 2020). Usually anaemia in coeliac disease is thought to be caused by decreased absorption of iron from the proximal small-intestine (duodenum), but there is evidence that it may be present in some patients even before the development of significant duodenal lesions (Kondala et al. 2016; Kurppa et al. 2010; Tosco et al. 2011). Other possible causes of anaemia in coeliac disease are chronic inflammation (Bergamaschi et al. 2008; Harper et al. 2007) and a depletion of folic acid or vitamin B12 (Kreutz et al. 2020), while rare cases of aplastic anaemia have also been reported (Maheshwari et al. 2012). These findings suggest the pathology of anaemia in coeliac disease to be multifactorial.

In recent decades, the understanding of human iron metabolism has taken huge steps, the most important discovery being the identification of the hepcidin-ferroportin (FPN) axis controlling iron release from body storages (Nemeth et al. 2004). The complex mechanisms of iron metabolism have also been studied increasingly in various diseases. In coeliac disease the detailed pathophysiological factors behind anaemia remain mostly obscure, although has been suggested that, for example, abnormal expression of iron transporter proteins in the duodenal mucosa could provide an explanation (Harper et al. 2007; Martín-Masot et al. 2019).

REVIEW OF THE LITERATURE

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1 IRON METABOLISM

1.1 Overview of iron metabolism

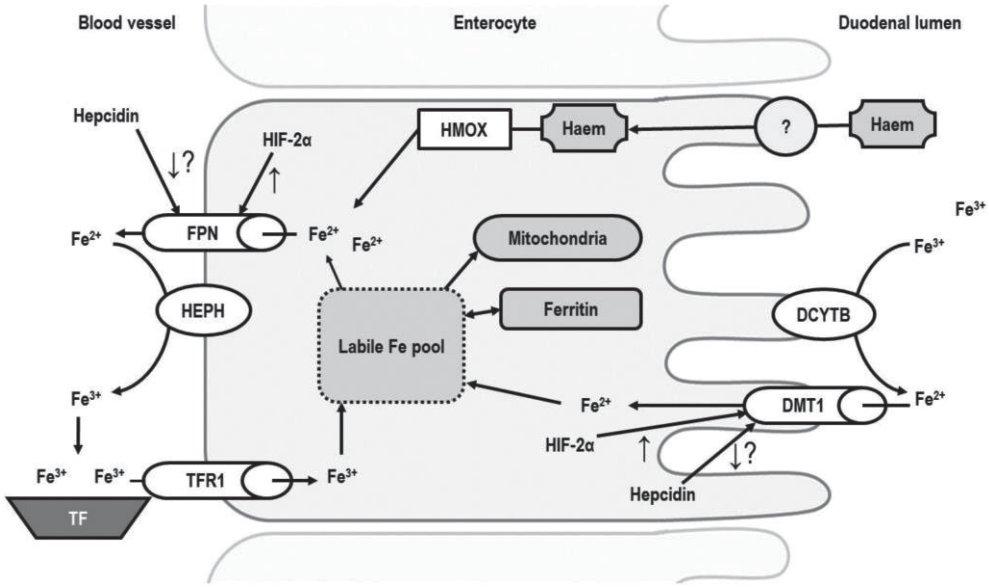
Iron balance is essential for all cellular life, as iron-containing proteins fulfil various vital functions, such as oxygen transport, cellular respiration, intermediary metabolism, regulation of transcription, host defence and DNA repair. On the other hand, iron excess is toxic as it generates harmful reactive oxygen species (Aisen et al. 1999; Camaschella 2019; Camaschella et al. 2020). The total iron balance in the body is maintained by controlling its intestinal absorption as there is no regulated pathway for iron excretion (Camaschella 2019; Evstatiev and Gasche, 2012). In recent decades, the understanding of iron metabolism in humans has undergone substantial developments, especially after identification of the central role of hepcidin-FPN axis in the control of systemic iron homeostasis (Aschemeyer et al. 2018; Nemeth et al. 2004).

1.2 Iron absorption

Iron is absorbed into the duodenal mucosa through various import proteins (Figure 1). Dietary inorganic iron is absorbed at the brush border of duodenal enterocytes via the divalent metal transporter 1 (DMT1) (Gunshin et al. 1997). Prior to this, the ferric Fe^{3+} form of iron must be reduced to ferrous Fe^{2+} form, either by the duodenal cytochrome B (DCYTB) or to a lesser extent by some other ferrireductases (McKie 2008). Iron bound to haem protein is transported independently by a currently unclear mechanism. Intracellular iron is released from the haem by haem oxygenase (HMOX, Ferris et al. 1999). Inside the enterocyte, iron comprises a labile pool. The free intracellular iron can be stored in ferritin or utilized in mitochondria in haem and iron-sulphur (Fe/S) cluster production. An additional role of the enterocytes is to carry iron into the circulation, which happens through the FPN in the basolateral membrane (Donovan et al. 2000; McKie et al. 2000). Before it can be loaded onto the plasma carrier protein transferrin (TF), two molecules at a time, iron is oxidized back to ferric form by hephaestin (HEPH) (Petrak and Vyoral 2005). TF binds to

the ubiquitous protein transferrin receptor 1 (TfR1) on the surface of the target cells, especially in the erythroid bone marrow, followed by endocytosis of the complex and finally iron release (Muckenthaler et al. 2017).

Figure 1. Intestinal iron absorption. In the apical membrane of enterocytes, the duodenal cytochrome B (DCYTB) reduces ferric iron (Fe3+) into ferrous form (Fe2+). Thereafter, divalent metal transporter (DMT1) transfers iron into the enterocyte, where it is either utilized in mitochondria, stored in ferritin, or transported to the circulation via basolateral transmembrane protein ferroportin (FPN). Before being able to bind to transferrin (TF), the main plasma iron carrier, iron must be reverted to ferric form by another basolateral protein called hephaestin (HEPH). Haem iron is transported into enterocytes by a currently unclear mechanism. Intracellularly iron is released from haem by haem oxygenase (HMOX). The enterocytes may also reuptake iron from circulation for their own metabolic functions through transferrin receptor 1 (TfR1). A key regulator of iron metabolism is liver-produced hepcidin thought to reduce iron uptake through enterocytes by inhibiting the function of DMT1 and possibly FPN. In contrast, in the hypoxic environment of the gut the hypoxia-inducible factor 2α (HIF-2α) increases the expression of these two proteins.



1.3 Iron trafficking

The total body iron content is on average 4 grams but is not normalized to body weight as it varies, depending on other factors such as age and gender. Approximately 0.5-2 mg of iron enters the circulation on a daily basis via duodenal absorption and a comparable amount leaves the body along with sloughing of intestinal epithelial cells, desquamation of skin and urinary cells, sweat and in women with menstruation. Iron circulates in blood bound to TF and is released to all tissues through TfR1 (Figure 1). The plasma iron concentration is just over 10% of the daily consumption so it must be replaced several times per day. Erythropoiesis takes place in bone marrow and daily 20-25 mg of iron is used in haemoglobin synthesis and recycled back to TF as monocyte-macrophage system phagocytizes senescent red blood cells (RBCs). Besides macrophages, excess iron is stored in liver in the form of ferritin (Hentze et al. 2010).

1.4 Iron homeostasis

As iron is needed in vital functions but its excess is toxic, physiological levels need to be carefully maintained. Since iron is not efficiently excreted from the body in mammals, the intra- and extracellular concentrations are kept in balance by regulating the intestinal absorption and release from the intracellular stores (Aisen et al. 1999). This is implemented through a complicated and partly still unidentified "orchestra" of which hepcidin is the conductor. For example, the origins and roles of the main iron deficiency laboratory markers, serum ferritin and soluble transferrin receptor (sTfR), remain unsolved. (Camaschella et al. 2020)

1.4.1 Systemic iron homeostasis

Bioactive mature hepcidin is a 25 amino acid long member of the defencin protein family produced in liver (Fleming 2008). It is secreted from hepatocytes and in plasma it is bound to α2-macroglobulin (Peslova et al. 2009). Besides being the major iron regulator, hepcidin is an acute phase protein that has strong, although still mostly unclear, links to innate immunity (Jordan et al. 2009; Weiss et al. 2019).

Hepcidin controls the iron export to the plasma from macrophages and hepatocytes, and possibly to some extent in enterocytes, by inhibiting its transport

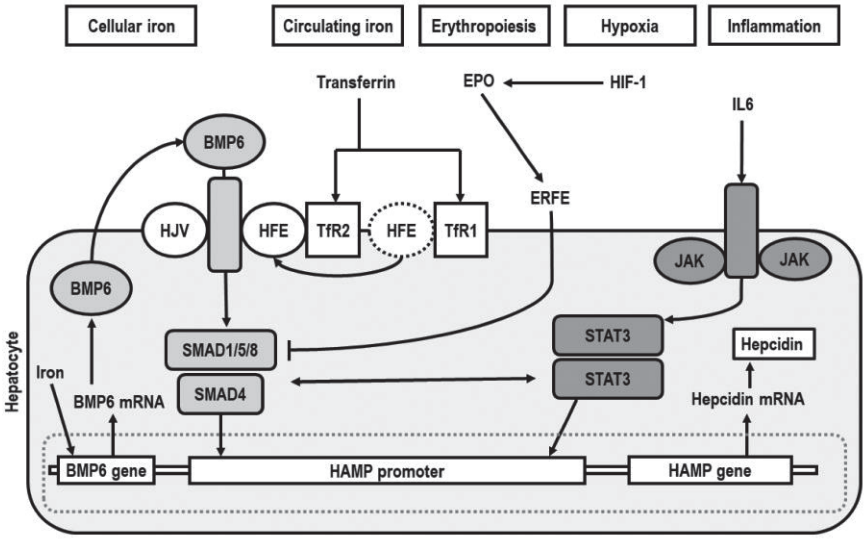
through FPN (Donovan et al. 2005; Drakesmith et al. 2015; Muckenthaler et al. 2017). This happens through two mechanisms: inducing the internalization and lysosomal degradation of FPN (Nemeth et al. 2004), and by occluding the central cavity of the transporter (Aschemeyer et al. 2018).

Hepcidin expression is regulated by multiple signals, including especially inflammation, systemic iron availability, hepatic iron stores, erythropoietic activity and hypoxia (Figure 2) (Canali et al. 2018; Kautz et al. 2008; Koch et al. 2017; Lim et al. 2019; Theurl et al. 2011). This regulation is most critically integrated transcriptionally by hepatocyte bone morphogenic protein (BMP)-SMAD pathway (Hentze et al. 2010) activated by TfR1, TfR2 and human homeostatic iron regulator proteins (HFE) (Goswami and Andrews 2006). Additionally, inflammatory cytokines, especially interleukin (IL) 6, upregulate hepcidin expression by activating the JAK-STAT pathway (Fleming 2007). This is thought to partially cause anaemia of inflammation, also known as anaemia of chronic disease, a common state e.g. in inflammatory bowel disease (IBD) and chronic kidney disease (Weiss et al. 2019).

Iron deficiency, expansion of erythropoiesis, hypoxia and testosterone have been found to inhibit hepcidin expression (Girelli et al. 2016; Hentze et al. 2010). The liver serine protease matriptase 2, which is encoded by TMPRSS6 and attenuates via BMP-SMAD signalling, has been found to be the most powerful inhibitor of hepcidin production (Du et al. 2008; Silvestri et al. 2008). Further, during stress erythropoiesis, such as after haemorrhage or in hypoxia, when more iron is needed, erythroblasts produce erythroferrone (ERFE) which suppresses hepcidin production in the liver (Kautz et al. 2014). Currently however, the regulatory mechanisms are only partially understood.

Apart from liver, macrophages themselves and intestinal dendritic cells (Bessman et al. 2020) may produce hepcidin in inflammation (Theurl et al. 2008), skin cells during necrotizing fasciitis (Malerba et al. 2020), and adipocytes in severe obesity (Bekri et al. 2006). Additionally, a tissue-specific hepcidin is found in heart (Lakhal-Littleton et al. 2016) brain (McCarthy and Kosman 2014) and kidneys (Kulaksiz et al. 2005). Currently the function of extra-hepatic hepcidin is unexplained, though a role in the local regulation of iron metabolism has been proposed (Camaschella et al. 2020).

Figure 2. A simplified figure of the major pathways of hepcidin regulation. The hepatocyte BMP-SMAD and JAK-STAT-pathways increase hepcidin mRNA transcription by binding on the hepcidin gene (HAMP) promoter areas. The BMP-SMAD pathway is upregulated by high intracellular and circulating iron. High hepatocyte iron concentration increases the production of bone morphogenic protein 6 (BMP6). Circulating transferrin binds to transferrin receptor proteins (TfR1 and TfR2) and, mediated by human homeostatic iron regulator protein (HFE), activates the pathway. A BMP coreceptor haemojuvelin (HJV) is also needed in the activation. The BMP-SMAD pathway is down-regulated in low oxygen levels when hypoxia-inducible factor 1 (HIF-1) increases erythropoietin (EPO) production in the kidneys. Thereafter, the EPO increases erythropoiesis in the bone marrow which in turn causes increased erythroferrone (ERFE) production. Additionally, inflammatory cytokines e.g. interleukin 6 (IL-6) activate the JAK-STAT and non-canonical BMP-SMAD pathways.



1.4.2 Cell iron homeostasis

Intracellular iron homeostasis is maintained by the iron regulatory protein-iron responsible element (IRP-IRE) system and is based on post-transcriptional control of relevant genes. More specifically, in iron deficiency TfR1 messenger ribonucleic acid (mRNA) is stabilized, and ferritin and FPN suppressed by IRP1 and IRP2, which bind to their conserved mRNA regions. Conversely, when cells are iron-replete, IRP1 is converted to aconitase by Fe/S clusters and IRP2 degraded. IRP2 acts in non-hypoxic tissue, whereas IRP1 acts in hypoxic tissues such as duodenum (Camaschella et al. 2020; Henderson 1996; Hentze et al. 2010).

1.4.3 Regulation of iron homeostasis in the intestine

In the intestinal mucosa, iron absorption is regulated by hepcidin, iron-dependent local IRP-IRE system and oxygen-dependent hypoxia-inducible factor 2α (HIF-2α). As well as systemic iron balance mechanisms, the local iron regulation and the role of the gut in whole-body iron homeostasis have been under rigorous investigation, but many details nevertheless remain unclear.

It has been suggested that in enterocytes, rather than inhibiting FPN, hepcidin reduces the iron absorption causing DMT1 internalization and proteasomal degradation (Brasse-Lagnel et al. 2011, Chaston et al. 2008, Chung et al. 2009, Mena et al. 2007). On the other hand, hepcidin has been shown to down-regulate the intestinal mRNA levels of DCYTB, DMT, FPN and HEPH (Bergamaschi et al. 2017).

In hypoxic duodenal environment, HIF-2α transcription is increased. HIF-2α upregulates the expression of both DMT1 and FPN (Mastrogiannaki et al. 2013). This process is thought to be enhanced by hepcidin downregulation, as this leads to increased FPN expression and consequently decreased iron concentration in enterocytes (Schwartz et al. 2019).

As previously mentioned, the IRP-IRE system also functions in enterocytes (Galy et al. 2008). However, both enterocytes and RBCs partially escape this regulation by producing FPN isoform lacking the 5'IRE, which ensures iron export to circulation even in iron deficiency (Zhang et al. 2009).

A great deal of iron loss from body occurs in the gut with the shedding of vigorously renewing enterocytes. This amount could be even higher in inflammation, when hepcidin is believed to decrease the amount of iron released from enterocytes. Interestingly, this excess of non-absorbed iron alters the gut microbiota by favouring the growth of iron-dependent enterobacteria (Bessman et al. 2020; Jaeggi et al. 2015).

A recent study revealed conventional dendritic cells in the gut of microbially stimulated mice and IBD patients to produce hepcidin even more than liver during intestinal inflammation (Bessman et al. 2020). This hepcidin did not deprive enterocyte FPN but acted on FPN-expressing phagocytes promoting local iron sequestration, limiting tissue-infiltrating microbes, and thus promoting mucosal wound healing.

1.4.4 Iron homeostasis and erythropoiesis

Two-hundred billion RBCs are produced daily in the bone marrow, requiring more than 2 x 10^{15} iron atoms every second to maintain adequate erythropoiesis. This means 20 ml of blood produced each day, containing six grams of haemoglobin and 20 mg of iron. The amount of iron in the body and erythropoiesis efficiency regulate each other at multiple levels (Hall 2016, Muckenthaler et al. 2017).

Haemoglobin synthesis begins in RBC precursors called proerythroblasts and continues in young RBCs called reticulocytes for another day after their release to circulation. Four separate polypeptide chains (α1, α2, β1, and β2) each containing an atom of iron form the haemoglobin molecule. The types of chains determine their capability to bind oxygen. Decreased circulating TF, for example due to iron deficiency, causes RBCs to contain less haemoglobin than normally, which makes them hypochromic and smaller, i.e. microcytic. Microcytic anaemia could also be caused by inherited disorders, such as thalassemia, in which there are defects in the synthesis of one or more of the haemoglobin chains (Muncie and Campbell 2009), and sickle cell disease with atypical haemoglobin molecules (Williams and Thein 2018).

Exposure to high altitude, severe blood loss and shortage of RBCs cause tissue hypoxia, which in the bone marrow is compensated by increased erythropoiesis stimulated by a renal hormone called erythropoietin (EPO). In iron deficiency, IRP1 binds to IRE of HIF-α2 and represses its translation and thus EPO production in the kidneys (Camaschella et al. 2020). Under hypoxia EPO production is increased in peritubular fibroblasts of the renal cortex by the binding of HIF-2 heterodimers to hypoxia-responsive elements of the EPO gene (Kuhrt and Wojchowski 2015).

Besides EPO, TfR2 can also bind to EPO receptor in the bone marrow and modify EPO sensitivity in an iron-dependent manner (Nai et al. 2015). Additionally, the HFE modulates erythroid iron homeostasis (Ramos et al. 2011). As mentioned before, erythropoiesis regulates hepcidin production by producing BNP-SMAD pathway inhibiting ERFE in response to EPO (Arezes et al. 2018).

Erythropoiesis is dependent on vitamin B12 and folic acid, which are vital for the synthesis of nucleotide acids. In deficiency of these essential nutrients, RBCs fail to proliferate rapidly and become larger than normal, i.e. macrocytic. Macrocytic RBCs have the normal capability to carry iron but are fragile and have a shortened life span (Hall 2016).

1.5 Special features of iron metabolism in children

Studies concerning on the regulation of iron metabolism have mostly been conducted in animal models and, to a limited extent, in human adults, whereas data on children is scarce (Lönnerdal 2017, Lönnerdal et al. 2015).

The current evidence suggests that to a large extent absent iron homeostasis regulation in early infancy, is possibly due to lack of regulation of DMT1 and FPN (Darshan et al. 2011; Domellöf et al. 2001; Domellöf et al. 2002; Domellöf et al. 2008; Frazer et al. 2007; Hicks et al. 2006; Leong et al. 2003a; Leong et al. 2003b; Lönnerdal 2017; Lynch et al. 2007). In animals, the mechanisms controlling intestinal iron absorption develop during the time of weaning (Darshan et al. 2011; Frazer et al. 2007; Leong et al. 2003a; Leong et al. 2003b). On the other hand, maturation of hepcidin regulation is a very early phenomenon, at least in children with inflammatory diseases (Berglund et al. 2011; Karaskova et al. 2018; Müller et al. 2012; Prentice et al. 2012).

2 ANAEMIA AND IRON DEFICIENCY

2.1 Overview

Anaemia is a condition characterized by a decreased quantity of RBCs and lower haemoglobin level, often accompanied by altered RBC morphology. Lower haemoglobin levels result in impaired delivery of tissue oxygen and consequently e.g. weakness and fatigue, but anaemia can also cause more long-lasting problems, for instance by impairing psychomotor and cognitive development and leading to defective immune system (Ekiz et al. 2005; Haas and Fairchild, 1989; Lozoff et al. 2007; Lozoff et al. 2013; McCann and Ames 2007; Sachdev et al. 2005; Shafir et al. 2008). Anaemia should not be considered a diagnosis; finding the cause(s) of it should always be further explored.

2.2 Epidemiology

Global anaemia prevalence has been shown to be over 30%, but this varies widely depending on the geographical location, age and sex of the population studied, and especially between high- and low-income countries (Kassebaum et al. 2015). As a general rule, children, women and people living in South Asia or Central, West, and East sub-Saharan Africa have higher prevalence of anaemia than do adult males and people living in Western countries (Kassebaum et al. 2015).

In children, anaemia is one of the most common chronic medical problems, affecting on average 16.7% of preschool children in Europe and as many as 64.6% in Africa (McLean et al. 2009). Although the overall percentage of people suffering from anaemia has been decreasing over time, in children under five years of age this number is still high and may in fact even be on the rise in developing countries (Kassebaum et al. 2015). Iron deficiency, which is the most common single nutrient deficiency in children, is also the most common reason for anaemia worldwide (McLean et al. 2008; Kassebaum et al. 2015; Rajantie, 2002).

2.3 Evaluation of anaemia in children

Paediatric anaemia is defined by sex- and age-specific haemoglobin reference values (Rfs, Table 1., (Marcdante and Kliegman 2019). The clinical presentation of anaemia varies significantly depending on its severity and speed of development, as well as on the cause and a child's age. For example, paleness, sweating, high heart rate and shortness of breath point to acute cause or severe anaemia, and bone pain, prolonged fever and poor wellbeing to malignancy (Rajantie 2002). In contrast, gradually developing anaemia is often asymptomatic, as young children are known to do well in their daily routines even with haemoglobin levels as low as 50 g/l (Lohi et al. 2014). The reason underlying anaemia should always be investigated and conditions requiring immediate treatment, such as leukaemia, haemolytic crises, and acute blood loss, should be recognized without delay.

Table 1. Finnish age- and sex -dependent paediatric reference values for haemoglobin and mean corpuscular volume (MCV). (Fimlab-ohjekirja, 2019)

Age	Haemoglobin, g/l	MCV, fl
1-7 days	150-230	88-126
7-30 days	100-206	85-123
1-2 months	95-130	80-103
2-6 months	95-141	76-97
6-12 months	100-141	72-87
1-2 years	100-142	73-87
2-4 years	100-142	73-87
4-13 years	110-155	73-95
Boys 13-17 years	130-160	76-98
Girls 13-17 years	125-160	78-102

Laboratory investigations in paediatric anaemia usually follow a fairly straightforward algorithm, in which the possible causes are categorized in accordance with specific blood count changes (Marcdante and Kliegman 2019). A change in another blood cell line besides erythrocytes indicates a need for immediate investigations to rule out leukaemia or aplastic anaemia. However, if the anaemia is the only blood cell line abnormality, the first step is to determine the age- and sex-dependent value of RBC size, in other words mean corpuscular volume (MCV, Table 1.). As already mentioned, various pathophysiological mechanisms and the anaemic

conditions behind them can lead to decreased or increased MCV, although the volume may also be normal (Table 2. See chapter 2.4).

Other important investigations in children with anaemia are the proportion and absolute count of young RBCs, called reticulocytes, which reflect the status of erythropoiesis in the bone marrow. The values are decreased or normal in iron deficiency anaemia (IDA), whereas increased reticulocyte count refers to the presence of RBC destruction (haemolysis) or haemoglobinopathies (Table 2.). RBC destruction may be due to RBC membrane disorders, enzyme deficiencies or immune-mediated haemolysis.

Besides the MCV and reticulocyte count, other important laboratory investigations in paediatric anaemia include complete blood count, RBC morphology and determination of iron status by sTfR and ferritin, as well as erythrocyte sedimentation rate (ESR), coeliac disease antibodies and possibly faecal blood occult test and/or calprotectin (Marcdante and Kliegman 2019). Furthermore, additional investigations such as bone marrow aspiration and biopsy, gastrointestinal endoscopies (See chapter 3.3) and imaging studies may be needed.

2.4 Specific causes of micro-, normo- and macrocytic anaemias and anaemias with reticulocytosis

Microcytic anaemias are caused by insufficient production of haemoglobin. By far the most common cause of microcytic anaemia is iron deficiency, but especially in certain geographic areas thalassemia is also quite frequent (Birgens and Ljung 2007; Kassebaum et al. 2015). Other possible causes of microcytic anaemia are e.g., chronic inflammatory diseases and prolonged infections, although these usually present with normocytic anaemia, and rarely copper deficiency, sideroblastic anaemia, hereditary pyropoikilocytosis, intoxication, haemoglobin C disease and tumours (Table 2.).

Most normocytic anaemias are associated with a systemic illness that impairs the synthesis of erythrocytes in bone marrow (Marcdante and Kliegman 2019). Chronic inflammatory diseases with systemic inflammation cause normocytic anaemia by three major pathophysiological pathways (Weiss et al. 2019). Increased production of inflammatory cytokines increases the production of hepcidin, which blocks intestinal iron absorption and causes retention of iron in reticuloendothelial cells resulting iron-restricted erythropoiesis. In addition, the production and biological activity of EPO is reduced impairing erythropoiesis, and RBC lifespan is shortened

by enhanced erythrophagocytosis as well as by mechanical damage caused by fibrin deposition in microvasculature.

Bone marrow infiltration by malignant cells moreover commonly leads to normocytic anaemia. In children this situation is related particularly to leukaemia. Additional causes for normocytic anaemia are, for example, transient erythroblastopenia and congenital hypoplastic anaemia (Table 2.).

Macrocytic anaemias are uncommon in children but, if present, are usually caused by a deficiency of vitamin B12 or folate. Other possible causes are bone marrow failures caused by aplastic anaemia or myelodysplastic syndromes, Fanconi anaemia, hypothyroidism and chronic liver disease or enzyme defects such as glucose-6-phosphate dehydrogenase deficiency or pyruvate kinase deficiency (Table 2.).

Besides those based on the size of RBCs, another anaemia category is those with reticulocytosis. These include haemoglobinopathies, enzymopathies and membranopathies, which are common in specific genetic backgrounds (Marcdante and Kliegman 2019). Additionally, different extrinsic factors, immune system reactions and drugs can cause the reticulocytosis (Table 2.).

2.5 Iron deficiency with and without anaemia

Iron deficiency, which is the most common cause of anaemia, is defined as depleted total-body iron, especially of macrophage and hepatocyte iron stores (Marcdante and Kliegman 2019). IDA is a systemic disorder affecting multiple aspects of health in various organs. It can be recognized first by lower ferritin values and elevated sTfR values. The microcytosis and particularly decreased haemoglobin values appear later as the iron deficiency continues and becomes more severe (Camaschella 2019; Evstatiev and Gasche 2012).

Table 2. Possible causes of anaemia in children categorized according to average red blood cell (RBC) size and presence of reticulocytosis. Modified from Marcdante and Kliegman 2019 and Rajantie 2002.

Microcytic RBC	Normocytic RBC	Macrocytic RBC	Reticulocytosis
Iron deficiency anaemia	Chronic inflammatory disease	Vitamin 12 deficiency	Haemoglobinopathy
Chronic blood loss	Persistent infection (e.g. *H. pylori*, parvovirus, cytomegalovirus)	Pernicious anaemia	Haemoglobin SS, S-C, S-β thalassemia
Malabsorption	Collagen-vascular disease	Ileal resection	Enzymopathy
Cow's milk allergy	Inflammatory bowel disease	Vegan diet	Glucose-6-phosphate dehydrogenase deficiency
Heavy menstrual bleeding	Acute blood loss	Abnormal intestinal transport	Pyruvate kinase deficiency
Chronic inflammatory disease	Malignancy/bone marrow infiltration	Congenital intrinsic factor or transcobalamin deficiency	Membranopathy
Persistent infection	Chronic renal failure	Folate deficiency	Hereditary spherocytosis
Thalassemia	Transient erythroblastopenia of childhood	Malnutrition	Elliptocytosis
β major, minor	Bone marrow aplasia/hypoplasia	Malabsorption	Ovalocytosis
α minor	Human immunodeficiency virus infection	Antimetabolite	Extrinsic factors
Sideroblastic anaemia	Hemophagocytic syndrome	Chronic haemolysis	Disseminated intravascular coagulation
Copper deficiency		Bone marrow failure	Haemolytic uremic syndrome
Aluminium or lead intoxication		Myelodysplasia	Thrombotic thrombocytopenic purpura
Hereditary pyropoikilocytoses		Fanconi anaemia	Abetalipoproteinemia
Haemoglobin C disease		Aplastic anaemia	Burns
Gastrointestinal cancer		Pearson syndrome	Wilson disease
		Hypothyroidism	Vitamin E deficiency
		Oroticaciduria	Immune haemolytic anaemia
		Chronic liver disease	Autoimmune
		Leasch-Nyhan syndrome	Isoimmune
		Hypersplenism	Drug-induced
		Drugs/alcohol	

The factors underlying iron deficiency are diverse and vary between different populations, ages and socioeconomic backgrounds. In developing countries, the main cause is poor nutrition and the situation is often exacerbated by recurrent haemorrhagic infections. In high-income societies iron deficiency more often results from disorders causing chronic bleeding and/or reduced iron absorption (Camaschella 2019, Kassebaum et al. 2015), although particularly among the youngest children nutritional causes such as excessive utilization of cow's milk, causing occult intestinal blood loss and inhibition of non-haem iron absorption, are not uncommon (Lohi et al. 2014; Ziegler 2011). Children undergoing rapid growth periods are particularly prone to iron deficiency, although their normal iron parameter values are poorly understood (Milman et al. 2009). Furthermore, iron supplementation for the first year of life is routinely advised in children born prematurely (Lohi et al. 2014).

Disorders causing decreased intestinal iron absorption include e.g. coeliac disease, IBD, autoimmune atrophic gastritis, *H. pylori* infection and uncommon genetic iron-refractory IDA, and those with chronic blood loss e.g. hookworm infestation, benign and malignant lesions in the gastrointestinal track, heavy menstruation and hereditary or drug-induced defects of haemostasis (Camaschella 2019; Lohi et al. 2014; Marcdante and Kliegman 2019). Sometimes IDA and anaemia of inflammation cannot be truly distinguished if a disease causes both reduced absorption of iron and/or chronic blood loss and depleted erythropoiesis. In these patients the size of RBCs may be normal and ferritin value normal or high, but sTfR is increased as a sign of iron deficiency (Camaschella 2019).

If the cause of IDA is not evident, the aforementioned disorders should be considered and additional laboratory tests, including coeliac disease antibodies, *H. pylori* antigen, faecal calprotectin, reticulocytes, ESR, full blood count and tests for haemorrhagic disorders, should be taken. Successful treatment of the causative disorder, when found, usually also improves the blood iron concentration, but additional supplementation for several months is usually recommended for more efficient repletion of iron reserves (Irwin and Kirchner 2001; Lohi et al. 2014; Powers and O'Brien 2019).

Whether to treat patients with a so-called subclinical iron deficiency, defined as low ferritin but normal haemoglobin, has been under debate in recent years. Iron depletion usually develops gradually and may thus remain asymptomatic for a long time, but it has also been suggested to be a common cause for unrecognized non-specific symptoms such as fatigue, sleep disorders and concentration difficulties even

without anaemia (Powers and O'Brien 2019). Accordingly, in pre-menopausal women with subclinical iron deficiency self-reported fatigue improves with iron supplementation (Krayenbuehl et al. 2014). In children, a marked subclinical iron deficiency causes cognitive difficulties and behavioural disorders, possibly even irreversibly (Lozoff et al. 2013; Pivina et al. 2019; Shafir et al. 2008). Additionally, the association between paediatric restless legs syndrome and subclinical iron deficiency and the benefits of iron supplementation in this condition have been demonstrated (Dosman et al. 2012; Grim et al. 2013; Tilma et al. 2013).

A recent review by Finnish paediatric haematologists recommends low dose oral iron supplementation for all children with IDA and, even without anaemia, for symptomatic children with serum ferritin less than 15 μg/l and asymptomatic children with serum ferritin less than 10 μg/l (Ryhänen et al. 2020). Intravenous iron supplementation is advised both in Finnish and international guidelines in case of disorders inhibiting intestinal iron absorption, or if immediate correction is needed. The procedure should be performed only in tertiary centres (Powers and O'Brien 2019; Ryhänen et al. 2020).

3 ANAEMIA IN GASTROINTESTINAL DISEASES

Several gastrointestinal disorders can lead to anaemia, either by chronic bleeding or by limiting the absorption of iron, vitamin B12 and folic acid from the duodenum as a result of mucosal damage and/or through inflammatory down-regulation of iron absorption (Bergamaschi et al. 2018; Weiss et al. 2019). The most common chronic gastrointestinal condition causing anaemia in children, at least in developed countries, is thought to be untreated coeliac disease.

3.1 Coeliac disease

3.1.1 Overview

Coeliac disease is a lifelong immune-mediated disorder in which ingestion of dietary gluten, naturally present in wheat, rye and barley, results in inflammation and structural damage in the duodenal mucosa, as well as possible extraintestinal manifestations in other organs (Green and Cellier 2008; Ludvigsson et al. 2013). The disease development requires genetic susceptibility, the most important predisposing genetic factors being the human leucocyte antigen (HLA) haplotypes HLA-DQ2 and HLA-DQ8, which are found in practically all coeliac disease patients (Karell et al. 2003, Kupfer and Jabri 2012, Sollid et al. 1989, Wolters and Wijmenga 2008). Besides dietary gluten and HLA susceptibility, other predisposing genetic and environmental factors, such as various microbial agents or gastrointestinal infections, have also been proposed to play a role in disease onset (Bouziat et al. 2017; Caminero et al. 2019; Kahrs et al. 2019; Kemppainen et al. 2017; Lindfors et al. 2019a).

Both the efficiency of clinical recognition and the true prevalence of coeliac disease seem to be rising (Kang et al. 2013; Lohi et al. 2007; Rubio-Tapia et al. 2009; Singh et al. 2018) and nowadays the disease is estimated to affect approximately 1% of the population worldwide (Singh et al. 2018) and 2% in Finland (Lohi et al. 2007). During the last two decades the incidence has continued to rise in many industrialized countries, whereas in Finland and Sweden a stabilization of incidence

has been seen (Ludvigsson and Murray 2019; King et al. 2020; Kivelä et al. 2015; Virta et al. 2017; Namatovu et al. 2014). The risk of coeliac disease is increased among first- and second-degree relatives of affected patients (Singh et al. 2015), as well as in patients with certain other immune-mediated conditions and genetic disorders (Collin et al. 2002; Freeman 2016).

3.1.2 Clinical features

The heterogenous clinical picture of coeliac disease varies from asymptomatic to the presence of various gastrointestinal and extraintestinal symptoms (Kivelä et al. 2015; Ludvigsson et al. 2013; Mäki et al. 1988; Volta et al. 2014). The so-called classic presentation is failure to thrive and diarrhoea in early childhood, but nowadays other gastrointestinal symptoms, such as abdominal discomfort and pain, bloating, indigestion and constipation, may be even more common (McGowan et al. 2009; Steens et al. 2005).

Extraintestinal manifestations of coeliac disease may affect almost any organ, and their pathogenesis is often obscure (Laurikka et al. 2018). In fact, even the definition is somewhat vague; for instance, frequently observed anaemia and reduced bone mineral density can be considered either as extraintestinal symptoms or as findings secondary to intestinal malabsorption and micronutrient deficiency (Heikkilä et al. 2015; Laurikka et al. 2018; Nurminen et al. 2015). One of the best-characterized extraintestinal presentations is dermatitis herpetiformis, in which a blistering and itching rash appears on elbows, knees and buttocks (Collin et al. 2017; Duhring 1884; Hervonen et al. 2014). Coeliac disease has also been associated to diverse neurological symptoms including gluten ataxia, peripheral neuropathy (Hadjivassiliou et al. 2006; Hadjivassiliou et al. 2008), migraine (Gabrielli et al. 2003) and epilepsy (Cooke and Smith 1966). Additionally, psychiatric and reproductive health problems (Slim et al. 2018), dental enamel defects (Aine et al. 1990) and abnormal liver values or even hepatic failure (Äärelä et al. 2016; Farre et al. 2002; Kaukinen et al. 2002a) have been reported.

Interestingly, over the years the clinical picture of newly diagnosed coeliac disease appears to have changed towards milder forms (Kivelä et al. 2015; Ukkola et al. 2011).

3.1.3 Pathophysiology

Gluten contains considerable amounts of proline amino acids, making it resistant to gastrointestinal digestive enzymes (Shan et al. 2002). In coeliac disease the poorly digested gluten peptides launch the activation of both adaptive and innate immune responses in the small intestines of individuals genetically susceptible to coeliac disease (Figure 3, Abadie et al. 2011; Sollid 2002).

An important step in coeliac disease pathogenesis is the deamination of gluten peptides by transglutaminase 2 (TG2) (Di Sabatino et al. 2012; Dieterich et al. 1997). According to a recent paper, this may already occur in the intestinal lumen (Iversen et al. 2020). The deamination increases the binding affinity of gluten to HLA-DQ2/DQ8 heterodimers expressed on antigen presenting cells of susceptible individuals (Molberg et al. 1998, Tollefsen et al. 2006). The HLA-bound gliadin peptides are recognized by gluten specific CD4+ T helper cells (Lundin et al. 1993; Van De Wal et al. 1998) which leads to their activation, clonal expansion and the secretion of interferon-γ, IL-21 and other cytokines creating an inflammatory milieu causing tissue damage in the duodenal mucosa (Bodd et al. 2010; Antonio Di Sabatino et al. 2012). The activated T cells may also emit signals causing both gluten- and TG2-specific B cells to activate and differentiate into plasma cells and to produce TG2-autoantibodies (TG2-abs) and antibodies targeting deaminated gluten peptides (du Pré et al. 2020; Stamnaes and Sollid 2015).

The triggers leading to the activation of the innate immune system in coeliac disease are obscure, but according to current understanding the mechanisms are mediated by IL-15 and other cytokines, which may cause increased epithelial permeability and cytotoxicity by activating intraepithelial lymphocytes (IELs) and non-suppressive T regulatory cells (Abadie et al. 2020; Lindfors et al. 2019b). Eventually the complex immunity responses lead to gradually developing small-bowel deterioration from a healthy mucosa to different stages of mucosal damage characterized by hyperplastic crypts, villous atrophy and increased number of IELs (Figures 3 and 4) (Kupfer and Jabri 2012; Kutlu et al. 1993; Marsh 1992).

Figure 3. A simplified illustration of the adaptive and innate immune responses involved in coeliac disease. **Adaptive immune mechanisms:** Insufficiently degraded gluten peptides cross the small-intestinal epithelium via trans- or paracellular pathway. In the lamina propria the peptides are deaminated by transglutaminase 2 (TG2). The deaminated peptides are presented to CD4+ T helper cells by HLA DQ2/DG8 molecules on the surface of antigen presenting cells. The activated CD4+ T helper cells then produce various inflammatory cytokines and activate B cells. The B cells differentiate into plasma cells and secrete antibodies against gluten peptides and TG2. **Innate immune mechanisms:** The inflammatory cytokines produced by stressed enterocytes and antigen presenting cells cause increased permeability and cytotoxicity by activating intraepithelial lymphocytes (IELs) and non-suppressive T cells. Figure adapted and modified from Lindfors et al. 2019.

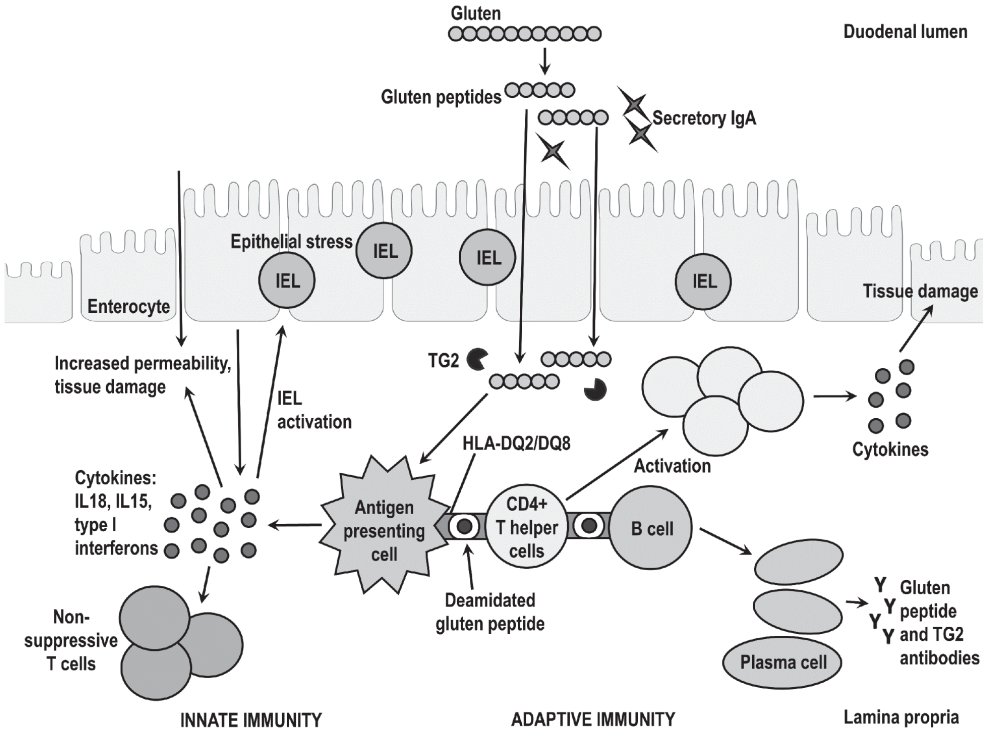
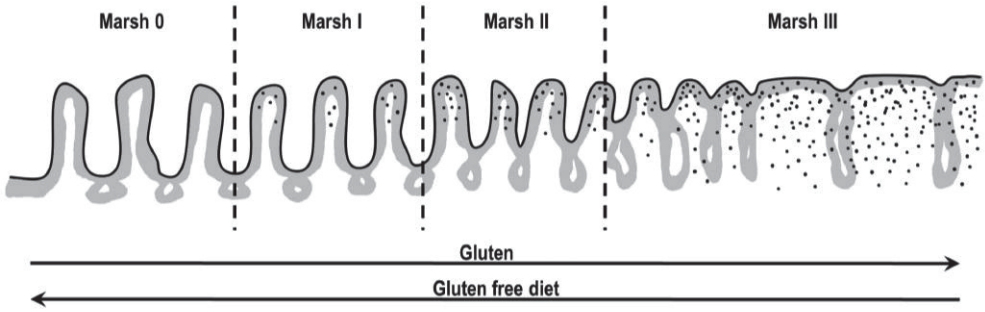


Figure 4. Gradual development of small-bowel mucosal inflammation and damage in an untreated coeliac disease patient consuming gluten. The changes can be reversed by gluten-free diet. Marsh 0 stands for a healthy mucosa with long villi and short crypts. Marsh I refers to an early state of coeliac disease where the mucosal architecture is normal, but an increased number of inflammatory cells can be detected. In Marsh II the crypts become elongated, and in Marsh III an overt villous atrophy and crypt hyperplasia can be seen. Adapted from Marsh, 1992.



3.1.4 Diagnosis and treatment

The diagnostics of coeliac disease has long been based on the determination of small intestinal mucosal morphology (Husby et al. 2012; Ludvigsson et al. 2014), but nowadays the investigations usually begin with the measurement of serum autoantibodies, particularly TG2-ab and endomysium antibodies (EmA). Additionally, once in a lifetime measurement of total immunoglobulin (Ig) A to exclude the possibility of selective IgA deficiency is usually recommended (Meini et al. 1996), at least in children (Husby et al. 2020). Traditionally, subjects with positive antibody tests have been referred to endoscopic confirmation of the duodenal damage and coeliac disease diagnosis (Ludvigsson et al. 2014). Other conditions should be considered in the case of negative autoantibodies (Gustafsson et al. 2020), although possible pitfalls for false negativity, including e.g. short duration of gluten consumption in infants, self-initiated gluten-free diet (GFD) and immunosuppression, should be kept in mind (Husby et al. 2020).

If it is decided to perform endoscopy, at least one representative mucosal biopsy should be taken from the anatomical duodenal bulb and four biopsies from the distal duodenum and sent for histopathologic evaluation (Husby et al. 2020; Ludvigsson et al. 2014). The mucosal morphology is usually classified according to either Marsh-Oberhuber (Figure 4) (Marsh 1992; Oberhuber et al. 1999) or Corazza-Villanacci

(Corazza and Villanacci 2005) criteria as follows: morphologically normal villi with or without mucosal inflammation (equivalent to Marsh 0–I or Corazza-Villanacci A), normal villi with crypt hyperplasia (Marsh II, Corazza-Villanacci A), partial and subtotal villous atrophy (P/SVA group, Marsh IIIa-b, Corazza-Villanacci B1), and total villous atrophy (TVA group, Marsh IIIc, Corazza-Villanacci B2). Marsh III or Corazza-Villanacci B1-B2 are considered to suffice for a diagnosis of coeliac disease. A more quantitative approach is to measure the villous height-crypt depth ratio, in which a ratio of <2.0 is equivalent to coeliac disease (Taavela et al. 2013). Regardless of the classification, only correctly oriented histological specimens with complete villus-crypt units and longitudinally cut crypts should be accepted for the histological analyses (Taavela et al. 2013). It must also be realized that villous atrophy is not pathognomonic to coeliac disease and other causes should be considered in inconclusive cases, particularly in the case of negative autoantibodies (Jansson-Knodell et al. 2018).

Modern antibody tests, especially positive EmA and high values of TG2-ab, possess close to 100% specificity for coeliac disease in both children and adults (Fuchs et al. 2019; Giersiepen et al. 2012; Lewis and Scott 2006; Werkstetter et al. 2017; Wolf et al. 2017). As a result, in 2012 the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) proposed for the first time that a paediatric coeliac disease diagnosis could in selective cases be established based on serology only, without no need for histological confirmation (Husby et al. 2012). In the recently revised guidelines this no-biopsy approach is allowed for all children with IgA class TG2-ab ≥10 times the upper limit of normal and positive EmA-IgA in a second serum sample (Husby et al. 2020). Only validated TG2-ab tests with a calibration curve should be applied. In all other circumstances endoscopy with duodenal biopsies is still required to confirm the diagnosis. In Finland, the Current Care Guidelines (Coealiac disease, Current Care Guidelines 2018) of 2018 specified a similar serology-based approach. What is more, unlike all the previous and even the most recent international guidelines (Al-Toma et al. 2019), the Finnish guidelines allow omitting the biopsy in some adults fulfilling the same criteria as children.

The issue of whether to treat so-called potential coeliac disease patients with positive coeliac autoantibodies but normal or only mildly damaged small bowel mucosa has been under discussion for years (Auricchio et al. 2014; Auricchio et al. 2019; Kurppa et al. 2009; Mandile et al. 2018; Tosco et al. 2011). It is known that these individuals may already present with various symptoms, including anaemia, before the development of diagnostic duodenal lesion (Kondala et al. 2016; Kurppa

et al. 2010; Shahriari et al. 2018; Tosco et al. 2011). Currently, however, there is no international consensus on whether potential coeliac disease should be treated with a GFD or if these individuals should only be monitored closely on a normal gluten-containing diet (Caio et al. 2019). The most recent ESPGHAN guidelines nevertheless give an option for a trial with a GFD in symptomatic cases after careful exclusion of other possible causes for the symptoms (Husby et al. 2020). The trial should be carried out under the supervision of a specialist with expertise in coeliac disease (Husby et al. 2020).

In the case of seronegative mucosal damage or in patients with borderline villous damage additional tools can be utilized. HLA typing can exclude coeliac disease if the person in question has neither HLA- DQ2 or HLA- DQ8 haplotype (Kaukinen et al. 2002b). Quantification of activated IELs in the duodenal mucosa and particularly detection of increased number of γδ-positive cells may provide additional value (Salmi et al. 2010). Furthermore, the recognition of TG2-targeted IgA antibodies bound to small bowel mucosa (IgA deposits) in tissue samples is a strong indication of active coeliac disease (Korponay-Szabó et al. 2004; Koskinen et al. 2008).

3.1.5 Anaemia in coeliac disease

Anaemia is one of the most common extraintestinal manifestations of untreated coeliac disease and may even be the only clinical finding (Çatal et al. 2015; Ludigsson et al. 2009; Martín-Masot et al. 2019). The reported prevalence of iron deficiency and anaemia in untreated coeliac disease has varied considerably between populations from nine to up to 93% (Table 3.).

Conversely, the risk of coeliac disease among individuals with IDA is also increased, the prevalence figures hitherto reported having been approximately 5% (Berry et al. 2018; Mahadev et al. 2018; Narang et al. 2018; Shahriari et al. 2018). As a result, many international guidelines recommend screening for coeliac disease in individuals with an unexplained anaemia (Goddard et al. 2011; Husby et al. 2020).

Both children and adult coeliac disease patients presenting with anaemia have been shown to suffer clinically, serologically and histologically from more severe disease than those presenting without anaemia (Berry et al. 2018; Singh et al. 2014) or with diarrhoea only (Abu Daya et al. 2013). Yet both iron deficiency and anaemia usually improve parallel with the histological recovery on GFD (Jericho et al. 2017).

Table 3. Prevalence of anaemia and iron deficiency in children and adults with untreated coeliac disease.

Reference	Number of patients	Country	Females, %	Anaemia %	Iron deficiency[1], %
Children					
Ben Hariz et al. 2007	26	Tunisia	77	15	-
Carroccio et al. 1998	130	Italy	55	70	-
Deora et al. 2017	140	Canada	62	11	35
Demir et al. 2000	104	Turkey	-	53	-
Dinler et al. 2009	87	Turkey	62	26	-
Garampazzi et al. 2007	307	Italy	-	21	-
Gokce and Arslantas 2015	191	Turkey	54	61	-
Kalayci et al. 2001	16	Turkey	44	25	-
Kochhar et al. 2012	434	India	75	84	-
Kuloğlu et al. 2009	109	Turkey	59	82	-
Jericho et al. 2017	157	USA	67	12	-
Mubarak et al. 2013	116	Netherlands	72	27	-
Nurminen et al. 2018	459	Finland	65	18	-
Oliveira et al. 2018	159	UK	69	23	63
Radlović et al. 2009	90	Serbia	62	52	-
Rashid et al. 2005	168	Canada	58	40	-
Roma et al. 2009	284	Greece	66	19	-
Sansotta et al. 2018	227	USA	66	12	-
Savilahti et al. 2010	197	Finland	68	24	43
Wessels et al. 2016	119	Netherlands	65	9	28
Adults					
Bergamaschi et al. 2008	132	Italy	64	34	-
Dahele et al. 2001	39	UK	82	41	-
Dickey and Hughes 2004	30	UK	60	83	-
Haapalahti et al. 2005	26	Finland	69	-	30
Harper et al. 2007	400	USA	67	21	24
Jericho et al. 2017	172	USA	80	48	-
Jones et al. 2006	32	UK	75	66	68
Kemppainen et al. 1998	40	Finland	70	30	33
Pulido et al. 2013	5912	Canada	75	68	-
Rampertab et al. 2006	590	USA	68	10	-
Sansotta et al. 2018	347	USA	78	48	-
Sategna-Guidetti et al. 2000	86	Italy	74	40	59
Schøsler et al. 2016	93	Denmark	76	30	40
Thomas et al. 2009	384	UK	73	54	70
Tikkakoski et al. 2007	14	Finland	86	-	14
Zanini et al. 2013	1382	Italy		41	-
Both children and adults					
Berry et al. 2018	103	India	53	93	82
Kochhar et al. 2012	298	India	66	94	-
Singh et al. 2014	338	India	53	85	-

USA, United States of America, UK, United Kingdom, [1]Serum iron, ferritin, transferrin iron saturation, transferrin receptor or transferrin receptor-ferritin index outside reference values.

Anaemia in coeliac disease is often attributed to damaged proximal small-intestinal mucosa and the resulting malabsorption of dietary iron. However, there are contradictory results about the correlation between the presence and severity of anaemia and the degree of histological damage (Abu Daya et al. 2013; Doyev et al. 2019; Harper et al. 2007; Mooney et al. 2016; Singh et al. 2014; Wierdsma et al. 2013), and even patients with potential coeliac disease may present with otherwise unexplained anaemia (Kondala et al. 2016; Shahriari et al. 2018; Tosco et al. 2013). These observations suggest that mechanisms other than malabsorption also contribute to the pathogenesis of anaemia in coeliac disease.

Deficiencies of folic acid and vitamin B12 are frequently seen in untreated coeliac disease and should thus be considered especially in case of macrocytic anaemia. A recent review reported folic acid depletion to have been observed in 11-75% of adults and 14-31% of children and vitamin B12 depletion in 5-19% adults and 1-14% of children at the time of diagnosis (Kreutz et al. 2020).

Even though coeliac disease is not characteristically associated with signs of major systemic inflammation, the gliadin-dependent activation of mononuclear cells may still lead to overproduction of proinflammatory cytokines and consequently promote hepcidin production (Cataldo et al. 2003; Ciccocioppo et al. 2005; A. Di Sabatino et al. 2006; Ludwiczek et al. 2003; Wang et al. 1995). Accordingly, four studies reported 4-17% of anaemic coeliac disease patients to present specifically with anaemia of inflammation, this being recognized by elevated levels of ferritin, reduced TF saturation, decreased sTfR, increased hepcidin, decreased EPO and/or increased interferon-γ (Bel'mer et al. 2014; Bergamaschi et al. 2008; Berry et al. 2018; Harper et al. 2007). Of note, sometimes inflammation and iron deficiency both contribute to the pathogenesis of anaemia in coeliac disease, and the possible presence of co-existing conditions such as IBD should also be born in mind (Martín-Masot et al. 2019).

Hepcidin has thus far been measured only in a few studies involving coeliac disease patients (Bel'mer et al. 2014; De Falco et al. 2018; Feruś et al. 2018). Bel'mer et al. showed decreased hepcidin in the majority of children with active coeliac disease, but also that 20% of them presented with elevated hepcidin levels suggesting the presence of anaemia of inflammation (Bel'mer et al. 2014). Interestingly, De Falco and colleagues found no differences in median hepcidin values between anaemic and non-anaemic coeliac disease patients at diagnosis, but the former group had lower levels on a GFD (De Falco et al. 2018).

Some case reports have also suggested aplastic anaemia to be associated with untreated coeliac disease (Badyal et al. 2014; Chatterjee et al. 2014; Irfan et al. 2018;

Maheshwari et al. 2012). In contrast, the evidence supporting the presence of increased faecal blood loss is weak, and the current understanding is that gastrointestinal bleeding is not an important contributor to anaemia and iron deficiency in coeliac disease (Fine 1996; Mant et al. 2006).

At intestinal level, it has been suggested that abnormal expression of iron transporter proteins could explain anaemia in coeliac disease. Currently, however, there are only a few studies on this issue, and even these have reported contradictory findings (Barisani et al. 2004a; Matysiak-Budnik et al. 2008; Sharma et al. 2009). Barisani et al. investigated DCYTB, DMT1, FPN, HEPH, TfR1 and IRP expression in duodenal biopsies and found all but DCYTB expression to be upregulated in anaemic coeliac disease patients similarly to anaemic non-coeliac controls (Barisani et al. 2004a). This suggests that the differential expression of the proteins is not associated with the disease itself but rather to constitute adaptive changes caused by iron deficiency. In contrast, Sharma et al. found DMT1 and FPN expressions to be increased in both anaemic and non-anaemic coeliac disease, suggesting that the changes reflect the immature epithelium of the duodenal mucosa (Sharma et al. 2009). Additionally, Tolone et al. showed increased DMT1 expressions in children with mild but not with severe mucosal atrophy when compared to normal mucosa (Tolone et al. 2017).

Of note, in 2008 Matysiak-Budnik et al. reported an interesting relationship between the iron metabolism and pathogenesis of coeliac disease, i.e. a retrotranscytosis of IgA-gliadin complexes through overexpressed TfR1 in duodenal enterocytes (Matysiak-Budnik et al. 2008). Later the possible role of TfR1 in gluten intake has also been supported by others (Heyman and Menard 2009; Lebreton et al. 2012; Papista et al. 2012).

Finally, mutations in genes modifying iron metabolism have been suggested as additional contributors to anaemia in coeliac disease. HFE mutations H63D and C282Y have been shown to have a protective effect against anaemia in coeliac disease patients (Butterworth et al. 2002; De Falco et al. 2018; Zanella et al. 2015), although this was not seen in a study by Barisani et al. (Barisani et al. 2004b). Furthermore, TMPRSS6 gene variant A736V has been found to be overrepresented in coeliac disease (Elli et al. 2015) and to predict inadequate oral iron supplementation response in anaemic patients (De Falco et al. 2018). Additionally, a polymorphism DMT1-IVS4+44AA in DMT1 gene has been shown to be significantly more frequent in anaemic than in non-anaemic children with coeliac disease and to confer a four-fold risk for the development of anaemia (Tolone et al. 2017).

3.2 Other gastrointestinal diseases causing anaemia in children

IBD is one of the most common gastrointestinal causes of anaemia, including children and adolescents who account for approximately 25% of IBD patients (Benchimol et al. 2011). IBD associated anaemia even has its own guidelines (Dignass et al. 2015; Gasche et al. 2007; Miele et al. 2018). As in coeliac disease, anaemia in IBD is thought to be multifactorial, including general and mucosal inflammation, blood loss via gastrointestinal bleeding and iron malabsorption as a result of hepcidin inhibited absorption through enterocytes (Weiss and Gasche 2010). The overall prevalence of anaemia in IBD is about 24%, but this varies markedly depending on the disease subtype and patient characteristics (Filmann et al. 2014). IBD is divided into three subtypes with certain general differences as regards anaemia. Crohn's disease is characterized by granulomatous inflammation in any part of the gastrointestinal tract, ulcerative colitis is a non-granulomatous form limited to the colon and IBD unspecified cannot be categorized to either of these two forms (Wilson and Russell 2017). Crohn's disease is the most common form in children and usually presents with anaemia of inflammation, whereas IDA is a typical feature of ulcerative colitis (Aljomah et al. 2018). Anaemia has been shown to adversely affect the quality of life of IBD patients (Wells et al. 2006) and either oral or intravenous iron supplementation is always recommended in case of active disease and low haemoglobin (Miele et al. 2018).

In high/moderate income countries a nowadays rare but in developing countries a frequent cause of anaemia in children is *H. pylori* gastritis (Fayed et al. 2008; Ferrara et al. 2006; Gulen et al. 2011; Huang et al. 2010). Untreated *H. pylori* infection has multiple ways to cause anaemia, including active haemorrhage secondary to gastritis and peptic ulcer, reduced secretion of ascorbic acid, increased iron utilization by the bacteria, production of proinflammatory cytokines and in the long run even gastric cancer (Franceschi et al. 2014). Additional causes of anaemia in developing countries are intestinal parasitic infections, particularly those caused by *Giardia duodenalis, Schistosoma sp., T. trichura* and hookworm (Hesham et al. 2004).

Other possible, although in children relatively uncommon, gastrointestinal causes of anaemia are autoimmune gastritis, eosinophilic gastrointestinal disorders, autoimmune enteropathy, hiatal hernia, long-standing use of proton-pump inhibitors or nonsteroidal anti-inflammatory drugs and gastrointestinal tumours (Bergamaschi et al. 2018; Stein et al. 2016; Zhu et al. 2010).

3.3 Gastrointestinal endoscopies in the evaluation of anaemia

The investigation of gastrointestinal disorders underlying anaemia often requires either OGD or colonoscopy, or both. Additionally, a wireless capsule endoscopy (WCE) or double-balloon endoscopy are sometimes needed in order to evaluate the whole small intestine. In children these invasive investigations, apart from the WCE, almost always require general anaesthesia. The ESPGHAN and European Society of Gastrointestinal Endoscopy guidelines recommend performing both OGD and colonoscopy in case of unexplained anaemia, but the evidence behind this approach currently remains weak (Thomson et al. 2017). As a possible means for better targeting of endoscopies, non-invasive biochemical markers, including particularly positive coeliac antibodies, low MCV, high ESR, faecal calprotectin, faecal blood occult test and *H. pylori* stool antigen test may predict gastrointestinal lesions in anaemic children (Fayed et al. 2008; Majid et al. 2008; Wang et al. 2017), but in many cases the decision whether to conduct the invasive investigations still relies solely on clinicians' experience. A couple of studies have suggested that symptoms such as haematemesis, dysphagia and vomiting in OGD and rectal bleeding in colonoscopy may increase the diagnostic yield (Noble et al. 2008; Wang et al. 2017).

THE PRESENT STUDY

4 AIMS OF THE STUDY

The main aims of the present study were to evaluate the diagnostic yield of gastrointestinal endoscopies in children with anaemia and to ascertain the pathogenesis and clinical significance of anaemia and iron deficiency in children in different stages of coeliac disease.

The specific aims were:

1. To investigate the diagnostic yield of gastrointestinal endoscopies in children with unexplained anaemia and the long-term prognosis of those who did not receive a diagnosis at the time of the first endoscopies(**I**).

2. To characterize the clinical, serological and histological manifestations in children with anaemia at coeliac disease diagnosis (**II**).

3. To investigate the prevalence of anaemia and markers of iron deficiency in children with potential and histologically verified coeliac disease (**III**).

4. To characterize the role of duodenal iron transporter proteins in the pathogenesis of anaemia in children with potential and histologically verified coeliac disease (**IV**).

5 PATIENTS AND CONTROL SUBJECTS

All studies were conducted at the Tampere Centre for Child Health Research and Celiac Disease Research Center, Tampere University, and the Department of Paediatrics, Tampere University Hospital. Additionally, Study IV was partly conducted in the National Institute for Mother and Child Health in Bucharest, Romania.

5.1 Patients in Study I

The retrospective cross-sectional study comprised all consecutive children who underwent gastrointestinal endoscopies (n=2395) at the Department of Paediatrics, Tampere University Hospital between 2007 and 2014. After excluding children with control endoscopies and without precise anaemia data, 864 children (age < 17 years) comprised the final study cohort, which was further divided into anaemic and non-anaemic subgroups. Relevant clinical and laboratory findings, as well as serological and histological data, were collected from medical records at the time of the first endoscopies. In addition, follow-up data from two to 11 years was collected, including the presence of possible new endoscopies and/or gastrointestinal diagnoses.

5.2 Patients in Study II

The study cohort comprised all the children (age <18 years) with biopsy-proven coeliac disease diagnosed at the Department of Paediatrics, Tampere University Hospital since the year 2000. Data on the clinical, histological and serological findings were collected systemically from patient records. From the year 2012 onwards, most of the participants were enrolled prospectively. Altogether 455 children comprised the final study cohort, which was further divided into anaemic and non-anaemic groups based on haemoglobin status at diagnosis. Follow-up data

regarding adherence and clinical response to the GFD was collected at six and 12 months from diagnosis.

5.3 Patients in Studies **III** and **IV**

The cohort for Study **III** included all consecutive children (age <16 years) with positive coeliac autoantibodies referred to the Department of Paediatrics, Tampere University Hospital from 2012-2015. In Study **IV,** 37 children with or without anaemia were selected from the same cohort. All children underwent a thorough clinical examination and blood sampling for relevant laboratory parameters and for coeliac disease serology and genetics. After OGD and histopathological evaluation of small-bowel mucosal biopsies, children with villous atrophy and crypt hyperplasia received a coeliac disease diagnosis. In Study **III**, these subjects were further categorized according to the degree of mucosal damage into those with P/SVA and TVA. In both Studies **III** and **IV**, children with positive coeliac autoantibodies but morphologically normal duodenal villi comprised the potential coeliac disease group.

In Study **III**, children with potential coeliac disease were monitored every three to six months during the study period (up to 2.5 years) and a new endoscopy was performed by decision of the clinician based on a marked increase in the antibody values or exacerbation of the symptoms. Additionally, after the patients with verified coeliac disease had been on a GFD for a minimum of three months, the values of iron parameters were re-measured.

5.4 Control children (**III, IV**)

Altogether 23 healthy children with no suspicion of coeliac disease and negative serum autoantibodies comprised the non-coeliac control group in Study **III**. They had participated as family members in a previous screening study in which coeliac disease serology, various laboratory parameters and genetics were evaluated (Kurppa et al. 2012). Four Finnish and two Romanian children with normal duodenal villi and negative autoantibodies comprised the non-coeliac control group in Study **IV**. Three of the children were anaemic and three non-anaemics. The control patients were endoscopied due to various abdominal complaints, and were diagnosed with gastroesophageal reflux disease (GERD), irritable bowel syndrome or remained without diagnosis.

6 STUDY PARAMETERS AND METHODS

6.1 Clinical data (**I-IV**)

The clinical data in Studies **I** and **II** was gathered from the medical records. In Studies **III** and **IV**, the corresponding data was recorded at the time of diagnosis and during the follow-up by the paediatrician in charge. The information collected included demographic and anthropometric data, clinical presentation and duration of the symptoms at presentation, as well as the possible presence of poor growth, coexisting chronic conditions (e.g. type 1 diabetes, Down syndrome, asthma and allergies) and other previously received diagnoses and family history of coeliac disease (in Study **I** also for IBD).

In Study **I**, the presenting symptoms were further categorized into abdominal pain, diarrhoea, melena/haematochezia, poor weight gain/growth failure, constipation, vomiting, reflux and dysphagia, and other (e.g. fatigue, persistent fever, dermatologic symptoms). In Studies **II** and **III**, the coeliac disease patients were categorised into those presenting with either gastrointestinal (e.g. diarrhoea, stomach pains, constipation, bloating) or extraintestinal symptoms (e.g. neurologic symptoms, rash, poor growth, anaemia, fatigue, arthralgia) and those detected by screening in at-risk groups (e.g. coeliac disease in relatives or previous type 1 diabetes). In addition, in Study **II** the gastrointestinal symptoms were further subcategorized into abdominal pain, constipation, diarrhoea, vomiting and other, and extraintestinal symptoms into aphthous ulcers, joint symptoms, liver abnormalities, neurological symptoms, poor growth, skin symptoms and other. Moreover, the severity of symptoms was categorized into "none", "mild" (occasional, somewhat bothersome gastrointestinal or extraintestinal symptoms) and "moderate/severe" (frequent and/or severe symptoms significantly disturbing daily life) (Kivelä et al. 2015).

In Study **II**, adherence to GFD was evaluated by a clinician by interviewing the patient and parents. Adherence was considered "strict" if they reported only a few minor lapses during the follow-up period. Less than one lapse in a month was rated as "occasional lapses" and more lapses as "no GFD".

6.2 Laboratory parameters and hepcidin (**I-IV**)

The following laboratory parameters were collected as available from the medical records: blood haemoglobin and MCV (Rfs presented in Table 1), plasma ferritin (Rf > 6-10 mg/L), total iron (Rf 6–25 mmol/L), albumin (Rf from 35-46 to 37-51 g/L), alanine aminotransferase (Rf < 40 U/L), alkaline phosphatase (Rf 115–460 to 80–445 U/L), thyroid-stimulating hormone (Rf 0.27–4.2 mU/L), thyroxine (Rf 11.0-12.6 to 21.0-32.0 pmol/L) and sTfR (age and gender-dependent Rfs from 1.6-2.0 to 5.1-7.0 mg/L), folate (Rf 10.4–42.4 nmol/L) and vitamin B12 (Rf 140–490 pmol/L) and TF iron saturation (Rf 15%–50%), ESR (Rf < 15 mm/ h) and faecal calprotectin (Rf < 100 mg/g) (Fimlab-ohjekirja, 2019). In Studies **III** and **IV**, the values of iron parameters (haemoglobin, sTfR, ferritin, plasma total iron, TF, TF iron saturation, vitamin B12 and folate) were measured with standard methods from frozen patient sera. To calculate the possible effect of the severity of anaemia on the likelihood of a subsequent diagnosis in Study **I**, haemoglobin values at the time of initial OGD and/or colonoscopy were further subclassified using the World Health Organization criteria (WHO, 2011).

For Studies **III** and **IV**, the serum bioactive hepcidin (hepcidin-25) levels were measured with parallel samples using a commercial solid-phase enzyme-linked immunosorbent assay (EIA-5258, DRG Diagnostics, Marburg, Germany).

6.3 Coeliac disease serology and HLA genotype (**I-IV**)

Serum EmA titres were measured by indirect immunofluorescence (in-house) with human umbilical cord as substrate (Ladinser et al. 1994). A dilution 1: ≥5 was considered positive and positive sera were further diluted 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:4000. The EliA Celikey test (Phadia, Uppsala, Sweden) was used to determine serum TG2-ab. Cut-off for TG2-ab positivity was >7.0 U/L according to the manufacturer's instructions. HLA DQ2/DQ8 genotyping was performed using the SSP low-resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden).

6.4 Gastrointestinal endoscopies and other diagnostic investigations (**I-IV**)

The patients with a suspected pathology in the upper alimentary tract in Study **I** and all patients in Studies **II-IV** underwent OGDs under general anaesthesia. If possible, the mucosal biopsies were collected systematically in each endoscopy regardless of the preceding clinical and laboratory findings. At least two specimens were taken from the lower oesophagus, and gastric corpus and antrum and at least four specimens from the duodenum. After 2012, the specimens were also obtained systematically from the anatomic duodenal bulb and middle/upper part of the oesophagus.

In Study **I**, the children with clinical suspicion of pathology in the lower alimentary tract underwent colonoscopy. A routine biopsy collecting protocol was also conducted during colonoscopy, including sampling at least from rectum, sigmoid/descending colon, ascending colon/cecum and terminal ileum. Additional biopsies were taken as indicated by clinical presentation or macroscopic findings during the endoscopy. All possible visual abnormalities were reported systemically by the endoscopist and, if considered significant, also photographed.

Other possibly conducted relevant investigations of the gastrointestinal tract, such as WCE, magnetic resonance enterography and oesophageal pH monitoring and their findings were also recorded in Study **I**.

6.5 Histological analysis of the gastrointestinal biopsies (**I-IV**)

In all studies, the endoscopically obtained duodenal specimens were paraffin-embedded, cut, stained with haematoxylin and eosin then evaluated by experienced pathologists. Only correctly oriented histological samples with complete villus-crypt units and longitudinally cut crypts were accepted for microscopic analyses (Taavela et al. 2013). The specimens of children with positive coeliac autoantibodies in Studies **I-IV** were further graded on the basis of the histological findings into those with morphologically normal villi with or without mucosal inflammation (potential coeliac disease group) and those with mucosal atrophy, and in Studies **II** and **III** into those with P/SVA and TVA (Marsh 1992; Oberhuber et al. 1999).

In Study **I**, the results from the evaluations of all other gastrointestinal biopsy specimens from upper and lower gastrointestinal tract were also recorded. All abnormalities reported by a pathologist, even if not diagnostic, were regarded as

histologic findings (e.g. unspecified duodenal inflammation, mildly increased number of oesophageal eosinophils and inactive chronic gastritis).

In addition, in Study **III** at least one biopsy from proximal and distal duodenum were freshly embedded in optimal cutting temperature compound (Tissue-Tec, Miles Inc, Elkhart, IN) and snap frozen in liquid nitrogen for the measurement of mucosal IELs and IgA deposits. The staining was performed using 5 μm thick frozen biopsy sections. The mucosal $CD3^+$ IELs were stained with monoclonal antibody Leu-4 (Becton Dickinson, San Jose, CA) and $\gamma\delta^+$ IELs with T-cell receptor-γ antibody (Endogen, Woburn, MA). A 100x flat field light microscope objective was used to count positive IELs and the results were expressed as cells/mm of epithelium (Järvinen et al. 2003). IgA deposits were measured by direct immunofluorescence (Koskinen et al. 2008; Tosco et al. 2008).

6.6 Measurements of the duodenal iron transporter proteins (**IV**)

The expressions of DCYTB, DMT1, FPN, HEPH and TfR1 were evaluated by immunohistochemistry. At first, 5 μm thick sections were cut from the formalin-fixed, paraffin-embedded duodenal specimens. The sections were deparaffinated and rehydrated antigens were exposed by heat-induced epitope retrieval. Thereafter non-specific staining was blocked followed by overnight incubation with primary antibodies (Supplementary Table 1 in the original publication **IV**). After washing the primary antibodies, the sections were incubated overnight with a secondary antibody (bionylated anti-rabbit, bionylated anti-goat or ImmPRESS peroxidase Anti rabbit Ig) prior to blocking of endogenic peroxidase and visualization of the staining with either ImmPRESS- or VECTASTAIN Elite ABC -reagent (Vector Laboratories Inc, Peterborough, United Kingdom). Finally, sections were counterstained with haematoxylin.

A SlideStrider scanner was used to scan all stained sections as whole-section images at a resolution of 0.16 μm per pixel (Jilab Inc., Tampere, Finland). Images were stored as JPX-files and viewed with Jilab's JVSview program from where they were exported to Fiji Image J program for further analysis (Schindelin et al. 2012). Of the DCYTB sections, where the staining took place mainly on the apical border of enterocytes, both the entire visible epithelial apical membrane and the DCYTB-stained membrane were drawn and measured. To assess the stained percentage of the apical border, the stained membrane length was divided by the whole membrane length. Thereafter, from the sections with DMT1, FPN, HEPH and TfR1, the

epithelium was selected, other parts cut out and the images consisting of only epithelium were stored as TIF-files. Subsequently, the files were transferred to Matlab-program (The MathWorks Inc. Natick, Massachusetts) and transformed from RGB to HSV images. To measure only the primary antibody staining, a red colour was chosen from the hue channel within values 0-0.1 and 0.9-1.0 (Supplementary Figures 1 and 2 in original publication **IV**). Next, the saturation channel was thresholded according to all sections in each stained protein series using Otsu's method (Otsu, 1979). Finally, the mean saturation of each section was divided by the maximum saturation of the protein series to measure the strength of the staining and the percentage of stained area was measured for each section.

6.7 Statistical analysis (**I-IV**)

Clinical characteristics and the prevalence of abnormal laboratory values and endoscopic and histologic findings are presented as percentage distributions. Chi-squared test or Fisher's exact test were used to compare the qualitative parameters as appropriate. The skewness of the quantitative data was assessed by the Shapiro-Wilk method and most of the variables in the studies were not normally distributed. For the sake of simplicity, all data are thus expressed as medians with quartiles except for ages, which are expressed as medians with ranges. Nonparametric quantitative parameters were compared using the Mann-Whitney U test or Kruskal-Wallis one-way analysis of variance as appropriate. The associations between symptoms/clinical signs and diagnoses in Study **I** were calculated using binary logistic regression analysis with 95% confidence intervals (CIs). Changes within the coeliac disease groups on a GFD in Study **III** were compared using either the paired t test or Wilcoxon signed-rank test as appropriate. In Study **IV** correlations between hepcidin, sTfR, serum ferritin and iron transporter saturations and stained areas/lengths were calculated with Spearman's rank (rS) correlation. P values <0.05 were considered significant in all studies **I-IV**. All statistical analyses were performed using IBM SPSS Statistics (Versions 23-26, IBM Corp. Armonk, NY, USA).

6.8 Ethical considerations (**I-IV**)

The study design and data collection in Study **I** were approved by the Department of Paediatrics, Tampere University Hospital. According to the national guidelines in

Finland, this registry-based study required no ethical approval. The ethics committee of Pirkanmaa Hospital District approved the study designs and data collection in Studies **II-IV**. In Study **IV**, similar approval was obtained from the Ethics Committee of the University of Medicine and Pharmacy "Carol Davila" and the National Institute for Mother and Child Health "Alessandrescu-Rusescu". In all studies, possibly identifiable personal data were pseudonymized and analyses performed anonymously. Furthermore, all prospectively recruited children and/or their guardians gave written informed consent (Studies **I-IV**). All studies followed the ethical guidelines of the Declaration of Helsinki (World Medical Association 1964).

7 RESULTS

7.1 Diagnostic yield of endoscopies and long-term prognosis in anaemic children (**I**)

Altogether 251 (29.1%) out of the 864 children with available haemoglobin/anaemia data in Study **I** had anaemia. Of these, 128 (51.0%) had undergone OGD alone and 10 (4.0%) colonoscopy alone, whereas the remaining 113 (45.1%) had undergone both OGD and colonoscopy. The corresponding figures for the 613 non-anaemic children were 311 (50.7%), 70 (11.4%) and 232 (37.8%) respectively. Additional alimentary tract investigations were conducted on 93 (37.1%) anaemic and 214 (35.0%) non-anaemic children, including WCE in 22 (8.8%) and 37 (6.0%) respectively.

Anaemic children were significantly older and had more often poor weight gain/growth failure and coexisting autoimmune diseases, whereas abdominal pain, reflux, dysphagia and other non-specific symptoms were more common among the non-anaemic patients (Table 4). In addition, the anaemia group showed more often high faecal calprotectin and low MCV values and had lower median ferritin and albumin values and higher sTfR and ESR values (Table 4).

In the histologic evaluations of OGD and colonoscopy biopsies, abnormalities were more common in anaemic than in non-anaemic children in all biopsy locations except the oesophagus (Table 2 in the original publication **I**). Anaemia also significantly increased the likelihood of receiving a diagnosis (anaemic children 73.0% vs. non-anaemic children 39.3% respectively, odds ratio (OR) 4.18, 95% CI 3.03-5.77). The most common diagnoses were IBD (anaemic 31.1% vs. non-anaemic 9.1%, $p<0.001$), coeliac disease (25.9% vs. 15.5%, $p<0.001$), *H. pylori* gastritis (4.4% vs. 2.0%, $p=0.044$), GERD, 2.8% vs. 4.8%, $p=0.165$) and gastrointestinal food allergy (2.0% vs. 2.3%, $p=0.791$) (Figure 5). During follow-up, an additional four anaemic (1.6%, Crohn's disease, GERD, angiodysplasia and Imerslund-Gräsbeck syndrome) and 33 non-anaemic (5.4%, celiac disease [n=9], Crohn's disease [n=6], GERD [n=5] and gastrointestinal food allergy [n=4]) children received diagnoses.

Predictors for a diagnosis in anaemic patients were age from five to twelve years and presence of diarrhoea, melena/haematochezia, poor weight/height gain,

positive coeliac antibodies, high calprotectin or ESR and hypoalbuminemia (Table 5). In contrast, children with reflux symptoms were less likely to receive a diagnosis (OR 0.20, 95% CI 0.08-0.49). Excluding diarrhoea and hypoalbuminemia, the above-mentioned findings and symptoms increased the probability of diagnosis also in non-anaemic children.

Table 4. Characteristics of 864 children with and without anaemia at the time of their first gastrointestinal endoscopies in Study **I**.

	Anaemia N=251		No anaemia N=613		P value
Categorical variables	**N**	**%**	**N**	**%**	
Girls	135	53.8	326	53.2	0.872
Autoimmune disease[1]	20	8.0	27	4.4	0.036*
Asthma, allergy, or atopy	44	17.5	116	18.9	0.632
Other chronic disease	32	12.7	58	9.5	0.151
Intestinal disease in relatives[2]	48	19.1	153	25.0	0.065
Positive EmA or TG2-ab	70[3]	39.5	132[3]	31.5	0.058
Faecal calprotectin > 100µg/g	65[4]	69.1	84[4]	33.1	<0.001*
Low MCV	91[5]	42.3	38[5]	6.8	<0.001*
Symptoms					
Abdominal pain	135	53.8	405	66.1	<0.001*
Diarrhoea	82	32.7	206	33.6	0.791
Melena/haematochezia	69	27.5	154	25.1	0.470
Poor weight gain/growth	64	25.5	96	15.7	<0.001*
Constipation	38	15.1	94	15.3	0.942
Vomiting	26	10.4	79	12.9	0.302
Reflux	23	9.2	90	14.7	0.029*
Dysphagia	1	0.4	24	3.9	0.005*
Other symptoms	101	40.2	307	50.1	0.009*
Continuous variables	**No. of data**	**Median (quartiles)**	**No. of data**	**Median (quartiles)**	
Age, years (range)	251	10.8 (0.0, 16.7)	613	8.3 (0.0, 17.7)	0.012*
Ferritin, µg/l	62	7.5 (5.0, 15.3)	70	20.5 (13.8, 31.0)	<0.001*
sTfR, mg/l	58	7.2 (5.0, 14.5)	37	4.3 (3.7, 5.2)	<0.001*
ESR, mm/hr	168	12.0 (5.0, 25.75)	434	7.0 (5.0, 11.3)	<0.001*
Alanine aminotransferase, U/l	172	19.5 (14.0, 29.0)	399	17.0 (14.0, 24.0)	0.189
Albumin, g/l	102	36.0 (33.0, 40.0)	305	40.0 (37.0, 43.0)	<0.001*

[1]Type 1 diabetes, rheumatic or thyroidal disease, coeliac disease, [2]Coeliac disease or inflammatory bowel disease, [3]Data from 177 anaemic and 419 non-anaemic patients, [4]Data from 94 anaemic and 254 non-anaemic patients, [5]Data from 215 anaemic and 557 non-anaemic patients, *P-value≤0.050.
EmA, endomysium antibodies, ESR, erythrocyte sedimentation rate, MCV, mean corpuscular volume, TG2-ab, transglutaminase 2 antibodies, sTfR, soluble transferrin receptor.

Figure 5. Diagnoses set in the initial investigations of anaemic and non-anaemic children in Study **I**. IBD, inflammatory bowel disease.

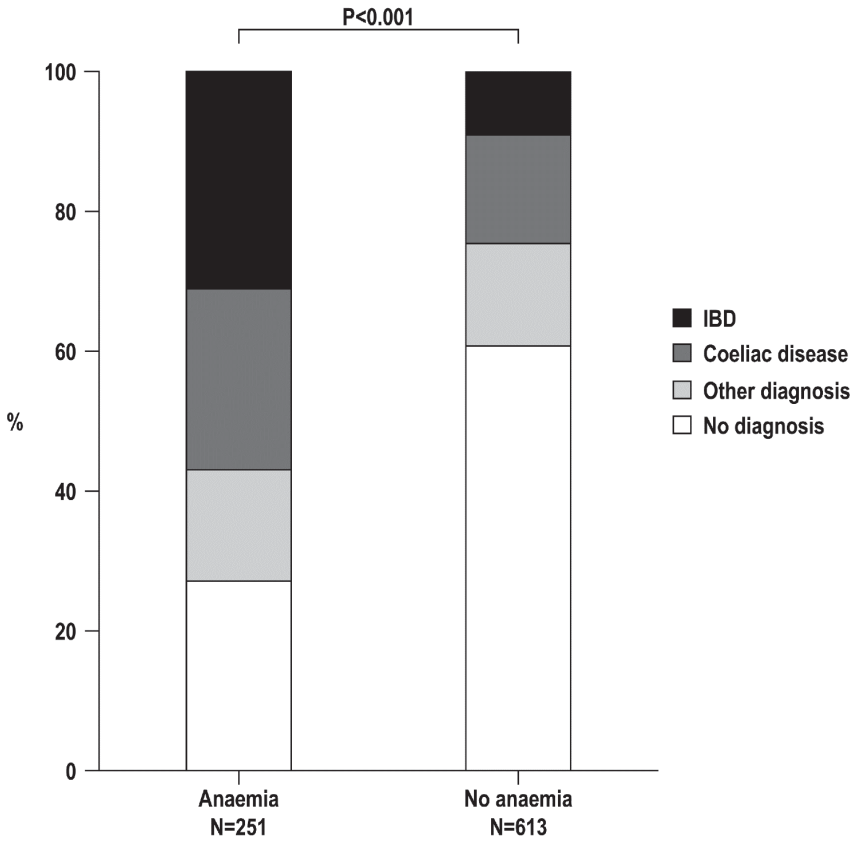


Table 5. Factors predicting a diagnosis in gastrointestinal endoscopies of 251 anaemic children in Study **I**.

	Diagnosis, %	Odds ratio[1]	95% CI
Age 5-12 years	79.5	3.52	1.27-9.75
Diarrhoea	81.7	2.04	1.07-3.90
Melena/haematochezia	84.1	2.40	1.17-4.92
Poor weight gain/poor growth	89.1	3.94	1.70-9.15
Positive coeliac autoantibodies[2,3]	95.7	11.81	3.47-40.12
Faecal calprotectin > 100µg/g[4]	92.3	12.86	4.00-41.32
Hypersedimentation[5]	82.1	2.65	1.29-5.44
Hypoalbuminemia[6]	91.7	5.05	1.56-16.34

[1]Calculated by binary regression analysis with 95% confidence intervals (CI) comparing age group 5-12 years with those younger than one year, and patients with different symptoms or abnormal laboratory parameters with those without such findings, [2]Type 1 diabetes, rheumatic or thyroidal disease, coeliac disease, [3]Inflammatory bowel disease or coeliac disease, [3]Endomysium antibodies or transglutaminase 2 antibodies, Data from [3]177, [4]94, [5]168 and [6]102 patients.

Only anaemia related (MCV, mean corpuscular haemoglobin, haematocrit, iron parameters) laboratory abnormalities were present in 45 (17.9%) of the anaemic children. Fifteen of them received a diagnosis in the initial investigations and two during the follow-up, whereas the remaining 28 cases were left without diagnoses. Furthermore, 30 (12.0%) of the anaemic children presented without additional gastrointestinal symptoms. Of these, 22 received an initial diagnosis, while eight children did not receive any diagnosis even during the long-term follow-up. All diagnosed children presented additional laboratory abnormalities, while only two of the non-diagnosed children had temporarily abnormal values (increased ESR, increased faecal calprotectin). Moreover, in six out the eight non-diagnosed children the haemoglobin values normalized during a subsequent iron supplementation.

7.2 Anaemia in histologically verified coeliac disease (II)

Altogether 18.0% of the 455 children with coeliac disease in Study **I** had anaemia at the time of diagnosis. Children with anaemia were significantly older and had less often family members with coeliac disease than those without anaemia (Table 1 in the original publication **II**). In addition, the anaemic patients had more often extra-intestinal presentation of coeliac disease and were less often screen detected (Figure 1A in the original publication **II**), and also showed more severe histological damage (Figure 1C and Table 2 in the original publication **II**) and higher median values of TG2-ab and EmA (Table 3 in the original publication **II**) at diagnosis.

After a median of 12 months from diagnosis, the anaemic children showed poorer adherence to the GFD assessed by interview than did the non-anaemic children (78.3% vs 87.5%, P=0.035), although only occasional lapses were reported in all patients. However, clinical response was similar (94.3% vs. 96.6% respectively, p=0.318), and serum autoantibody and blood haemoglobin values improved significantly in both groups (Table 4 original publication **II**). Although the median haemoglobin levels rose more in anaemic children (2.0 g/l vs. 0.4 g/l respectively), their levels still remained lower than in the non-anaemic group after one year of follow-up (Table 4 in the original publication **II**). Four children did not recover from anaemia during the follow-up despite a strict GFD and a good clinical and serological response. After the coeliac disease diagnosis, altogether 25 of the anaemic children were prescribed oral iron supplementation, including three of those with persistent anaemia.

7.3 Anaemia and markers of iron deficiency in potential and histologically verified coeliac disease (**III**)

Coeliac disease was diagnosed in 83 children in Study **III**. Altogether 16 of them had TVA and 67 P/SVA. Furthermore, nineteen seropositive children with normal duodenal villi comprised the potential coeliac disease group and 23 seronegative children the non-coeliac control group. Anaemia was detected in 62.5% of those with TVA, in 22.4% of those with P/SVA, in 15.3% of those with potential coeliac disease and in none of the controls ($p<0.001$, Table 6). The levels of EmA, TG2-ab, alanine aminotransferase and IELs and the proportion of patients with low total iron, high sTfR, low ferritin and low TF saturation increased parallel with the mucosal damage when compared among children with TVA, P/SVA and potential coeliac disease (Table 6 and Table 1 in the original publication **III**). No differences in the levels of iron parameters were seen between children with potential coeliac disease and P/SVA, whereas the levels differed significantly between TVA and all the other groups (Figure 1 in the original publication **III**). The hepcidin levels did not differ between any of the aforesaid study groups or between the subgroups of anaemic and non-anaemic patients.

After an average of seven months on a GFD, a significant improvement was seen in the median haemoglobin, sTfR1, ferritin and albumin values in the TVA group, and in the median haemoglobin, total iron, ferritin and albumin values in the P/SVA group (Figure 2 in the original publication **III**). Of note, the median albumin levels in the TVA group on a GFD were significantly higher than the baseline levels of those with potential coeliac disease ($p=0.037$, Figure 2 in original publication **III**). After a thorough discussion with the family, three children with potential coeliac disease started on a GFD. During the follow-up, an increase in haemoglobin and total iron levels was observed in two of them, including one with originally low and one with normal haemoglobin. None of the other potential coeliac children started on a GFD during the follow-up, although all remained seropositive.

Table 6. Baseline characteristics and anaemia-related laboratory parameters in 19 children with potential coeliac disease, 83 children with histologically verified coeliac disease and in 23 seronegative controls (Study **III**).

	Potential coeliac disease, n=19	Coeliac disease n=83		Controls, n=23	P-value
		P/SVA, n=67	TVA, n=16		
	%	%	%	%	
Age, median (range), years	6.3 (3.5-16.9)	7.5 (1.6-15.2)	6.1 (3.7-15.6)	6.0 (2.1-11.4)	0.532
Girls	74	73	69	44	0.062
Positive EmA/TG2-ab	100	100	100	0	**<0.001**
HLA DQ2/8	100	100	100	59	**<0.001**
Anaemia	15.3	22.4	62.5	0	**<0.001**
Low total iron	0	13.6	50.0	4.8	**<0.001**
High TfR1	15.8	20.4	46.7	4.8	**0.002**
Low ferritin	21.1	35.2	86.7	0	**<0.001**
Low transferrin saturation	11.1	40.5	71.4	9.5	**<0.001**
Low vitamin B12	0	0	0	0	1.000
Low erythrocyte folate	0	2.3	0	0	1.000

P/SVA, partial or subtotal villous atrophy, TVA, total villous atrophy, EmA, endomysium antibodies, TG2-ab, transglutaminase 2 antibodies, HLA DQ2/8, coeliac disease-associated human leucocyte antigen

7.4 Expressions of duodenal iron transporter proteins in potential and histologically verified coeliac disease (**IV**)

Altogether 27 children in Study **IV** had histologically verified coeliac disease and 10 had potential coeliac disease. The two groups did not differ in demographic data or median hepcidin values, or in the frequency of anaemia and low MCV (Table 1 in the original publication **IV**). The coeliac disease patients had nevertheless higher median EmA (1:1000 vs. 1:50, p<0.001) and TG2-ab (120 U/l vs. 17 U/l, p=0.001) values as well as higher sTfR and lower ferritin values (Table 1 in the original publication **IV**).

The stained area of FPN was increased (median 66% [with interquartile range 54-75%] in coeliac patients vs. 45% [22-57%] in controls, Supplementary Figures 1. and 2. in the original publication **IV**) and the saturation of HEPH decreased (27% [25-29%] vs. 31% [27-37%], correspondingly) in coeliac disease patients compared to controls, and a similar but non-significant trend was seen in the saturation of FPN and in the stained area of HEPH (Table 2 in the original publication **IV**). Additionally, a moderate positive correlation between ferritin values and TfR1 saturations (rS=0.594, p=0.015) and stained area (rS=0.761, p=0.001) was seen in

children with coeliac disease. A moderate negative correlation was also found between sTfR values and HEPH saturation (rS =-0.349, p=0.046) when evaluated in all patients, but not in separate analysis of those with coeliac disease. No other significant correlations or differences between the study groups or between anaemic and non-anaemic patients were observed (Tables 2 and 3 in the original publication **IV**).

8 DISCUSSION

8.1 Gastrointestinal endoscopies in the evaluation of paediatric anaemia (**I**)

Study **I** showed anaemic children to have higher frequency of abnormal histologic findings and to receive more often diagnoses than the non-anaemic children. Although anaemia alone increased the likelihood of diagnosis, this was the case especially when it was presented with certain (one or more) specific clinical findings, i.e. hypersedimentation, hypoalbuminemia, increased faecal calprotectin, positive coeliac antibodies, diarrhoea and melena/haematochezia. On the contrary, presence of constipation, abdominal pain or vomiting did not affect the probability of diagnosis in anaemic children, and presence of reflux symptoms even reduced the likelihood.

The predictors of pathologic findings and diagnoses in paediatric gastrointestinal endoscopies have been investigated in a few studies, but only in older children or in a more general level instead of focusing on anaemia. In a small Turkish study by Gulen et al. altogether 44 anaemic children between 9.5 and17.5 years of age underwent an OGD and abnormal findings were found in 57% (Gulen et al. 2011). A study from the United Kingdom reported anaemia to be the most common laboratory abnormality in children who underwent either colonoscopy alone or both colonoscopy and OGD (Wang et al. 2017). Furthermore, anaemia, rectal bleeding and hypersedimentation were the strongest predictors of histological abnormalities in colonoscopy, and positive coeliac autoantibodies, vomiting and dysphagia in OGD. In contrast, only a minority of subjects with abdominal pain or reflux symptoms and none of those with diarrhoea were found to have abnormal histology in OGD. A corresponding Canadian study found age ≥13 years, vomiting, and hypoalbuminemia to be associated with OGD findings, and age ≥10 years, rectal bleeding, failure to thrive, anaemia, thrombocytosis, hypoalbuminemia and hypersedimentation with colonoscopy findings (Noble et al. 2008). In contrast, haematemesis, dysphagia, reflux symptoms, failure to thrive, epigastric tenderness, anaemia, eosinophilia, hypersedimentation and - quite surprisingly - positive coeliac autoantibodies did not predict OGD abnormalities, and diarrhoea and abdominal

pain were only borderline significant predictors in colonoscopy. Furthermore, positive coeliac autoantibodies, dysphagia, gastrointestinal bleeding, and age groups between 5-12 and 13-18 years, but not age 1-4 years, reflux symptoms, failure to thrive, diarrhoea, emesis and epigastric pain, predicted histologic OGD abnormalities in a study from the United States of America (USA) (Sheiko et al. 2013). A recent study from India reported quite different results compared with the aforementioned Western studies, as unexplained anaemia was the main indication for OGD in only 9% of the children and of those only 23.0% presented any abnormality in endoscopy (Wani et al. 2020). Instead, an endoscopic abnormality was found most often in children whose OGD indication was variceal surveillance, gastrointestinal bleeding, haematochezia, corrosive injury, or biliary ascariasis.

The fact that here dysphagia and vomiting did not increase the probability of diagnosis could be due to the small number of children presenting with these symptoms and the subsequent lack of statistical power. Furthermore, the high proportion of coeliac disease diagnoses conducted at the average age of 7.1 years may explain why children between five and 12 years were more likely to receive a diagnosis than were the older children. Besides the differences in study designs, an additional plausible explanation for the discrepancy between earlier research and the present study is that we included only initial diagnostic endoscopies, and that OGDs and colonoscopies were not analysed separately as was done in the studies by Noble et al. and Wang et al. (Noble et al. 2008; Wang et al. 2017). Furthermore, surgical endoscopies were not included in our data, and rectal bleeding and haematemesis were not listed as categorized symptoms.

The most frequently established diagnoses in Study **I** were coeliac disease and IBD. This is in line with the findings of Noble et al. and Wang et al. who also included both OGDs and colonoscopies in their study (Noble et al. 2008; Wang et al. 2017). Ferrara et al. and Fayed et al. also showed coeliac disease, but not IBD, to be a common finding in anaemic children (Fayed et al. 2008; Ferrara et al. 2006). However, like Gulen et al. and Huang et al. they also found *H. pylori* infection to be a particularly frequent cause of anaemia (Gulen et al. 2011; Huang et al. 2010). These discrepancies in the main causes of anaemia likely reflect geographical variation in the general nutrition level, the rapid decrease of *H. pylori* infection especially in Western countries (Elitsur et al. 2009; Kawakami et al. 2008; Oona et al. 2004) and the worldwide increase in the incidence of immune-mediated diseases (Vegh et al. 2014; Catassi et al. 2014).

The diagnosis-predicting symptoms and laboratory markers noted here (Table 5) in anaemic children are in fact classic findings of untreated IBD and coeliac disease

(Husby et al. 2020; Levine et al. 2014). When these signs/symptoms are present, the diagnostic algorithm and the decision on whether to conduct OGD and/or colonoscopy is usually fairly straightforward and independent of the presence of anaemia, although the haemoglobin level may affect the urgency of the investigations. Of note, in coeliac disease a serology-based diagnosis has become possible after the introduction of the revolutionary 2012 ESPGHAN guidelines (Husby et al. 2012) and the number of OGDs performed due to this indication is decreasing rapidly. It can actually be calculated that 62% of the coeliac disease diagnoses in the present study could have been established without OGD by applying the serology-based criteria.

The decision on whether to conduct an endoscopy is more difficult in the case of more unspecific symptoms. In fact, in the study by Wang et al. most of the endoscopies were found to be normal after excluding the children with suspicion of IBD or coeliac disease (Wang et al. 2017). In the present study the co-occurrence of abdominal pain, constipation and vomiting did not affect, and reflux symptoms even decreased, the likelihood of endoscopic diagnosis in anaemic children. The findings on reflux symptoms and abdominal pain are in line with those of earlier studies (Noble et al. 2008; Sheiko et al. 2013; Wang et al. 2017), but Noble et al. and Wang et al. found vomiting and Wang et al. and Sheiko et al. dysphagia also to predict findings in OGD. Constipation was not categorised as a symptom in the aforementioned studies. Our findings support the recent ESPGHAN guidelines, where uncomplicated reflux symptoms, functional gastrointestinal disorders and constipation as such are not indications for gastrointestinal endoscopy (Thomson et al. 2017).

Although presence of anaemia increased the probability of endoscopic diagnoses in general, this was not the case in children lacking additional laboratory and clinical findings. Furthermore, a later gastrointestinal diagnosis explaining the anaemia after an endoscopy with negative results appears to be exceptional even in the long term. These findings indicate that a short period of non-invasive surveillance, possibly with iron supplementation, could be a safe option in children having anaemia as a sole finding or when presenting with abdominal pain, constipation or reflux symptoms and no additional laboratory abnormalities. However, an endoscopy should be conducted in the case of melena, haematochezia, prolonged diarrhoea, or failure to thrive, or when increased faecal calprotectin, hypersedimentation, hypoalbuminemia, or positive coeliac autoantibodies (when not fulfilling the non-biopsy criteria) are present.

8.2 Association between anaemia and the clinical, histological, and serological presentation of coeliac disease (**II-III**)

Anaemia was present in 18% and 43% of the children at the time of coeliac disease diagnosis in Studies **II** and **III**, respectively. The smaller number of patients in Study **III** may account for this difference in percentages between the studies, but overall, the figures are in line with those reported (9%-52%) in North American and European studies since the year 2000 (Table 3). Markedly higher frequencies of anaemia, up to 94%, have been reported in studies from India and Turkey. This likely reflects the wide variation in the incidence of anaemia between the low/moderate-income countries and high-income countries (Kassebaum et al. 2015). In any case, in both settings, anaemia seems to be overrepresented in children with coeliac disease, as the reported prevalence in general population has been 11% in high-income countries and approximately 70% in low/moderate-income countries (Stevens et al. 2013).

There was an association between the presence of anaemia and more severe damage of the small bowel mucosa in both Studies **II** and **III**. More specifically, in Study **II** anaemic children presented more often with TVA than did non-anaemic patients, and in Study **III** the prevalence of anaemia increased parallel to the severity of mucosal atrophy. Moreover, in Study **II** the median coeliac autoantibody values were higher in the anaemic than in the non-anaemic children. These findings are in line with an adult study from USA in which anaemic coeliac disease patients presented with more severe clinical, serologic and histologic features than did those having diarrhoea as the main clinical presentation (Abu Daya et al. 2013). Also, another adult study from the USA has reported an association between the prevalence of anaemia and severity of mucosal damage (Thomas et al. 2009).

The anaemic coeliac disease patients in Study **II** also presented more often with additional extra-intestinal symptoms and were less often screen detected. However, there was no difference in the severity of the symptoms, which is somewhat inconsistent with a prospective birth cohort study reporting an association between histologically and serologically advanced disease and more severe clinical picture (Agardh et al. 2015). Although more studies on this issue are needed, it seems that the clinical presentation of coeliac disease is also affected by factors other than only intestinal malabsorption (Lindfors et al. 2019b).

Interestingly, although the haemoglobin values of the majority (92%) of the anaemic coeliac disease patients in Study **II** were normalized after one year on a GFD, their median value remained lower than those who were non-anaemic in the

first place. All children with persistent anaemia were on a strict GFD and showed good clinical and serological response, indicating that the incomplete recovery was not due to poor dietary adherence or non-responsive coeliac disease. Previously normal haemoglobin levels have been reported in 94% of Turkish children within 48 months (Çatal et al. 2015) and in 91% of Serbian children within one to nine years (Radlović et al. 2009) on a GFD. In adult studies from Italy the corresponding prevalences were 94% and 70% (Annibale et al. 2001; Bergamaschi et al. 2008) and in a Finnish study 75% (Kemppainen et al. 1998) after one year on a GFD respectively. In a Canadian adult study 55.2% of anaemic coeliac disease patients recovered within one year of starting on a GFD and 83.5% within five years (Pulido et al. 2013),

There are several plausible explanations for these discrepancies in recovery from anaemia on a GFD. For example, the possible use of iron supplementation and the adherence to GFD were often poorly reported and may have differed. Furthermore, the populations studied may have had dissimilar genetic background, particularly relevant being the polymorphisms in HFE, DMT1 and TMPRSS6 genes affecting predisposition to anaemia and response to iron supplementation (Butterworth et al. 2002; De Falco et al. 2018; Elli et al. 2015; Tolone et al. 2017; Zanella et al. 2015).

Of note, Nestares et al. (Nestares et al. 2020) also recently reported lower levels of iron parameters in treated coeliac disease children compared to healthy controls and, as a plausible explanation, observed the patients to have reduced intake of iron and other micronutrients. This suggests that a suboptimal nutritional value of a typical GFD may contribute to incomplete recovery from anaemia. Additionally, the ongoing duodenal inflammation processes even among coeliac patients on GFD may compromise the absorption of iron in the intestine (Dotsenko et al. 2020). The fact that recovery from anaemia in coeliac disease may be protracted even on a strict GFD emphasizes the importance of dietitian visits and a structured follow-up.

8.3 Anaemia and iron deficiency in potential coeliac disease (**III**)

The main finding in Study **III** was that, even if less often that in patients with an established coeliac disease, anaemia and signs of iron deficiency may already appear in children with potential coeliac disease. In fact, otherwise unexplained anaemia was almost as common among them (15%) as in patients with P/SVA (22%). Moreover, these two groups did not differ in the median values of haemoglobin, sTfR, total

iron, ferritin, TF iron saturation and hepcidin, and median ferritin was lower in both groups than among the non-coeliac controls.

By way of comparison, Tosco et al. found none of 106 Italian children with potential coeliac disease to suffer from anaemia at baseline, but four of them had low ferritin and four also developed anaemia during a follow-up of up to three years on normal diet (Tosco et al. 2011). In a previous Finnish adult study three out of 27 subjects with mild enteropathy (Marsh 1–2) and positive coeliac serology presented with malabsorption or anaemia, although no significant increase in the haemoglobin levels of these adults was detected during one year on a GFD (Kurppa et al. 2010). Shahriari et al. recently showed the frequency of potential coeliac disease to be increased in anaemic children not responding to iron supplementation (Shahriari et al. 2018). These findings suggest that the distinction between potential coeliac disease and P/SVA is partly artificial and more based on simplified histological classification than the actual biological nature of the disease.

ESPGHAN has recently given increased recognition to this entity, as their updated guidelines permit starting a GFD in symptomatic children with potential coeliac disease after a careful discussion with the family (Husby et al. 2020). In asymptomatic individuals, normal diet and follow-up with repeated serology is still recommended. No specific definition of "symptom" has been provided, but since IDA is listed as a sign suggestive of coeliac disease it could be considered to justify the diagnosis and treatment. In fact, even subclinical iron deficiency, present in some of the patients here and in the study by Tosco et al. (Tosco et al. 2011), may impair psychomotor and cognitive development and lead to a malfunctioning of the immune system (Ekiz et al. 2005; Lozoff et al. 2007; Lozoff et al. 2013; Shafir et al. 2008). At the moment, however, the position of potential coeliac disease in the diagnostic guidelines remains debated, and further studies on the natural history of this condition are called for (Popp and Mäki 2019; Trovato et al. 2019). On the other hand, anaemia and iron deficiency may be due to a number of reasons which should always be ruled out before considering them to result from untreated/inadequately treated coeliac disease.

8.4 The role of duodenal iron transporter proteins in coeliac disease-associated anaemia (**IV**)

The presence of otherwise unexplained anaemia or subclinical iron deficiency already in children with potential coeliac disease indicates that other factors than decreased

iron absorption from damaged mucosa areas also play a role in these circumstances. This finding, together with some previously published - although somewhat disputed - results (Barisani et al. 2004a; Matysiak-Budnik et al. 2008; Sharma et al. 2009; Tolone et al. 2017) led us to study the hypothesis that the expressions of duodenal iron transporter proteins contributes to this pathogenic process.

An anaemia-independent increase in the expression of FPN and a decrease in the expression of HEPH was observed in Study **IV** in children with histologically verified coeliac disease compared to seronegative controls. The down-regulation of HEPH has not been reported before, but the upregulation of FPN is in line with an adult study by Sharma et al. who also reported increased expressions of FPN and DMT1 in the duodenal biopsies of both iron replete and iron deficient patients (Sharma et al. 2009). In contrast, Barisani et al. found the mRNA and protein expressions of DMT1, FPN, HEPH and TfR1, but not DCYTB, to be increased in iron deficient but not in iron replete adult patients. However, they included both untreated and treated coeliac patients in their analyses (Barisani et al. 2004a). Tolone et al. (Tolone et al. 2017) also reported upregulation of DMT1 mRNA levels in children with coeliac disease compared to those with only potential coeliac disease and non-coeliac controls, although this was seen only when patients with mild (Marsh 3a) duodenal atrophy were included.

The current understanding of the functions and roles of FPN and HEPH remains limited (Rescigno 2020). Generally, the expression of FPN in hepatocytes and macrophages has been shown to be altered by systemic iron requirements by hepcidin induced internalization and degradation (Aschemeyer et al. 2018, Nemeth et al. 2004). However, it has been suggested that in enterocytes the regulation of FPN is more dependent on local iron and oxygen concentrations (Brasse-Lagnel et al. 2011; Chaston et al. 2008; Chung et al. 2009; Drakesmith et al. 2015; Mena et al. 2007; Zhang et al. 2009). HEPH has been thought not to play a crucial regulatory role in iron absorption, although its mRNA expressions have been shown to increase slightly in iron deficiency (Anderson et al. 2002). Interestingly, even though HEPH has been shown to appear mostly intracellularly, its functions inside the enterocytes remain unidentified (Anderson et al. 2002; Gulec et al. 2014). In Study **IV** no increase of HEPH in anaemic children was seen.

Notably, in 2008 Matysiak-Budnik et al. suggested a link between iron metabolism and pathogenesis of coeliac disease, as they reported a retrotranscytosis of IgA-gliadin complexes through overexpressed TfR1 in duodenal enterocytes (Matysiak-Budnik et al. 2008). However, no such overexpression was observed here and further studies on this issue are needed.

The somewhat conflicting results between the aforementioned reports and the present study could be caused by differences in the number and clinical characteristics of the study participants, especially as expressions of iron transporter proteins may differ between children and adults (Lönnerdal 2017). Another explanation may be variation in the use of laboratory methods, staining protocols and primary antibodies.

To conclude the findings of Study **IV**, it appears that the changes in FPN and HEPH expressions rather reflect the immature epithelium of the atrophic duodenal mucosa (Oittinen et al. 2017) than the pathophysiologic mechanisms of anaemia in coeliac disease.

8.5 Hepcidin and coeliac disease (**III-IV**)

Apart from the altered expression of iron transporter proteins, a possible inhibition of intestinal iron absorption by hepcidin has been suggested to play a role in the development of anaemia in coeliac disease (Martín-Masot et al. 2019). However, in Study **IV** no differences were found between iron transporter protein expressions or hepcidin values between children with potential or histologically verified coeliac disease and non-coeliac controls. Bergamaschi et al. likewise previously found no correlation between levels of hepcidin and other iron parameters or frequency of anaemia in adult coeliac disease patients (Bergamaschi et al. 2008). However, they measured the less accurate pro-hepcidin instead of the actual functional hepcidin-25 applied in the present study (Zipperer et al. 2013).

There was nevertheless quite a wide variation in the hepcidin values in Study **III**, especially among the children with potential coeliac disease. As far as we know, the only previous study on this issue reported a subgroup (20%) of coeliac children to present with increased hepcidin levels, leading the authors to speculate that inflammation could still be behind their anaemia (Bel'mer et al. 2014). This theory is supported by some other studies reporting an association between coeliac disease and anaemia of inflammation (Bergamaschi et al. 2008; Berry et al. 2018; Harper et al. 2007).

8.6 Strengths and limitations

In all four studies (**I-IV)**, particular attention was paid to the precise morphometric analysis of the duodenal biopsies and thus correct histological grouping of the participants (Taavela et al. 2013). Another strength was the opportunity to use large and well-characterized patient cohorts in Studies **I** and **II**, and a partly prospective design in Study **III.**

The retrospective designs in Studies **I** and **II** are a clear limitation, particularly since laboratory parameters were not systemically taken from all patients. In addition, the higher number of OGDs compared to colonoscopies and low ileum intubation rate (anaemic children 65.3% and non-anaemic children 67.1%) in Study **I** may have biased the results. Additional limitations were the fairly short follow-up time, and missing laboratory results and other than GFD-related dietary data from a substantial part of the patients in Study **II**. These shortcomings were nevertheless counterbalanced by the availability of comprehensive medical data in Studies **I-II** and the long-term follow-up and systematic biopsy sampling in Study **I**.

The main limitation in Studies **III** and **IV** was the relatively small number of participants, especially controls. There was also a rather short follow-up time in Study **III**, and the Romanian control children in Study **IV** may have differed e.g. genetically and/or nutritionally from their Finnish counterparts. Another limitation was that neither the mRNAs of the iron transporters nor the locations of proteins in the enterocytes were investigated in Study **IV**. However, since the measurements of iron transporter protein saturations and stained areas were not observer-dependent and the results were consistent within and between the study groups, we believe the findings to reflect the true state of the expressions.

Considering the generalizability of our results, the substantial countrywide variation in the aetiology and prevalence of anaemia has to be kept in mind (Kassebaum et al. 2015; Stevens et al. 2013). Furthermore, as the study comprised children from a single Finnish tertiary centre, the results may not fully represent the clinical heterogeneity of coeliac disease and anaemia seen at other health care levels.

9 SUMMARY AND CONCLUSIONS

The present results showed that, in general, anaemia increases the likelihood of a diagnosis in paediatric gastrointestinal endoscopies, the most common of these being coeliac disease and IBD. However, the yield of endoscopies was low in children without specific symptoms or additional laboratory abnormalities besides anaemia, suggesting that in such cases a period of non-invasive surveillance, possibly together with oral iron supplementation, might be considered. Additionally, a subsequent diagnosis after a non-diagnostic endoscopy appears to be exceptional even in the long term.

In children with coeliac disease, the presence of anaemia at diagnosis was associated with more severe serological and histological presentation. On the other hand, low haemoglobin and subclinical iron deficiency were frequently present even in children with potential coeliac disease. These observations demonstrate the complexity of iron metabolism in coeliac disease, contrasting with the traditional theory of decreased iron absorption via damaged duodenal mucosa being the sole causative factor behind anaemia. Of note, recovery from anaemia in some children was shown to take a rather long time despite a strict GFD. Together the aforesaid results call for an early diagnosis and intensified follow-up of coeliac disease in paediatric patients presenting with anaemia.

As a further proof of the complexity of iron metabolism in coeliac disease, altered expression of small-bowel iron transporter proteins did not explain the pathophysiology of anaemia in coeliac disease, but rather reflected the immature nature of epithelium in atrophic phase of the disease. It is evident that further studies aiming to decipher the mechanisms of abnormal iron metabolism in different stages of coeliac disease are needed.

Taken together, this dissertation emphasises the high frequency of anaemia in gastrointestinal diseases, as well as its importance as an alarm symptom. One of the most common diseases underlying anaemia is coeliac disease, where anaemia may be present even in the so-called potential state of the disease. This supports early diagnosis and dietary treatment of this specific condition.

10 FUTURE DIRECTIONS

More precise predictive models and recommendations on whether and when to conduct OGD, colonoscopy or both in anaemic children should be generated. The present dissertation paves the way to achieve these goals but eventually prospective studies with a sufficiently large numbers of children, long enough follow-up and standardized definitions of the clinical variables and endoscopy outcomes are called for.

Our results support individual consideration on whether to start GFD in children with potential coeliac disease presenting with anaemia or subclinical iron deficiency. However, further research on this issue is again needed, particularly in asymptomatic potential coeliac disease patients. The importance of this patient group will likely continue to increase due to the widening of risk group screenings.

Notwithstanding the active research during recent years, many unanswered questions about human iron metabolism remain. To mention a few, the mechanism of intestinal haem absorption and roles of circulating sTfR and ferritin, as well as the development of mechanisms maintaining iron homeostasis in childhood, are poorly understood. Likewise, the pathophysiology behind anaemia in coeliac disease, especially the role of genetic factors and the local iron regulatory mechanisms in the intestine, need additional exploration. A better understanding of iron metabolism could enable more efficient and personalised treatments of anaemia-related disorders in general.

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PUBLICATIONS

PUBLICATION I

Diagnostic findings and long-term prognosis in children with anemia undergoing GI endoscopies

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Diagnostic findings and long-term prognosis in children with anemia undergoing GI endoscopies

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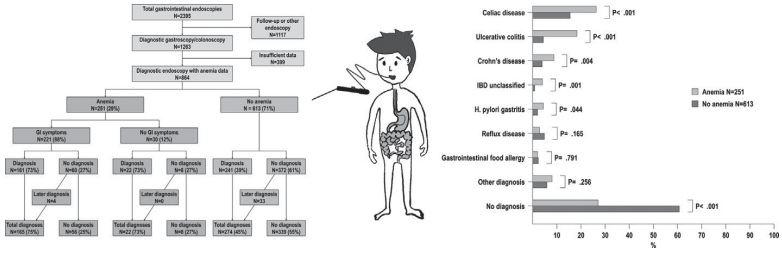
GRAPHICAL ABSTRACT



Background and Aims: Intestinal diseases are regarded as a common cause of anemia, but the diagnostic outcomes of children with anemia undergoing endoscopic investigations are unclear. We investigated this issue in a large cohort of children.

Methods: Indications for and findings of consecutive gastrointestinal (GI) endoscopies were collected. Clinical presentation and diagnostic outcomes were compared between anemic and nonanemic patients and between anemic patients with and without a diagnosis. Diagnoses received during follow-up were collected.

Results: Of 2395 consecutive endoscopies, 251 children with and 613 children without anemia had undergone either diagnostic esophagogastroduodenoscopy (EGD) (51.4% and 51.4%, respectively), colonoscopy (4.0% and 11.4%), or both (45.8% and 37.8%). Children with anemia more often received diagnoses (72.9% vs 39.3%; odds ratio [OR], 4.18; 95% confidence interval [CI], 3.03-5.77), particularly of celiac disease (26.3% vs 15.5%, $P < .001$) and of inflammatory bowel disease (31.1% vs 9.1%, $P < .001$), than did nonanemic children. The diagnosis in anemic patients was predicted by age 5 to 12 years (OR, 3.52; 95% CI, 1.27-9.75), presence of diarrhea (OR, 2.04; 95% CI, 1.07-3.90), melena/hematochezia (OR, 2.40; 95% CI, 1.17-4.92), poor growth (OR, 3.94; 95% CI, 1.70-9.15), positive celiac serology (OR, 11.81; 95% CI, 3.47-40.12), high calprotectin (OR, 12.86; 95% CI, 4.00-41.32), hypersedimentation (OR, 2.65; 95% CI, 1.29-5.44), and hypoalbuminemia (OR, 5.05; 95% CI, 1.56-16.34). Thirty children with anemia (12.0%) had no GI symptoms, and 22 of them (73.3%) were given diagnoses at the time of the endoscopies. All 22 had additional laboratory abnormalities, whereas these were present in only 2 of 8 undiagnosed children. None of them was diagnosed later in the follow-up of up to 11 years, in contrast to 4 (6.7%) of all anemic and 33 (8.9%) of all nonanemic patients.

Conclusions: Anemia increased the probability of being given a diagnosis, emphasizing its importance as an alarm symptom. However, endoscopies in anemic patients without additional symptoms or laboratory abnormalities seldom improved the diagnostic yield. (Gastrointest Endosc 2020;91:1272-81.)

(footnotes appear on last page of article)

Anemia is one of the most common chronic medical problems in children, affecting on average 16.7% of preschool children in Europe and as many as 64.6% in Africa.[1] Although in developing countries pediatric anemia is usually caused by inadequate nutrition and deficits of essential micronutrients, in developed countries the leading cause is iron deficiency, which may be either dietary or secondary to a gastrointestinal (GI) or hematologic disease or other chronic conditions.[2] Importantly, anemia is not a diagnosis as such but a clinical sign, the underlying cause of which should always be investigated.

Alimentary tract diseases are regarded as a common reason for anemia. The development of anemia in these conditions is multifactorial, including, for example, insidious blood loss and inadequate iron absorption.[3,4] It has been suggested that GI pathologies should always be ruled out in children and adolescents with unexplained anemia.[5] Unfortunately, a reliable examination of the intestine requires invasive methods, particularly esophagogastroduodenoscopy (EGD) and colonoscopy, which in children are usually performed with the patient under general anesthesia. Optimal targeting of these invasive procedures in children would be of particular importance, but currently the evidence on this issue is scarce.[6-9]

METHODS

Study ethics

The study design and data collection were duly approved by the Department of Pediatrics, Tampere University Hospital. All identifiable personal data were coded, and analyses were performed anonymously. According to the national guidelines in Finland, this registry-based study required no ethical approval.

Patients and study design

This retrospective cross-sectional study of a tertiary center was conducted at Tampere University and Tampere University Hospital. We have long maintained comprehensive medical records on all children undergoing GI endoscopies and performed systematic mucosal sampling irrespective of macroscopic findings. These practices afforded us an opportunity to investigate the diagnostic yield of endoscopic investigations and the long-term prognoses in children with unexplained anemia.

The medical data on children (age <17 years) who had undergone consecutive EGDs and/or colonoscopies in 2007 to 2014 were recorded. Only patients who had undergone diagnostic endoscopy(ies) and for whom anemia/hemoglobin data were available were included for further analyses, whereas follow-up and other endoscopies (rectosigmoidoscopies) were excluded. These children were divided into anemic and nonanemic groups, and the anemia group were divided further into those presenting with and without additional GI symptoms (Fig. 1). All study data were compared between these groups. Further, patient characteristics predicting diagnosis were identified. In addition to medical information before and at the time of the endoscopic studies, follow-up data were collected from 2 to 11 years.

Clinical and laboratory data

The information recorded included demographic parameters, clinical presentation, duration of symptoms, presence of poor growth, chronic conditions, and family history of GI diseases. Other possibly conducted relevant investigations of the GI tract, such as wireless capsule endoscopy, magnetic resonance enterography, or esophageal pH monitoring, were also recorded, as well as all diagnoses set in either the primary or follow-up investigations. Children with minor unspecific and apparently clinically insignificant endoscopic or histologic abnormalities, such as lymphonodular hyperplasia common in children or inactive chronic gastritis, and those with only functional abdominal symptoms were assigned to the no-diagnosis group.

The results of the following laboratory tests were collected as available: blood hemoglobin and mean corpuscular volume (MCV; reference values [Rfs][10] presented in Supplementary Table 1, available online at www.giejournal.org), plasma ferritin (Rf > 6 μg/L), plasma transferrin receptor (Rf for children age <1 years, 1.6-7.0 mg/L; 1-3 years, 2.7-5.4 mg/L; and 4-6 years, 2.4-6.3 mg/L; for boys ages 7-12 years, 2.4-5.7 mg/L; girls 7-12 years, 2.0-5.1 mg/L; boys 13-17 years, 2.0-6.8 mg/L; and girls 13-17 years 1.6-5.2 mg/L), erythrocyte sedimentation rate (ESR; Rf < 15 mm/h), plasma albumin (Rf from 35-46 to 37-51 g/L), plasma alanine aminotransferase (Rf < 40 U/L), serum antiendomysial antibodies (Rf titer 1: <5) and IgA-class antibodies against transglutaminase 2 (Rf < 7.0 U/L), and fecal calprotectin (Rf < 100 μg/g). To calculate the possible effect of the severity of anemia on the likelihood of a subsequent diagnosis, hemoglobin values at the time of endoscopy were further subclassified using the World Health Organization criteria.[11] Failure to thrive was defined as reduced growth velocity in the longitudinal evaluation of growth charts and/or impaired height or weight development when compared with the gender- and age-dependent Rfs or to target height calculated using midparental height.[12]

Histology

Endoscopic abnormalities were reported systemically and photographed if considered potentially significant. During EGD, at least 2 mucosal biopsy specimens were taken systemically, regardless of the preceding clinical and laboratory findings, from the lower esophagus, antrum, and gastric corpus and antrum and at least 4 specimens from the duodenum. Since 2012, biopsy specimens were also obtained from the duodenal bulb and middle part of the esophagus. During colonoscopy, biopsy

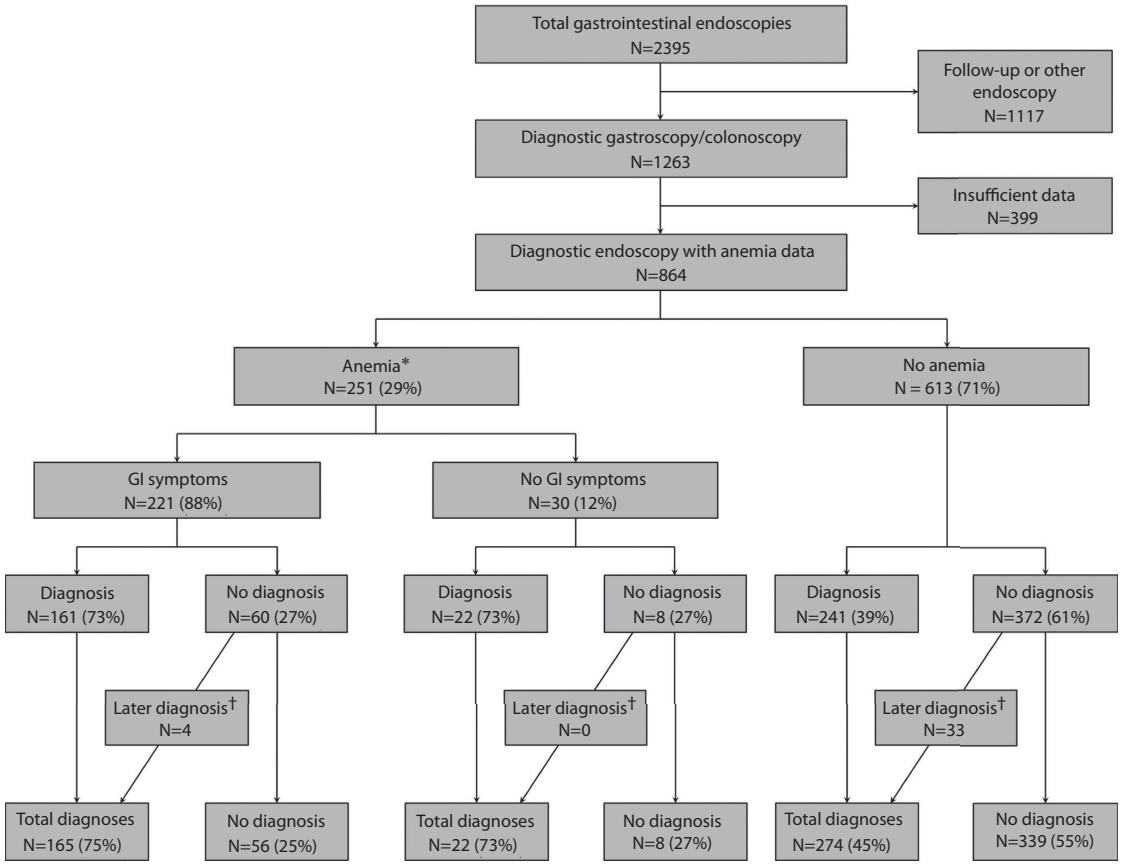


Figure 1. A flowchart of the study. *A patient was considered anemic if the hemoglobin value was below the age- and sex-dependent reference at the time of endoscopy. †For up to 11 years of follow-up.

specimens were taken from the rectum, sigmoid/descending colon, ascending colon/cecum, and terminal ileum. In both EGD and colonoscopy, additional specimens were taken as clinically indicated. Endoscopies were considered adequate when biopsy specimens were taken systemically from each abovementioned bowel section.[7]

Specimens were cut, stained, and evaluated by standard histopathology methods. If needed, special stainings were performed. Only representative and correctly oriented and cut histologic specimens were accepted for quantitative morphometric analyses of duodenal samples.[13] All abnormalities reported by a pathologist were regarded as histologic findings even if not diagnostic, including, for example, unspecified duodenal inflammation, mildly increased number of eosinophils in the esophagus,[14] and inactive chronic gastritis.

Individual diagnoses were set according to international consensus[14-20] or, if no specific guidelines existed, based on previous literature and clinical experience. During the study period the European guidelines on celiac disease were revised in 2012 but the new criteria[21] were not implemented in our clinical practice until 2015. Additionally, the guidelines on food allergy[22] and *Helicobacter pylori*[23] were both updated in 2011 and the guidelines of reflux disease[24] in 2009, and the new criteria were subsequently used to establish the diagnoses. The diagnosis of GI food allergy was set by experienced clinicians based on a combination of clinical and histologic findings, allergy testing, and elimination diet followed by food challenge.[19,22]

Statistical analysis

Clinical characteristics, abnormal laboratory parameters, and endoscopic and histologic findings are presented as percentage distributions. Statistical significances of differences in qualitative parameters were calculated using the χ^2 test or the Fisher exact test. Most quantitative variables were found to be skewed by the Shapiro-Wilk method and

TABLE 1. Characteristics of 864 children with and without anemia at the time of their first GI endoscopies

	Anemia (n = 251)		No anemia (n = 613)		
Categorical variables	**N**	**%**	**N**	**%**	***P* value**
Girls	135	53.8	326	53.2	.872
Autoimmune disease*	20	8.0	27	4.4	.036†
Asthma, allergy, or atopy	44	17.5	116	18.9	.632
Other chronic disease	32	12.7	58	9.5	.151
Intestinal disease in relatives‡	48	19.1	153	25.0	.065
Positive EmA or TGA-IgA	70§	39.5	132§	31.5	.058
Fecal calprotectin > 100 μg/g	65¶	69.1	84¶	33.1	<.001†
Low MCV	91‖	42.3	38‖	6.8	<.001†
Continuous variables	**N**	**Median (quartiles)**	**N**	**Median (quartiles)**	
Age, y (range)	251	10.8 (.03, 16.7)	613	8.3 (.02, 17.7)	.012‡
Ferritin, μg/L	62	7.5 (5.0, 15.3)	70	20.5 (13.8, 31.0)	<.001†
Transferrin receptor 1, mg/L	58	7.2 (5.0, 14.5)	37	4.3 (3.7, 5.2)	<.001†
ESR, mm/h	168	12.0 (5.0, 25.75)	434	7.0 (5.0, 11.3)	<.001†
Alanine aminotransferase, U/L	172	19.5 (14.0, 29.0)	399	17.0 (14.0, 24.0)	.189
Albumin, g/L	102	36.0 (33.0, 40.0)	305	40.0 (37.0, 43.0)	<.001†

EmA, Antiendomysial antibodies; *ESR*, erythrocyte sedimentation rate; *MCV*, mean corpuscular volume; *TGA-IgA*, IgA-antibodies against transglutaminase 2.
*Type 1 diabetes, rheumatic or thyroidal disease, celiac disease.
†$P \leq .050$.
‡Celiac disease or inflammatory bowel disease.
§Data from 177 anemic and 419 nonanemic patients.
¶Data from 94 anemic and 254 nonanemic patients.
‖Data from 215 anemic and 557 nonanemic patients.

included outliers. The variables were thus analyzed by nonparametric Mann-Whitney test and expressed as medians and quartiles.[25] The associations between symptoms/clinical signs and diagnoses were calculated using binary logistic regression analysis with 95% confidence intervals (CIs). Odds ratios (ORs) were calculated comparing boys with girls, age groups with those younger than 1 year, and patients with autoimmune disease, intestinal disease in relatives, different symptoms, or abnormal laboratory parameters with those without such findings. A $P < .05$ was considered statistically significant. All statistical analyses were performed using SPSS Statistics version 23 (IBM Corp, Armonk, NY, USA).

RESULTS

Altogether, 2395 consecutive GI endoscopies were conducted during the study period. Of these, 1117 were follow-up or other endoscopies, and 399 children lacked hemoglobin values and were therefore excluded. Of the remaining 864 children with hemoglobin and anemia data available, altogether 251 (29.1%) had anemia (Fig. 1). Of these, 128 (51.0%) had undergone EGD alone, 10 (4.0%) had undergone colonoscopy alone, and the other 113 (45.1%) had undergone both EGD and colonoscopy. The corresponding figures for nonanemic children were 311 (50.7%), 70 (11.4%), and 232 (37.8%), respectively. The duodenum was reached in 98.8% and 99.1% and the ileum in 65.3% and 67.1% of the anemic and nonanemic children, respectively. Subsequently, combined with missing samples in some cases, respectively, 83.0% and 85.1% of EGDs and 62.1% and 67.2% of colonoscopies of anemic and nonanemic children were considered adequate according to our strict definition. Additional alimentary tract investigations were conducted in 93 (37.1%) anemic and 214 (35.0%) nonanemic children, including wireless capsule endoscopy in 22 (8.8%) and 37 (6.0%).

Children with anemia were significantly older (median 10.8 vs 8.3 years, $P = .012$) and more often had concomitant autoimmune disease, positive calprotectin, and low MCV than did those without anemia (Table 1). They also presented with higher plasma transferrin receptor and ESR and lower ferritin and albumin values (Table 1), whereas there were no differences between the groups in gender distribution, presence of other diseases, familial history, celiac antibody, or alanine aminotransferase levels. Failure to thrive was significantly more common and abdominal pain, reflux, and dysphagia less common in children with anemia (Fig. 2). Children with anemia also had more often endoscopic findings in both EGD (60.3% vs 47.9%, $P = .001$) and

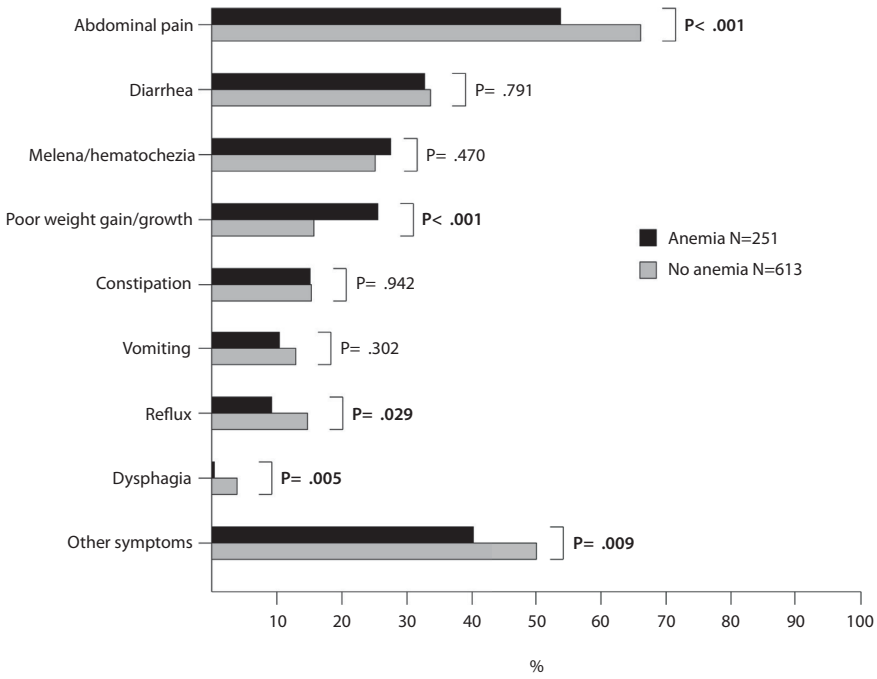


Figure 2. Comparison of the prevalence of different symptoms in children with and without anemia. Other symptoms included, for example, fatigue, persistent fever, and dermatologic symptoms.

TABLE 2. Distribution of histologic abnormalities in the endoscopic biopsy samples of patients with and without anemia

	Anemia (n = 251)		No anemia (n = 613)		
Biopsy sample location	**No. of samples**	**Abnormalities* (%)**	**No. of samples**	**Abnormalities* (%)**	***P* value**
Total EGD	242	72.7	543	51.6	<.001†
Esophagus	236	22.5	538	19.7	.382
Stomach	241	49.0	542	26.8	<.001†
Duodenum	239	36.8	538	22.1	<.001†
Total colonoscopy	124	71.8	300	33.0	<.001†
Ileum	81	28.4	208	13.5	.003†
Ascending colon	110	60.0	274	20.4	<.001†
Descending/sigmoid colon	122	62.3	297	21.5	<.001†
Rectum	116	57.8	277	20.9	<.001†

*Subjects could have histologic abnormalities in 1 or more sampling site.
†$P \leq .050$.

colonoscopy (72.1% vs 48.3%, $P < .001$) as well as histologic abnormalities at each biopsy site excluding the esophagus (Table 2).

Altogether, 183 (73.0%) anemic and 241 (39.3%) nonanemic patients received a diagnosis during the primary investigations (OR, 4.18; 95% CI, 3.03-5.77) (Fig. 1). Eight of 10 children (80.0%) with severe anemia, 91 of 111 children (82.0%) with moderate anemia, and 77 of 119 children (64.7%) with mild anemia received a diagnosis ($P = .011$). Altogether, anemia had 43.2% sensitivity, 84.6% specificity, 72.9% positive predictive value, 60.7% negative predictive value, and 64.2% overall accuracy for the subsequent diagnosis. Seven children with anemia (2.8%) with normal histologic findings in EGD and/or colonoscopy received a diagnosis in other concomitant investigations, including 1 with Meckel's diverticulum on technetium scan and 1 with Burkitt's lymphoma in magnetic resonance enterography. No endoscopic

TABLE 3. Relationships between clinical features, presence of endoscopic and histologic findings, and diagnosis received in initial investigations in 251 patients with anemia

		Abnormal findings		Diagnosis		
	No. of cases	Endoscopic (%)	Histologic (%)	%	Odds ratio*	95% Confidence interval
Sex						
Female	135	72.6	80.7	70.4	—	—
Male	116	80.2	82.8	75.9	1.32	.75-2.32
Age						
<1 y	21	61.9	61.9	52.4	—	—
1-4 y	52	73.1	82.7	73.1	2.47	.86-7.07
5-12 y	78	78.2	87.2	79.5	3.52	1.27-9.75
13-17 y	100	79.0	81.0	72.0	2.34	.89-6.11
Autoimmune disease†	44	88.6	75.0	77.3	0.86	.32-2.33
Intestinal disease in relatives‡	48	81.3	85.4	79.2	1.52	.71-3.25
Symptoms						
Abdominal pain	135	73.3	83.7	71.9	.89	.51-1.56
Diarrhea	82	84.1	92.7	81.7	2.04	1.07-3.90
Melena/hematochezia	69	89.9	89.9	84.1	2.40	1.17-4.92
Poor weight gain/growth	64	84.4	92.2	89.1	3.94	1.70-9.15
Constipation	38	71.1	76.3	65.8	.29	.32-1.40
Vomiting	26	73.1	73.1	69.2	.82	.34-1.98
Reflux	23	52.2	43.5	39.1	.20	.08-.49
Other	101	80.2	82.2	74.3	1.12	.63-1.99
Laboratory parameters						
Positive celiac serology§¶	70	80.0	97.1	95.7	11.81	3.47-40.12
Fecal calprotectin > 100 μg/g‖	29	87.7	95.4	92.3	12.86	4.00-41.32
Hypersedimentation**	78	84.6	88.5	82.1	2.65	1.29-5.44
Hypoalbuminemia††	48	83.3	95.8	91.7	5.05	1.56-16.34

—, Reference group in binary regression analysis.
*Calculated by binary regression analysis with 95% confidence intervals comparing boys with girls, age groups with those younger than 1 year, and patients with autoimmune disease, intestinal disease in relatives, different symptoms, or abnormal laboratory parameters with those without such findings.
†Type 1 diabetes, rheumatic or thyroidal disease, celiac disease.
‡Inflammatory bowel disease or celiac disease.
§Antiendomysial antibodies or IgA antibodies against transglutaminase 2.
¶Data from 177 patients.
‖Data from 94 patients.
**Data from 168 patients.
††Data from 102 patients.

abnormalities were reported in 24 anemic (13.1%) and 59 nonanemic (24.5%) children who received a diagnosis.

Anemic patients with diagnoses presented more often with positive celiac serology, positive calprotectin, and higher ESR and lower albumin values than those without diagnoses (Supplementary Table 2, available online at www.giejournal.org). Predictors for initial diagnoses in anemic patients were age from 5 to 12 years, presence of diarrhea, melena/hematochezia, poor weight gain, positive celiac antibodies, high calprotectin or ESR values, and hypoalbuminemia, whereas children with reflux symptoms were less likely to receive a diagnosis (Table 3). In nonanemic patients, symptoms significantly increasing the likelihood of diagnosis were comparable with those in anemic patients excluding diarrhea and hypoalbuminemia (data not shown). In a multivariate binary regression analysis, anemia, melena/hematochezia, and failure to thrive predicted diagnosis independently of other GI symptoms (Supplementary Table 3, available online at www.giejournal.org).

An additional 4 anemic (1.6%) and 33 nonanemic (5.4%) patients received diagnoses later in the follow-up (Fig. 1). Follow-up diagnoses in anemic children were Crohn's disease, GERD, angiodysplasia, and Imerslund-Gräsbeck syndrome, whereas in nonanemic patients the most common diagnoses were celiac disease (n = 9), Crohn's disease (n = 6), gastroesophageal reflux disease (GERD) (n = 5), and GI food allergy (n = 4).

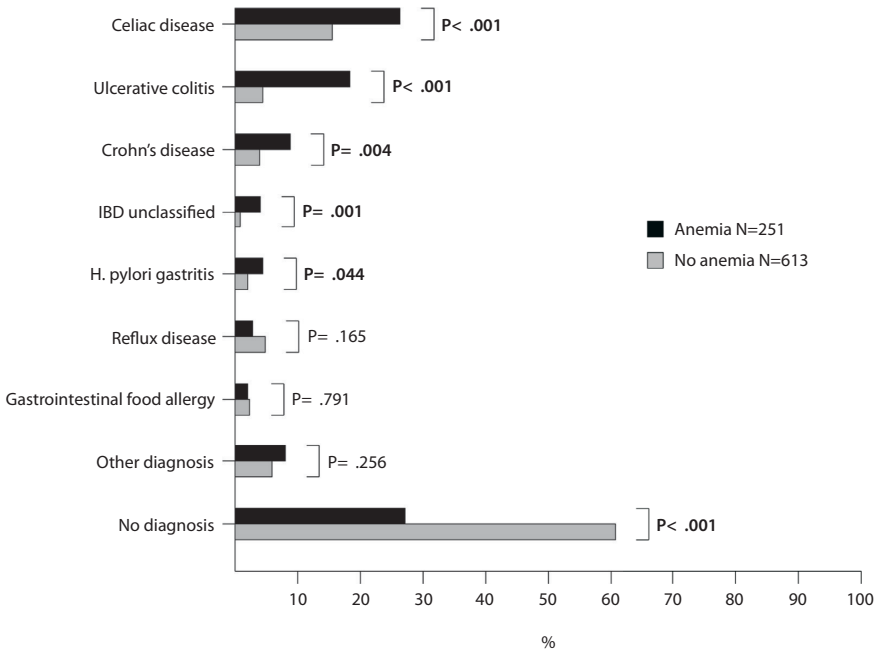


Figure 3. Comparison of the diagnoses received in the initial investigations between patients with or without anemia. *H. pylori, Helicobacter pylori*; *IBD* inflammatory bowel disease.

The most common initial diagnoses in the anemia group were celiac disease and inflammatory bowel disease (IBD), which were also more common in anemic than in nonanemic patients (Fig. 3). Other GI diagnoses found in more than 1 anemic child were *H pylori* gastritis (n = 11), GERD (n = 7), GI food allergy (n = 5), gastric or duodenal ulcer (n = 3), juvenile polyps (n = 2), and undefined colitis (n = 2), whereas GERD (n = 30), *H pylori* gastritis (n = 12), GI food allergy (n = 14), esophagitis (n = 6), mastocytosis (n = 5), pinworms (n = 5), juvenile polyps (n = 3), polyposis syndrome (n = 2), gastric/duodenal ulcer (n = 3), and rectal prolapse (n = 2) were found in nonanemic children.

Of the anemic children, 45 (17.9%) presented with only anemia-related laboratory abnormalities. Of these, 23 (51.1%) had undergone EGD alone, 2 (4.4%) colonoscopy alone, and 20 (44.4%) both EGD and colonoscopy. Fifteen (33.3%) of them received a diagnosis in the initial investigations, including 6 IBD, 1 fungal esophagitis, 3 reflux esophagitis, 1 GERD diagnosed based on esophageal pH monitoring, 2 gastric/duodenal ulcers, 1 Burkitt's lymphoma, and 1 eating disorder. Two children received a diagnosis during subsequent follow-up (Crohn's disease and angiodysplasia).

Thirty anemic children had no additional GI symptoms. All 30 underwent EGD and 7 also colonoscopy. Initial endoscopies revealed a diagnosis in 22 cases (Fig. 1), including 13 celiac disease, 4 *H pylori* gastritis, 2 IBD, 1 GI stromal tumor, 1 autoimmune gastritis, and 1 Epstein-Barr virus infection of the colon. Additional laboratory abnormalities besides anemia and/or low MCV were present in all 22 (Supplementary Table 4, available online at www.giejournal.org). None of the remaining 8 children underwent repeated endoscopies or received a diagnosis during a follow-up of up to 11 years (Fig. 1). After endoscopy, a wireless capsule endoscopy was conducted in 4 children and a technetium scan in 2, with normal findings. One of them had elevated ESR (21 mm/h) and 1 elevated calprotectin (2160 μg/g) that later normalized. In 6 of these 8 children, hemoglobin values normalized during iron supplementation.

Forty-four nonanemic children (7.2%) had signs of iron deficiency based on low MCV and/or ferritin value and/or high plasma transferrin receptor value. Twenty of these 44 (45.5%) received a diagnosis compared with 39% of the remaining nonanemic children ($P = .570$).

DISCUSSION

We found abnormal endoscopic and histologic findings at almost every biopsy sampling location and the number of final diagnoses to be more frequent in anemic than in nonanemic children. In addition, the anemia group presented significantly more often with growth problems ($P < .001$) and previously diagnosed autoimmune diseases

($P = .036$) and, of the laboratory parameters, hypersedimentation, hypoalbuminemia, increased fecal calprotectin ($P < .001$ each), and positive celiac antibodies with borderline significance ($P = .058$). Co-occurrences of these symptoms and markers, alongside diarrhea and melena/hematochezia, also further increased the likelihood of diagnoses in children with anemia. Although classically considered an "alarm signal," data on the actual significance of anemia when considering pediatric GI endoscopies have been limited.[5] More evidence on this issue is particularly important in children because even if permanent adverse effects are relatively rare, these invasive and expensive procedures should be carried out only after careful assessment of the benefits and risks involved.[5,26]

Although studies with a similar design are lacking, our results can, to some extent, be compared with studies investigating the yield of GI endoscopies at a more general level. In a small study by Wang et al,[9] anemia was the most common laboratory finding in children undergoing EGD and/or colonoscopy and up to 75% of the 20 subjects with anemia were found to have abnormal histology. Nevertheless, neither the prevalence of anemia in patients undergoing EGD nor histologic changes in nonanemic subjects were presented. Noble and colleagues[27] reported anemia as well as rectal bleeding, hypoalbuminemia, and hypersedimentation to predict endoscopic and/or histologic findings in colonoscopy, whereas age $\geq$13 years, hematemesis, dys/odynophagia, vomiting, and hypoalbuminemia, but not anemia, were associated with EGD abnormalities. Vomiting and dysphagia did not increase the likelihood of diagnoses here, but this may be because of the different study designs and the low frequency of these symptoms in our cohort. Moreover, in our setting systematic sampling from the middle part of the esophagus was not done before 2012. We also found children with anemia between 5 and 12 years old to be more likely to be given a diagnosis than were older children. This is at least partially explained by the fact that the median age of patients with anemia with celiac disease, our most common diagnosis, was 7.1 years. The constantly increasing number of pediatric IBD diagnoses may change the age distribution in the future.

Celiac disease and IBD were the most frequent cause for anemia in our study. This differs from reports from Turkey,[28] Taiwan,[29] Egypt,[30] and Italy,[3] where *H pylori* gastritis (11%-43%) and/or inadequate iron intake (16%-39%) were the leading causes, whereas celiac disease was common (19%) only in the Italian study[3] and IBD reported only in 1 Taiwanese child.[29] Here dietary cause was rare, and *H pylori* was present in only 4.4% of children, which likely reflects improved nutrition, rapid decrease of *H pylori* infection,[31-33] and concurrent worldwide increase in immune-mediated diseases.[34] The differences observed between countries regarding the cause of pediatric anemia may thus reflect temporal variation in these changes. Interestingly, up to 16% of Italian children with anemia were diagnosed with cow's milk allergy, the severe enteropathic form,[35] which we currently find exceptional.

The decision to proceed to endoscopy in children with suspicion of celiac disease or IBD is usually quite straightforward based on, respectively, either serology or specific symptoms and high blood and fecal inflammatory markers. In fact, the symptoms and markers we found to best predict diagnoses in children with anemia are the classic signs of these 2 conditions.[21,36] In these circumstances, the presence of anemia rarely influences the decision to perform endoscopy. Nevertheless, anemia predicts more severe histopathology in celiac disease[37-39] and could thus support EGD in cases with borderline positive serology. In case of IBD suspicion, the hemoglobin level may affect the urgency of endoscopies. Although good noninvasive antigen tests for *H pylori* are available[40] and the infection is a possible cause of unexplained anemia, other causes of anemia should be ruled out before testing by noninvasive or invasive methods.[41] Then again, many other possible causes can only be ruled out by endoscopic studies, including colonoscopy. This brings us to the broader question of when to perform only EGD or colonoscopy or both on children with anemia. The guidelines by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition consider unexplained anemia to be an indication for both, but the evidence is scarce.[5] Resolving this issue was not among the aims of our study, and further research on it is needed.

The decision on endoscopy is more difficult in the presence of less specific symptoms, particularly when anemia is the sole presenting sign. We found the coexistence of constipation, abdominal pain, or vomiting with anemia did not affect the likelihood of diagnoses, and reflux symptoms even reduced the probability. Consequently, uncomplicated reflux symptoms, "functional GI disorders," and constipation as such are not indications for endoscopy in the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) guidelines.[5] Evidence on the endoscopic yield in anemic children without any GI symptoms is almost nonexistent, and we thus deem our results important. Notably, all 22 children without GI symptoms who received a diagnosis presented with additional laboratory abnormalities, whereas these were (temporarily) present in only 2 nondiagnosed children. This suggests that in children with mild or moderate anemia as a sole clinical sign/laboratory finding, a short period of observation with possible iron supplementation could be a safe option, particularly because GI malignancies are extremely rare in this age group. Instead, when "red flag" GI symptoms such as melena/hematochezia or failure to thrive are present, an endoscopy and possible adjunct testing should be conducted even without anemia or other laboratory abnormalities.

The main limitations of the study are the retrospective design and data collection from only 1 tertiary center.

Although the retrospective design enabled a large study cohort and a long-term follow-up, it limited our opportunities to evaluate the significance of individual laboratory parameters separately, because in clinical practice they, with the exception of almost always measured hemoglobin, are usually taken selectively based on the clinical scenario. This also hampered the opportunity to evaluate the subtypes of anemia and children presenting with signs of iron deficiency despite normal hemoglobin. Another problem is that the pediatric reference values for iron-related parameters vary and remain controversial.[42-45] It is also important to realize that some laboratory parameters, such as anemia and ESR, may interact. These limitations were, however, counterbalanced by the large number of consecutive endoscopies with systematic biopsy sampling regardless of previous laboratory test results and endoscopic findings as well as by the availability of comprehensive medical information, including long-term follow-up data. The importance of systematic sampling is emphasized by the fairly high number of children receiving a diagnosis despite macroscopically normal endoscopy. The study population was also clinically and socioeconomically diverse and represented all pediatric age groups. Although several endoscopists and pathologists were involved during the study period, the diagnostic heterogeneity should be reduced by our unified clinical practices and regular discussion of problematic cases in multidisciplinary meetings. It is nevertheless possible that the higher number of EGDs conducted in children with anemia biased the results. Furthermore, some histologic abnormalities reported may not have any major clinical relevance, and the percentage of ileum intubation rate was unacceptably low. It must also be mentioned that we did not evaluate the significance of fecal occult blood testing, because the benefits of this approach in children remain controversial.[46] Altogether, when considering the generalizability of our results, the substantial countrywide variation in the diagnostic approach to and etiology of anemia has to be kept in mind.[1]

To conclude, anemia increased the likelihood of a diagnosis in pediatric GI endoscopies. However, in a subgroup of children with nonspecific symptoms and no additional laboratory abnormalities, especially with anemia as the sole presenting sign, a period of noninvasive surveillance with a possible treatment trial might be considered. Furthermore, subsequent diagnosis after endoscopy with negative results appears to be exceptional even in the long term.

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Abbreviations: CI, confidence interval; ESR, erythrocyte sedimentation rate; IBD, inflammatory bowel disease; MCV, mean corpuscular volume; OR, odds ratio; Rf, reference value; GI, gastrointestinal; EGD, esophagogastroduodenoscopy; GERD, gastroesophageal reflux disease; Hb, hemoglobin.

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SUPPLEMENTARY TABLE 1. Finnish age- and sex-dependent pediatric reference values[10] for hemoglobin and mean corpuscular volume

	Hemoglobin (g/L)	Mean corpuscular volume (fl)
1-7 days	150-230	88-126
7-30 days	100-206	85-123
1-2 months	95-130	80-103
2-6 months	95-141	76-97
6-12 months	100-141	72-87
1-2 years	100-142	73-87
2-4 years	100-142	73-87
4-13 years	110-155	73-95
Boys 13-17 years	130-160	76-98
Girls 13-17 years	125-160	78-102

SUPPLEMENTARY TABLE 2. Baseline characteristics of 251 anemic children who received or did not receive diagnoses after their first GI endoscopies

	Diagnosis (n = 183)		No diagnosis (n = 68)		
Categorical variables	**N**	**%**	**N**	**%**	***P* value**
Girls	95	51.9	40	58.8	.329
Autoimmune disease*	14	7.7	6	8.8	.760
Asthma, allergy, or atopy	34	18.6	10	14.7	.473
Other chronic disease	22	12.0	10	14.7	.571
Intestinal disease in relatives†	38	20.8	10	14.7	.278
Positive EmA or TGA-IgA	67‡	48.9	3‡	7.5	<.001§
Fecal calprotectin > 100 μg/g	60¶	81.1	5¶	25.0	<.001§
Low MCV	74‖	45.7	17‖	32.1	.082
Continuous variables	**N**	**Median (quartiles)**	**N**	**Median (quartiles)**	
Age, years, (range)	183	10.8 (.03, 16.4)	68	9.8 (.04, 16.7)	.757
ESR, mm/hr	121	16.0 (7.0, 28.5)	47	8.0 (3.0, 15.0)	.002§
Alanine aminotransferase, U/l	133	18.0 (13.0, 28.5)	39	21.0 (14.0, 31.0)	.236
Albumin, g/l	81	35 (33, 39)	21	39 (37, 42)	.008§

EmA, Antiendomysial antibodies; *ESR*, erythrocyte sedimentation rate; *MCV*, mean corpuscular volume; *TGA-IgA*, IgA antibodies against transglutaminase 2.
*Type 1 diabetes, rheumatic or thyroidal disease, celiac disease.
†Celiac disease or inflammatory bowel disease.
‡Data from 137 diagnosed and 40 nondiagnosed patients.
§$P \leq .050$.
¶Data from 74 diagnosed and 20 nondiagnosed patients.
‖Data from 162 diagnosed and 53 nondiagnosed patients.

SUPPLEMENTARY TABLE 3. Relationships between symptoms and diagnosis adjusted for presence of anemia in 251 children with and 613 without anemia

	Odds ratio* for the diagnosis	95% Confidence interval	*P* value
Anemia	3.50	2.50-4.91	<.001†
Abdominal pain	.91	.67-1.23	.539
Diarrhea	.85	.62-1.16	.302
Melena/hematochezia	2.51	1.53-4.14	<.001†
Poor weight gain/growth	3.24	1.69-6.20	<.001†
Constipation	.74	.49-1.10	.137
Vomiting	.92	.59-1.45	.302
Reflux	.78	.50-1.21	.270
Other‡	.82	.61-1.10	.177

*Calculated by multivariate binary regression analysis with 95% confidence intervals.
†$P \leq .050$.
‡Other symptoms included, for example, fatigue, persistent fever, and dermatologic symptoms.

SUPPLEMENTARY TABLE 4. Characteristics of 30 children who had anemia without GI symptoms and received or did not receive diagnoses after their first GI endoscopies

	Diagnosis (n = 22)		No diagnosis (n = 8)		
Categorical variables	**N**	**%**	**N**	**%**	***P* value**
Girls	15	68.2	3	37.5	.210
Autoimmune disease*	3	13.6	1	12.5	1.000
Asthma, allergy, or atopy	3	13.6	0	0	.545
Other chronic disease	3	13.6	1	12.5	1.000
Intestinal disease in relatives†	2	9.1	1	12.5	1.000
Positive EmA or TGA-IgA	13‡	72.2	0‡	0	.007§
Fecal calprotectin > 100 μg/g	5¶	100	1¶	33.3	.107
Low MCV	12‖	75.0	3‖	50.0	<.001§
Continuous variables	**N**	**Median (quartiles)**	**N**	**Median (quartiles)**	
Age, y (range)	22	9.4 (1.4, 15.3)	8	12.3 (.04, 15.1)	.963
ESR, mm/h	9	12.0 (5.5, 21.0)	5	5.0 (3.0, 16.5)	.180
Alanine aminotransferase, U/L	11	22.0 (17.0, 29.0)	5	17.0 (11.0, 81.5)	.609
Albumin, g/L	7	28.0 (32.0, 42.0)	2	31.5 (21.00, —)	.766

EmA, Antiendomysial antibodies; *ESR*, erythrocyte sedimentation rate; *MCV*, mean corpuscular volume; *TGA-IgA*, IgA-antibodies against transglutaminase 2; —, upper quartile could not be calculated because of the low number of patients.
*Type 1 diabetes, rheumatic or thyroidal disease, celiac disease.
†Celiac disease or inflammatory bowel disease.
‡Data from 18 diagnosed and 5 nondiagnosed patients.
§$P \leq .050$.
¶Data from 5 diagnosed and 3 nondiagnosed patients.
‖Data from 16 diagnosed and 6 nondiagnosed patients.

PUBLICATION II

Anemia in children with celiac disease: association with the clinical, serological and histological findings and response to the gluten-free diet

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Anemia in pediatric celiac disease: association with clinical and histological features and response to gluten-free diet

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Abstract

Goals: To compare clinical, serological and histological manifestations between children with anemia and without anemia at celiac disease (CD) diagnosis.

Background: Despite being a common finding, the association between the presence of anemia and clinico-histopathological presentation of CD in children remains obscure.

Study: 455 celiac disease patients <18 years of age were divided into those with anemia and those without anemia at diagnosis. The groups underwent comparisons of a variety of clinical, serological and laboratory parameters and severity of small-bowel mucosal damage. Further, adherence and clinical and serological response to the gluten-free diet (GFD) were compared.

Results: Anemia was detected in 18.0% of the patients. Children with anemia had higher values for transglutaminase 2 antibodies (120.0 U/l vs. 88.0 U/l, $p<0.001$) and, by definition, lower values for hemoglobin (10.5 g/dl vs. 12.8 g/dl, $p<0.001$) and other iron parameters. They were also less often screen-detected (13.4% vs 34.6%), had more severe histological damage ($p=0.048$) and poorer dietary adherence (78.3% vs 87.5%, $p=0.035$) than the non-anemic patients. Anemia recovered in 92% after a median of one year on a GFD, but hemoglobin values remained significantly lower compared with the non-anemic group (12.5 g/dl vs. 13.2 g/dl, $p=0.045$). There was no difference between the groups in the clinical and serological response to the GFD ($p=0.318$).

Conclusions: Anemia at CD diagnosis is associated with more severe histological and serological presentation in children. Further, low hemoglobin may not fully recover even after a median of one year on a strict GFD.

Keywords: Celiac disease, children, anemia, serology, histology

What's Known on This Subject

- Celiac disease (CD) is one of the most common chronic diseases in children
- A very common manifestation in untreated CD is anemia
- The association between the presence of anemia and clinico-histopathological presentation of CD is unclear

What This Study Adds

- Anemic children with CD have more severe clinical, serological and histological disease than non-anemic children
- Low hemoglobin levels may not fully recover even after one year on a gluten-free diet
- Early diagnosis of anemia at CD diagnosis is important

Introduction

Several recent screening studies have revealed that, with a prevalence of up to 2%, celiac disease (CD) is one of the most common lifelong disorders in children.[1,2] Simultaneously the condition has been found to have a very heterogeneous clinical presentation, including classical gastrointestinal symptoms such as diarrhea and abdominal pain, and a variety of extraintestinal symptoms such as arthralgia, dermatitis herpetiformis, poor growth and hypertransaminasemia.[3-6] Arguably one of the most common manifestations of CD in children is iron-deficiency anemia, of which the prevalence in untreated patients has varied from 16 % up to 84 % depending on the study.[7-12] Even mild or subclinical anemia can be detrimental to health since, besides the direct effects of reduced oxygen transport capacity, it predisposes children for example to poor cognitive and psychomotor development and to impaired immune defense.[13-15] Despite being such a prevalent finding, however the association between anemia and the clinico-histopathological presentation of CD remains poorly established. Interestingly, a recent study has shown adult CD patients with anemia as a prominent symptom to have more severe clinical and histological disease than those presenting with diarrhea.[16] Evidently, this issue should also be carefully investigated in children, in whom anemia may have markedly different causes and consequences compared with adults. Here we sought to address this issue by comparing a variety of clinical, histological, serological and laboratory findings and dietary response between children presenting with and those without anemia at CD diagnosis.

Materials & Methods

Patients and study design

The study was carried out at the Department of Pediatrics, Tampere University Hospital and at the Tampere Center for Child Health Research. Data on the clinical, histological and serological findings were collected systemically from the medical records of all children (age

less than 18 years) with biopsy-proven CD diagnosed at the Department of Pediatric Gastroenterology from the year 2000 onwards. From the year 2014 onwards most of the study children have been enrolled prospectively. After data collection and preliminary analysis children were divided into those with and those without anemia at the time of CD diagnosis, and all study variables (see below) were compared between these two groups. Anemia at CD diagnosis was defined as a Hb value lower than the age- and sex-specific reference.[17] Moreover, follow-up data regarding adherence and clinical response to treatment with a gluten-free diet (GFD) were collected as available.

The Ethics Committee of The Pirkanmaa Hospital District approved data collection from the medical records and prospective patient recruitment. In addition, all children and/or their parents recruited prospectively gave written informed consent.

Data analyses

Clinical characteristics

The following clinical information was gathered on all study children: demographic and anthropometric data, presence of CD associated or other co-morbidities (e.g. type 1 diabetes, Down's syndrome, asthma, allergies) and CD in the family. The type of the clinical presentation at diagnosis was classified as gastrointestinal, extraintestinal or screen-detected, and severity of presentation as no symptoms, mild symptoms (occasional, somewhat bothersome gastrointestinal or extraintestinal symptoms) or moderate/severe symptoms (frequent and/or severe symptoms significantly disturbing daily life). Gastrointestinal symptoms were further sub-categorized into abdominal pain, constipation, diarrhea, vomiting and other, and extraintestinal symptoms and signs into aphtous ulcers, joint symptoms, liver abnormalities, neurological symptoms, poor growth, skin symptoms and other.

Serology and laboratory parameters

Serum IgA-class transglutaminase 2 antibodies (TG2-ab) were measured in the hospital laboratory either by traditional ELISA (Phadia, Uppsala, Sweden, before 2011) or by an automatized recombinant-based EliA assay (Phadia). In our settings values ≥7.0 U/l for TG2-ab are considered positive and the maximum provided value is 120.0 U/l. Serum IgA-class endomysial antibodies (EmA) were measured in the Tampere Center for Child Health Research by a well-defined immunofluorescence method as described elsewhere.[18] EmA titer 1:5 was considered positive and further diluted up to 1:4000 or until negative. In case of a selective IgA-deficiency the corresponding IgG antibodies were determined.

The following iron-related laboratory parameters measured as a part of routine clinical practice were recorded for each child when available: blood hemoglobin (Hb) (g/dl), erythrocyte mean corpuscular volume (MCV) (fl), plasma total iron (µmol/l), plasma transferrin receptor 1 (TfR1) (mg/l) and plasma ferritin (µg/l). Further, plasma alanine aminotransferase (ALT) (U/l), plasma alkaline phosphatase (ALP) (U/l), plasma albumin (g/l), plasma thyroid-stimulating hormone (TSH) (mU/l) and plasma thyroxine (pmol/l) were recorded in order to further elucidate the overall severity of the disease. For consistency we decided to accept only laboratory values taken in our hospital laboratory at the day of endoscopy for the baseline comparisons between the groups. Further, values other than Hb were started to be taken systemically only during the latter part of the study period.

Histology

In our clinical practice a minimum of four distal duodenal biopsies are taken upon upper gastrointestinal endoscopy in all cases with CD suspicion. From the year 2011 onwards 2-3

additional biopsies have been routinely taken from the anatomical duodenal bulb as recommended in recent guidelines.[19] The biopsies are processed in the hospital pathology unit and only well-oriented and representative cuttings are accepted for further microscopic analyses.[20] The severity of small-bowel mucosal damage is systemically graded by pathologist into partial (PVA), subtotal (SVA) and total villous atrophy (TVA). These grades correspond approximately Marsh-Oberhuber grades IIIa, IIIb, and IIIc, respectively.

Adherence and response to the gluten-free diet

All children/parents received personal guidance for the GFD from a dietitian after the celiac disease diagnosis. Adherence to the diet was evaluated after 6-12 months, and was considered strict if only a few minor lapses were reported; lapses less than once in a month were rated as "occasional lapses"; and more lapses as "no GFD". Good clinical response was defined as disappearance of symptoms, negative seroconversion or marked decrease in CD autoantibodies and improvement in possible abnormalities in growth and laboratory values. In addition, the EmA, tTG2-ab and hemoglobin values measured after a median 12 months on GFD were recorded and compared between the study groups.

Statistical analysis

The variables are presented either as medians with lower and upper quartiles or as percentage distributions. In addition, the figure for available data in each variable is reported in the tables. Non-parametric variables were compared with Mann-Whitney U test and categorical variables were examined by cross tabulation with χ^2-test. A p-value <0.05 was considered significant in all analyses. All statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS Inc. Chicago, IL, USA).

Results

The final study cohort comprised 455 children of whom 82 (18.0 %) were found to have anemia and 373 (82%) had normal hemoglobin levels at CD diagnosis. Children with anemia were significantly older and had fewer CD in the family compared with the non-anemic patients (Table 1). No differences were seen between the study groups either in gender or in the prevalence of any of the CD associated and other co-morbidities (Table 1).

The main clinical presentation at diagnosis was more often extraintestinal and less often screen-detected in the anemic children than those in the non-anemic group (Figure 1A). Of specific gastrointestinal symptoms the anemic patients suffered less from vomiting, and there was also a non-significant trend towards more constipation and less diarrhea (Table 2). In contrast, there were no differences between the groups in the prevalence of specific extraintestinal symptoms (Table 2). Anemic children also had a somewhat higher percentage of moderate/severe symptoms, though the difference was not significant (Figure 1B). The results remained unchanged when adjusted by age (data not shown).

The median values for both TG2-ab and EmA were higher in the anemia group at diagnosis (Table 3). Furthermore, by definition, Hb and all iron-related variables were poorer in the anemic children, while there were no differences between the groups in the other laboratory parameters (Table 3). Anemic children also evinced more severe small-bowel mucosal damage, the difference being caused by the higher proportion of total villous atrophy and the lower proportion of partial villous atrophy (Figure 1C).

Children in the anemia group showed significantly poorer adherence to the GFD at follow-up (78.3% vs 87.5%, p=0.035). However, in all cases there were only occasional lapses and none

reported unrestricted gluten consumption. After a median of 12 months on GFD celiac autoantibody and Hb values were significantly improved (median change anemia group 2.0g/dl (16% increase) and non-anemic group 0.4g/dl (3% increase), respectively) in both groups compared with the baseline (Table 4). Anemia recovered in 92% of the children, but the anemia group still yielded significantly lower median Hb levels than the non-anemic group (Table 4). All four (8%) subjects with persistent anemia (baseline Hb values 6.5 g/dl, 9.7 g/dl, 11.2 g/dl and 11.6 g/dl) were on a strict GFD and showed good clinical and serological response. Altogether 25 anemic children (30 %) initiated oral iron supplementation after the CD diagnosis, including three out of the four with persistent anemia. Clinical response to the GFD was seen in 94.3% of anemic and 96.6% of non-anemic children (p=0.318).

Discussion

The main finding in the present study was that children with anemia at the time of CD diagnosis had more severe disease in terms of serology and small-bowel mucosal histology compared with those presenting without anemia. In addition, the anemic children showed partly incomplete recovery of Hb values even after a median of one year on a GFD.

Altogether 18% of the CD patients here had anemia, which is a relatively low figure compared with most previous studies conducted in children.[7-9,11] For example, de Vizia and colleagues[21] observed anemia to be present in 34% and Demir and colleagues[22] in 53% of pediatric CD patients at diagnosis, and the highest prevalences of anemia in untreated CD children, up to 85%, have been reported in studies from India.[8,9] However, in some of the more recent studies figures closer to ours have been reported. In a British study[23] the prevalence of anemia in CD children was 20%, in a U.S. study[24] it was 20%, and in a previous smaller study from Finland[10] 25%. This global variation in anemia prevalences possibly reflects differences in clinical

presentation, as in many developed countries the previously common severe infantile CD has almost disappeared, while in less developed countries the classical malabsorptive disorder still prevails.[8] Moreover, multiple nutritional defects, including iron deficiency, are very common in the third world nations and may further contribute to the high prevalence of anemia. In contrast, anemia is very rare in native Finnish pediatric population, and even the 18% seen in celiac patients here was a clear overrepresentation[25], in particular as there were no immigrants in our study cohort.[26] Interestingly, we have recently shown that the prevalence of anemia as an initial presentation of CD could be again slightly increasing in developed countries.[27] This somewhat surprising phenomenon might be attributable to the increasing activity of clinicians to seek out CD in children with unexplained anemia.

Perhaps the most conspicuous difference between the groups here was the presence of more severe histological damage among the anemic children. Our results are in agreement with those of a recent study conducted among adult CD patients from the USA by Abu Daya and colleagues.[16] The authors compared patients presenting with diarrhea to those presenting with anemia and, as here, observed more advanced mucosal atrophy in the latter group. Moreover, in both the present and the U.S. study anemic patients had higher CD autoantibody values at diagnosis. This is in line with previous CD findings showing a correlation between the results of serology and histology[28-30], and further demonstrates the presence of more advanced disease in anemic patients. It seems logical that severe enteropathy should predispose to iron-deficiency anemia in view of the reduced absorptive surface of the intestine.[7] On the other hand, one would also assume more diarrhea and other gut-related symptoms, this being the case neither here nor in the U.S. study.[16] Also, despite the more severe mucosal damage in the anemic patients, in the present study there was no difference between the groups in the prevalence of poor growth. Evidently, the eventual clinical picture in CD is a result of a much

more complicated process than only a secondary effect of the mucosal damage.[31] Interestingly, previous results obtained by Matysiak-Budnik and colleagues[32] indicate that anemia might not be merely an outcome but also an active player in the CD pathogenesis. The authors showed that gluten may enter the body via enterocyte transferrin receptors, whose expression increases during iron deficiency.[33] As a result, anemia could further promote gluten influx, leading to a kind of self-perturbing pathogenic loop. The iron metabolism is further complicated by the presence of mucosal inflammation, which may again affect the iron intake via hepcidin upregulation.[34,35] Obviously, more studies are needed to elucidate these intriguing issues, but in any case our results indicate that an early detection of anemic CD patients is important to prevent possible permanent complications related to advanced CD.

Both groups here showed excellent clinical and serological response to the GFD, further confirming the overall good dietary adherence of the study children. Although, for some obscure reason, adherence was still somewhat lower in the anemia group, none of the patients had major dietary lapses and the few with ongoing anemia maintained a strict GFD and had good clinical response. Thus, neither the persistent anemia nor the lower median Hb value in the anemia group on follow-up seem to be fully explained by compliance problems.[36] Previously Annibale and colleagues[37] have reported anemia to recover in 94% of adult patients after 12 months on diet, and in a pediatric study by Catal et al.[38] this was seen in 94% after a median of 48 months. In contrast, in a study by Bergamaschi et al.[39] recovery was seen in 70% of patients after 12 months, and in a study by Pulido et al.[40] in only 55% after one and in 78% after five years. Annibale et al. reported no additional iron supplementation, in the study by Catal et al. supplementation was given in all anemic patients, while the other reports did not provide this information. Besides supplemental iron, another factor potentially affecting these variable recovery results is dietary adherence, on which no precise definition was reported in

the aforementioned studies. It is also possible that some CD patients have a genetic predisposition to low Hb and thus both fulfil the criteria for anemia more easily and also evince seemingly incomplete recovery.[41,42] In any case, when designing the follow-up of these children, clinicians should bear in mind that the recovery of anemia may be protracted even on a strict GFD.

Major strengths of the present study are the well-defined and large cohort of children with histologically confirmed CD, and the large number of different variables investigated. On the other hand, limitations are the retrospective design and lacking long-term follow-up results. In addition, even the short-term follow-up results were missing from quite many patients, and we did not have detailed data of dietary habits possibly affecting to the iron intake and hemoglobin values. Another limitation is lacking values of vitamin B12 and folate, which are not routinely taken in our clinical practice. Further, many laboratory parameters were not taken systemically during the whole study period and were thus missing from a substantial part of the children. Also, in order to reduce possible bias caused by variation in time and place of the Hb measurement a part of these values were omitted from the Table 3. It must also be emphasized that the study children were very often screen-detected and diagnosed in a single tertiary center, and thus may not fully represent the wide spectrum of CD seen in everyday clinical practice. CD is well-known among physicians and gluten-free products are easily available in Finland; the follow-up results might thus be better compared with countries where awareness is lower and the diet more difficult to maintain.[43]

To conclude, our results demonstrate that the presence of anemia at the diagnosis of CD is associated with more advanced clinical and histological presentation. Therefore, in order to prevent excessive burden and possible permanent complications caused by ongoing untreated

CD, it is important to identify and treat these anemic children as early as possible. Clinicians should also be aware that the recovery of anemia may take a rather long time despite a strict GFD, and thus special emphasis should be placed on the follow-up of this patient group.

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Table 1. Demographic data, presence of celiac disease in the family and co-morbidities in 82 children with anemia and 373 children without anemia at celiac disease diagnosis.

	Anemia n=82		No anemia n=373		p value
Variable	n	%	n	%	
Girls	58	70.7	241	64.6	0.307
Celiac disease in the family[1]	15	27.3	104	43.5	0.033
Co-morbidities					
Type 1 diabetes	4	5.1	33	9.0	0.276
Thyroidal disease	2	2.5	5	1.4	0.361
Down´s syndrome	0	0.0	3	0.8	1.000
Epilepsy	0	0.0	2	0.5	1.000
Asthma	4	5.1	35	9.5	0.273
Any allergy	18	22.0	86	23.1	0.885
Other chronic illness[2]	8	9.8	43	11.5	0.645
Age, median (Q_1, Q_3), years	8.5 (5.0, 13.4)		7.4 (4.7, 11.0)		0.038

Data were available in >95% of the patients in each variable except [1]in 55 and 239 patients, respectively

[2]E.g. Asperger's syndrome, ulcerative colitis, migraine, systemic juvenile idiopathic arthritis, chronic neutropenia, anorexia nervosa

Q_1, Q_3, lower and upper quartiles

Table 2. Distribution of different gastrointestinal and extra-intestinal symptoms in 82 children with anemia and 373 children without anemia at celiac disease diagnosis.

	Anemia n=82		No anemia n=373		p value
Variable	n	%	n	%	
Gastrointestinal symptoms					
Diarrhea	16	19.5	112	30.0	0.060
Abdominal pain	38	46.3	166	44.5	0.383
Constipation	16	19.5	58	15.5	0.063
Vomiting	0	0.0	10	2.7	0.021
Other[1]	7	10.0	39	11.3	0.838
Extra-intestinal symptoms[2]					
Poor growth	19	23.2	85	22.8	1.000
Rash	5	6.1	21	5.6	0.796
Neurological symptoms	1	1.2	9	2.4	1.000
Joint symptoms	3	3.7	21	5.6	0.594
Aphtous ulcers	2	2.4	8	2.1	0.698
Liver abnormalities	3	3.7	8	2.1	0.426
Other[3]	4	4.9	23	6.2	0.800

[1]E.g. flatulence, abnormal/bloody stools, gastroesophageal reflux, abdominal distension
[2]Excluding anemia
[3]E.g. dental enamel defects, fatigue, alocipea, muscle pains, brittle nails

Table 3. Baseline laboratory parameters and anthropometric data in 82 children with anemia and 373 children without anemia at celiac disease diagnosis.

	Anemia n=82		No anemia n=373		p value
Variable	n[1]	Median (Q_1, Q_3)	n[1]	Median (Q_1, Q_3)	
EmA, titer	64	1:500 (1:500, 1:4000)	249	1:500 (1:100, 1:1000)	<0.001
TG2-ab, U/l[2]	63	120.0 (80.7, 120.0)	271	88.0 (29.0, 120.0)	<0.001
Hemoglobin, g/dl	76	10.5 (9.3, 11.5)	264	12.8 (12.1, 13.3)	<0.001
MCV, fl	68	74.0 (68.0, 79.0)	227	81.0 (78.0, 84)	<0.001
TfR1, mg/l	34	6.2 (4.4, 12.0)	52	3.8 (3.0, 4.8)	<0.001
Ferritin, µg/l	39	7.0 (3.5, 13.0)	70	15.0 (9.8, 26.0)	<0.001
Total iron, µmol/l	18	5.6 (3.7, 11.9)	24	14.1 (10.0, 19.9)	0.002
ALT, U/l	41	22.0 (16.0, 29.0)	106	19.5 (15.5, 24.5)	0.172
ALP, U/l	25	212.0 (137.0, 242.0)	81	196.0 (165.0, 235.0)	0.861
Albumin, g/l	24	37.5 (35.8, 39.3)	70	39.0 (37.0, 41.0)	0.128
TSH, mU/l	40	2.8 (1.4, 3.6)	122	2.5 (1.7, 3.2)	0.650
Thyroxine, pmol/l	16	14.7 (13.6, 16.0)	48	14.4 (13.0, 16.3)	0.561
Height, SD	42	-0.1 (-0.8, 1.0)	210	0.1 (-0.7, 1.0)	0.659
Weight, SD	33	-0.3 (-1.5, 0.5)	160	-0.4 (-1.2, 0.4)	0.728

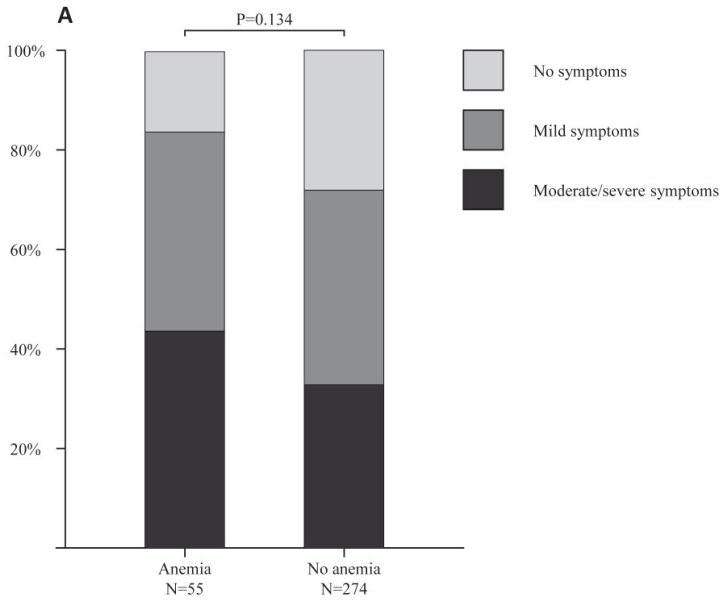
[1]Data available; [2]Upper limit of the assay is 120.0 U/l
Q_1, Q_3, lower and upper quartiles; EmA, endomysial antibodies; TG2-ab, transglutaminase 2 antibodies; MCV, mean corpuscular volume; TfR1, transferrin receptor 1; ALT, alanine aminotransferase; ALP, alkaline phosphatase, TSH, thyroid-stimulating hormone; SD, standard deviation

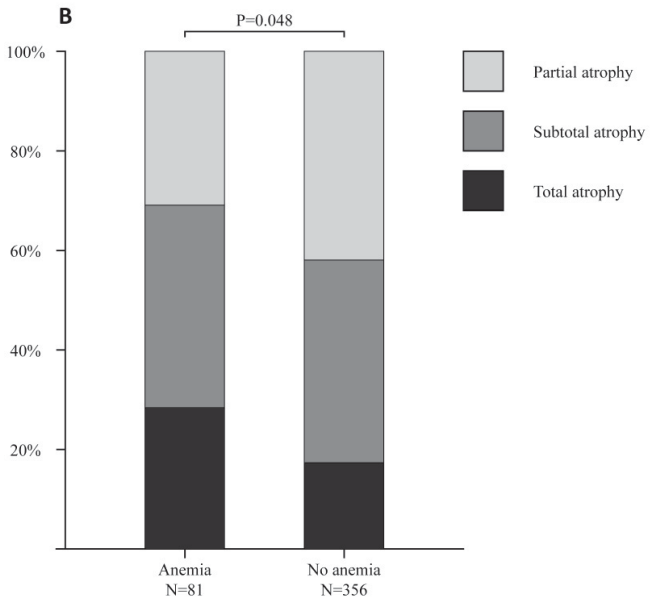
Table 4. Recovery of serum celiac disease autoantibodies and hemoglobin after a median of 12 months on a gluten-free diet (GFD) in children with anemia and those without anemia at celiac disease diagnosis

	Anemia		No anemia		p value
	n[1]	Median (Q_1, Q_3)	n[1]	Median (Q_1, Q_3)	
TG2-ab, U/l[2]					
At diagnosis	54	120.0 (120.0, 120.0)	204	120.0 (30.0, 120.0)	0.002
On a GFD	54	6.3 (2.5, 14.0)	204	4.4 (2.3, 9.5)	0.093
EmA, titer					
At diagnosis	46	1:1000 (1:500, 1:4000)	164	1:200 (1:100, 1:1000)	< 0.001
On a GFD	46	1: <5 (1: <5, 1:5)	164	1: <5 (1: <5, 1:5)	0.144
Hemoglobin, g/l					
At diagnosis	50	10.5 (9.0, 11.5)	158	12.8 (12.1, 13.3)	< 0.001
On a GFD	50	12.5 (12.1, 13.4)	158	13.2 (12.5, 13.6)	0.045

[1]Value available both at diagnosis and on GFD; [2]Upper limit of the assay is 120.0 U/l
The change within the groups on GFD was significant ($p<0.001$ in each) in all variables

Figure 1. The main clinical presentation (A), severity of the symptoms (B) and degree of small-bowel mucosal atrophy (C) in children presenting with or without anemia at CD diagnosis. The total number of study patients was 82 in the anemia group and 373 in the non-anemia group, but some of them were omitted from the figure owing to unclear or contradictory medical information.




B
P=0.048
100%
80%
60%
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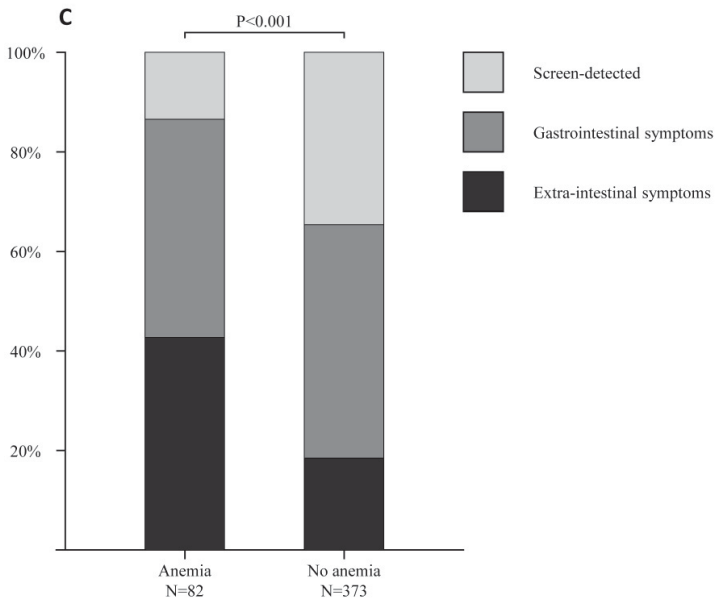
20%
Partial atrophy
Subtotal atrophy
Total atrophy
Anemia
N=81
No anemia
N=356


C
P<0.001
100%
80%
60%
40%
20%
Screen-detected
Gastrointestinal symptoms
Extra-intestinal symptoms
Anemia
N=82
No anemia
N=373

PUBLICATION III

Anemia and iron deficiency in children with potential celiac disease

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ANEMIA AND IRON DEFICIENCY IN CHILDREN WITH POTENTIAL CELIAC DISEASE

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Marleena Repo contributed the conception and the design of the work, performed a part of the laboratory tests, analyzed the study data and wrote the first draft of the article. Katri Lindfors, Markku Mäki, Heini Huhtala, Kaija Laurila, Marja-Leena Lähdeaho, Päivi Saavalainen, Katri Kaukinen and Kalle Kurppa contributed the conception and design of the work and revised the text for important intellectual content. All authors approved the final version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Abstract

Objectives: Active screening for celiac disease frequently detects seropositive children with normal villous morphology (potential celiac disease). It remains unclear whether these subjects should be treated. We here investigated the prevalence of anemia and iron deficiency in children with potential and mucosal atrophy celiac disease.

Methods: The prospective study involved 19 children with potential disease, 67 with partial or subtotal villous atrophy (P/SVA) and 16 with total villous atrophy (TVA). Twenty-three healthy children comprised the control group. The groups were compared for various clinical, histological and laboratory parameters and hepcidin.

Results: The prevalence of abnormal parameters was as follows (controls, potential celiac disease, P/SVA and TVA, respectively): anemia 0%, 15%, 22% and 63%; low iron 5%, 0%, 14% and 50%; increased transferrin receptor 1 (TfR1) 5%, 16%, 20% and 47%; low ferritin 0%, 21%, 35% and 87%; and low transferrin saturation 10%, 11%, 41% and 71%. One subject had low folate and none had low vitamin B12. The median values for hemoglobin, total iron, ferritin and transferrin saturation were significantly lower and TfR1 values higher in TVA group compared with other groups. After a median of seven months on a gluten-free diet hemoglobin, total iron, ferritin and albumin in children with P/SVA exceeded the baseline values in the potential celiac disease group.

Conclusions: The development of anemia and iron deficiency in celiac disease is a continuum and may already be present in children with normal villous morphology, advocating an early diagnosis and possible dietary treatment of these patients.

Key words: Hepcidin, hemoglobin, gluten-free diet, mucosal damage

What is known

- An increasing number of children with positive celiac autoantibodies but morphologically normal small-bowel mucosa are detected by active case-finding and screening
- It remains unclear whether subjects with this so-called potential celiac disease should be treated with a gluten-free diet

What is new

- Celiac disease-associated anemia and iron deficiency is a continuum and may appear in seropositive children even before morphological villous damage
- These children may benefit of an early diagnosis and dietary treatment

Introduction

Celiac disease is a life-long disorder characterized by a heterogeneous clinical picture.[1] Recent screening studies have revealed the prevalence to be as high as 1-2% (2, 3). The only treatment for the condition is a permanent gluten-free diet, which usually results in beneficial clinical, histological and serological response (4). In all current criteria the diagnosis is still based on the morphological damage of the small-bowel mucosa (6-8), although in the latest pediatric guidelines the actual biopsy is not necessary in all cases (5). However, accurate serological tests measuring antibodies against transglutaminase 2 (TG2-ab) and endomysium (EmA) are nowadays available (5,9,10). Widespread utilization of these tests has resulted in the identification of an increasing number of seropositive subjects who still have morphologically normal mucosa and are thus not diagnosed (2, 11). Recent studies have produced a growing body of evidence that this so-called potential celiac disease may in fact already cause gluten-dependent symptoms before villous atrophy with crypt hyperplasia, the end-stage of the disease, develops (11-13). Further, most of these cases will eventually develop atrophy when continuing on a gluten-containing diet (12, 14-16). Interestingly, clinical experience suggests that children with potential celiac disease evince signs of anemia or subclinical iron deficiency. Besides a role in oxygen transport, iron plays a crucial part in many biological functions such as energy production, DNA synthesis and cell proliferation, and iron deficiency can impair psychomotor and cognitive development and lead to a defective immune system (17-21). Thus, if abnormal iron parameters may be present already in children with potential celiac disease, this would strongly support active diagnosis of seropositive patients even before the development of advanced mucosal lesion.

The aim of the present prospective study was to investigate the prevalence of anemia and abnormal iron parameters in children with potential and mucosal atrophy celiac disease. Further, these variables and a variety of other clinical and laboratory features were

compared between these seropositive patients with different stages of mucosal damage and healthy controls.

Materials and methods

Patients and study design

The study was conducted at the Tampere Centre for Child Health Research, University of Tampere and Tampere University Hospital. It involved consecutive children (age <16 years) referred to our tertiary referral center due to celiac disease suspicion and evincing positive celiac disease serology. Exclusion criteria were study refusal and negative or lacking serology. All participants underwent a thorough clinical examination and blood sampling for serology and laboratory parameters and for celiac disease genetics. Gastrointestinal endoscopy with duodenal biopsies was performed under general anesthesia. Children with established villous atrophy with crypt hyperplasia received a celiac disease diagnosis and were placed on a gluten-free diet, while those with positive autoantibodies but morphologically normal villi continued on a normal diet and comprised the potential celiac disease group (for details see below). Children with potential celiac disease were monitored every 3-6 months during the study period (up to 2.5 years) and a new endoscopy was performed in case of marked increase in the antibody values or worsening of the symptoms. Finally, all study parameters were compared between the study groups at the time of diagnosis. After the patients with a celiac disease diagnosis had been on a gluten free diet for a minimum of three months, the values of iron parameters were re-measured. Twenty-three healthy children with no celiac disease suspicion and negative EmA and TG2-ab comprised the non-celiac control group for comparisons of the study parameters. These children had participated as family members in a celiac disease screening study in which blood samples

were drawn for laboratory parameters and celiac disease serology and genetics as previously described (10).

Written informed consent was obtained from all study participants and/or their parents according to the Helsinki Declaration. The study protocol and patient recruitment were approved by the Ethics Committee of the Pirkanmaa Hospital District.

Clinical Evaluation

The clinical presentation of celiac disease was recorded and categorized into gastrointestinal symptoms (e.g. diarrhea, stomach pains, constipation, bloating), extra-intestinal symptoms (e.g. neurologic symptoms, rash, poor growth, anemia, fatigue, arthralgia) or screen-detected in at-risk groups (e.g. celiac disease in relatives or previous type 1 diabetes). In addition, family history of celiac disease and presence of disease-associated (e.g. type 1 diabetes, autoimmune thyroidal disease) or other chronic conditions were recorded. Height and body mass index (BMI) at celiac disease diagnosis were expressed as standard deviation scores (SDS) for age and sex.

Small-Bowel Mucosal Histology and IgA deposits

A minimum of five small-bowel mucosal biopsies were taken from the distal duodenum and three biopsies from the anatomical duodenal bulb. The paraffin-embedded specimens were cut, stained with hematoxylin and eosin and evaluated by experienced pathologists. Only correctly oriented histological specimens with complete villus-crypt units and longitudinally cut crypts were accepted for microscopic analyses (22). The specimens were further graded on the basis of the histological findings as follows: morphologically normal villi with or without mucosal inflammation (potential celiac disease group, equivalent to Marsh 0-1 or Corazza-Villanacci A), normal villi with crypt hyperplasia (Marsh 2, Corazza-Villanacci A),

subtotal and partial villous atrophy (P/SVA group, Marsh 3a-b, Corazza-Villanacci B1) and total villous atrophy (TVA group, Marsh 3c, Corazza-Villanacci B2) (23, 24).

At least one biopsy from both the distal duodenum and the bulb area were freshly embedded in optimal cutting temperature compound (OCT) (Tissue-Tec, Miles Inc, Elkhart, IN, USA) and snap-frozen in liquid nitrogen. Staining of intraepithelial lymphocytes (IELs) was performed with 5-μm-thick frozen biopsy sections. The mucosal CD3+ IELs were stained with monoclonal antibody Leu-4 (Becton Dickinson, San Jose, CA, USA) and T-cell receptor-γ antibody (Endogen, Woburn, MA, USA) was used to stain γδ+ IELs. A 100x flat field light microscope objective was used to count positive IELs and counted cells were expressed as cells/mm of epithelium (25). Celiac disease-specific mucosal TG2-targeted autoantibody deposits (IgA deposits) were measured by direct immunofluorescence from the frozen specimens as previously described (26, 27). In celiac disease the deposits are found along the villous and crypt epithelium and around mucosal vessels, whereas in healthy individuals IgA is detected only inside plasma and epithelial cells (27).

Celiac Disease Serology and HLA Genotype

Serum IgA class EmA titers were measured by indirect immunofluorescence (in-house) with human umbilical cord as substrate. A dilution ≥ 1:5 was considered positive and positive sera were further diluted 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:4000. The EliA Celikey test (Phadia, Uppsala, Sweden) was used to determine serum TG2-abs. Cut-off for TG2-ab positivity was >7.0 U/l according to the manufacturer's instructions. Human leucocyte antigen (HLA) DQ2/DQ8 genotyping was performed using the SSP low-resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden).

Laboratory Parameters and Hepcidin

The following associated laboratory parameters were measured by standard methods: hemoglobin (Hb, reference value (Rf) from 100-141 g/l to 130-160 g/l depending on age and sex), plasma transferrin receptor 1 (TfR1, Rf from 1.6-5.2 mg/l to 2.0-6.8 mg/l), serum total iron (Fe, Rf 6-25 µmol/l), plasma ferritin (Rf >10 µg/l), transferrin iron saturation (Rf 15-50 %), serum folate (Rf 10.4-42.4 nmol/l) and serum vitamin B12 (Rf 140-490 pmol/l). Serum bioactive hepcidin (hepcidin-25) levels were measured with parallel samples using a commercial solid-phase enzyme-linked immunosorbent assay (EIA-5258, DRG Diagnostics, Marburg, Germany). Furthermore, values for plasma albumin (Rf from 35-46 g/l to 37-51 g/l depending on age and sex), plasma alkaline phosphatase (ALP, Rf from 115-460 U/l to 80-445 U/l), plasma alanine aminotransferase (ALT, Rf <40 U/l) and plasma thyroid-stimulating hormone (TSH, Rf 0.27-4.2 mU/l) were measured by standard methods. Laboratory parameters other than iron were measured in order to further elucidate the overall severity of the disease in each group.

Statistics

Clinical characteristics and prevalence of abnormal blood parameters are presented as percentage distributions. The skewedness of the quantitative data was assessed by the Shapiro-Wilk method and most of the variables were not normally distributed. For simplicity, all data are thus expressed as medians with quartiles or with range. Laboratory values between groups were compared using the Kruskal-Wallis one-way analysis of variance or by Fisher's exact test. Changes within the groups on a gluten-free diet were compared using either the paired t-test or Wilcoxon signed-rank test as appropriate. Chi-squared test or Fisher's exact test were used to compare the proportions of abnormal laboratory parameters between the groups. P-values <0.05 were considered significant.

Results

Altogether 102 children participated the study. Of them TVA was detected in 16, P/SVA in 67, and potential celiac disease in 19 subjects. None presented with crypt hyperplasia and normal villi (Marsh 2). There were no differences in age or gender between the groups, except a trend with a borderline significance (P=0.062) for lower number of girls in the control group (Supplemental Table 1, supplemental digital content). Gastrointestinal presentation was more common in the TVA than in the P/SVA or potential celiac disease, while the latter two groups comprised more screen-detected children and those with extra-intestinal presentation. Associated conditions and family history of celiac disease were more common in potential celiac disease. By definition, all 102 patients with celiac disease suspicion and none of the controls had positive TG2-ab and/or EmA. Also, HLA DQ2/8 was present in all seropositive children and 59% of the controls (Supplemental Table 1, supplemental digital content).

Both EmA and TG2-ab differed significantly between the study groups, as their concentrations increased parallel with the severity of mucosal damage (Table 1). Similarly, the densities of CD3+ and γδ+ IELs increased gradually from potential celiac disease to TVA. There were no differences between the groups in any of the non-iron related laboratory parameters or in height and BMI-SDS, but in ALT there was an increase towards significantly higher values in TVA. TG2-targeted mucosal IgA deposits were positive in all seropositive children except two with P/SVA and one with potential celiac disease; in the three negative cases there was possible patchy distribution of the deposits (Table 1).

Anemia, low iron, high TfR1, low ferritin and decreased transferrin saturation were more common in children with advanced histological damage (Table 2). Nevertheless, anemia and abnormal TfR1, ferritin and transferrin saturation were also present in a number of subjects with potential celiac disease (Table 2). Furthermore, all these laboratory abnormalities except low iron were more common in potential celiac disease group than in

controls. Of the three potential celiac disease patients presenting with anemia one had Marsh 0 (CD3+ IELs 25 cell/mm) and the others Marsh 1 lesion, but all had increased γδ+ IELs (10.5 cells/mm, 25.1 cells/mm and 12.6 cells/mm) and positive EmA (1:200, 1:50 and 1:100) and IgA deposits. Their ferritin values were 28 µg/l, 6 µg/l and 6 µg/l. Besides these three with anemia, two potential celiac disease patients (Marsh 0, EmA 1:50 and Marsh 1, EmA 1:200) had high TfR1 (6.5 mg/l and 5.1 mg/l) and low/borderline low ferritin (7 µg/l and 10 µg/l) and transferrin iron saturation (14.2% and 17.3%). These children were between 3.5-7.2 years of age, except one girl age of 15 years of whom there was no information of excessive or prolonged menstruation, and no other pathological or dietary causes of anemia except potential celiac disease were detected. A low folate value was seen only in one patient with SVA and low vitamin B12 in none of the study children (Table 2).

The median values for hemoglobin, total iron, ferritin and transferrin iron saturation were significantly lower and TfR1 higher in TVA group compared with the other groups (Figure 1). In contrast, there were no differences in any of these parameters between the potential celiac disease and P/SVA groups, and both had significantly lower ferritin than the control group. There were no differences between the study groups in the median hepcidin levels (Figure 1). Furthermore, there were no differences in hepcidin concentrations between subgroups with or without anemia, or with or without low ferritin (data not shown). In the clinical presentation between the potential celiac disease children with or without anemia there were no significant differences as 78% of those presenting with anemia/iron deficiency suffered from gastrointestinal or extraintestinal symptoms while the corresponding figure was 90% in those with normal hemoglobin and iron parameters. There were also no correlations between hepcidin and hemoglobin or ferritin values (data not shown).

After an average of seven (range 3-13) months on a gluten-free diet the hemoglobin, TfR1, total iron, ferritin and albumin values improved significantly within both

the TVA and the P/SVA groups (Figure 2). When these follow-up values were compared with the baseline values in the potential celiac disease group, albumin in TVA group (p=0.037) was found to be significantly higher. A similar but non-significant trend was also seen in P/SVA group in total iron, ferritin and albumin (Figure 2). Anemia was still present in one (2.5%) of the P/SVA patients and none of the TVA patients in whom it was re-evaluated while on the dietary treatment.

Three children with potential celiac disease started a gluten-free diet while still having normal villi. One of them was anemic at baseline and while on diet had increased hemoglobin and total iron values. Another child experienced similar improvements even though he was not anemic at baseline. In the third case there were no marked changes in the laboratory values. All but two of the remaining 16 children have remained seropositive during a follow-up of up to 2.5 years. Furthermore, two potential celiac disease children with anemia started an iron substitution without gluten-free diet and also experienced improved hemoglobin and iron parameters.

Discussion

The main finding in the present prospective study was that, even if more common in children with severe mucosal atrophy, celiac disease-associated anemia and iron deficiency is a continuum and may appear even before morphological villous damage. Further, there were no significant differences in the iron parameters between the seropositive subjects with diagnostic PVA or SVA (Marsh 3a-b) and those with the currently non-diagnostic potential celiac disease (Marsh 0-1).

Anemia and iron deficiency are among the most common abnormal findings in untreated celiac disease (28). Accordingly, anemia was seen here in approximately one fifth of children with P/SVA and two thirds of those with TVA. Notwithstanding this correlation

with the severity of the histology, it is noteworthy that anemia was also present in 15% of the otherwise healthy potential celiac disease patients with no other obvious reason for the low hemoglobin. Moreover, while still evincing normal villi these seropositive children also had abnormalities in many other iron parameters more often than controls, of whom almost none presented with even subclinical anemia. This issue has not hitherto been investigated systematically, but a few earlier studies have in fact pointed in the same direction. In a prospective Italian study by Tosco and colleagues none of the 106 children with potential celiac disease had anemia at baseline, but four presented with low ferritin, and during a follow-up of up to three years on a gluten-containing diet four developed anemia (14). In our previous adult study three out of 11 seropositive subjects with mild enteropathy (Marsh 1-2) presented with malabsorption or anemia (29), this again being in line with the present findings.

Compatible with the proportion of abnormal iron parameters, also the absolute values were inferior in the TVA group compared with the other three groups. Notably, however, there were no significant differences between potential celiac disease and P/SVA, while in contrast both groups had lower median ferritin than the control children. In addition, on a gluten-free diet several of the iron parameters within the P/SVA and TVA groups improved to levels even better than those in the potential celiac disease patients on a normal gluten-containing diet, suggesting that the latter group already had sub-optimal values. In line with this we have previously observed improved hemoglobin values in EmA-positive children and adults who had mild enteropathy (Marsh 1-2) and were placed on an experimental gluten-free diet for one year (11, 12). These results demonstrate that, despite the differences in histology and serology, the distinction between potential celiac disease and in particular P/SVA is at least partly artificial and based more on simplified histological classification than on the actual biological nature of the disease.

Somewhat surprisingly we found neither differences in hepcidin values between the groups nor correlations between hepcidin and other iron parameters. Hepcidin is the key player in iron metabolism, as it regulates both intestinal absorption and release of iron from the body storages (18). Hitherto only a few studies have investigated hepcidin in the context of celiac disease. Bergamaschi and co-authors also found no correlation between hepcidin and anemia or other iron parameters in adults (30). However, they measured the less accurate pro-hepcidin instead of the actual functional hepcidin-25 used here (30, 31). In a sole pediatric study (only abstract available) the majority of children with villous atrophy celiac disease had low hepcidin, but in 20% it was increased and the authors speculated that they had anemia of chronic disease (32). This type of anemia is associated with high levels of pro-inflammatory cytokines, as seen for example in inflammatory bowel disease, in which a correlation between anemia and high hepcidin values has been observed (21, 33). Interestingly, although no differences between the groups were found in our study, there was a wide variation in hepcidin values, indicating that anemia in individual patients is caused by variable factors, as previously shown (34). Furthermore, the fact that two children here presented with anemia or low ferritin despite apparently completely normal duodenal mucosa (Marsh 0) suggests that iron metabolism might be disturbed even before marked intestinal inflammation. Indeed, Borrelli and colleagues (35) recently demonstrated activation of different immunoregulatory reactions even in such early potential celiac disease which could possibly affect iron metabolism (36). This could also explain the changes observed in sensitive iron parameters, especially the significantly lower ferritin values in potential celiac disease group compared with the controls (37). Further studies of the mechanisms of anemia in early developing celiac disease are evidently needed.

Currently the diagnosis of potential celiac disease remains unclear. For example, in the new ESPGHAN guidelines measurement of γδ+ IELs and IgA deposits in patients with

Marsh 1 is suggested, but there is no conclusion as to their diagnosis (5). As a result of active screening for celiac disease the number of these seropositive individuals is constantly rising and, in consequence, also their natural history has been increasingly investigated (12, 14, 38-40). In two recent Italian studies symptomatic children with potential celiac disease were placed on a gluten-free diet, while the rest continued on a normal diet (14, 38). All patients on diet became seronegative and most had a positive clinical response, while after nine years 67% of those on gluten still had normal villi (14, 38). None of the 16 children continuing on a gluten containing diet in the present study have yet developed mucosal atrophy, although control endoscopy was not done systemically to all. This is in contrast with our previous study where this was in most cases already seen after one year (12). However, the children in the earlier study had more advanced clinical and histological disease at baseline. It is also noteworthy that two of the potential celiac disease patients here seroconverted to negative. Such a fluctuation in low antibody levels has been shown before, especially in asymptomatic cases (39, 41). In these circumstances additional evidence of celiac disease, such as the appropriate genetics, positive EmA, IgA deposits and refined antibody tests are of particular importance (40-42). In sum, it is evident that the presentation and natural history of potential celiac disease is variable, but it would appear that a part of these subjects may present with clinical symptoms and signs and benefit from early dietary therapy. Considering the essential role of iron in human health, our further support at least a possibility of an early diagnosis and treatment of these children.

The major strengths of the present study are the prospective design and the variety of clinical, histological and serological parameters measured. Furthermore, particular attention was paid to the precise histomorphological analysis of the biopsies and thus correct histological grouping of the participants. The main limitation is the relatively small number of children with potential celiac disease which hampers statistical evaluation. In addition, since

currently it is not recommend to set an official diagnosis in children with potential celiac disease in Finland, it was not possible to systematically evaluate the effect of a gluten-free diet on their hemoglobin and iron parameters. Since the main aim of the study was not to investigate the natural history of potential celiac disease patients the overall duration of the study was rather short, and obviously careful long-term follow-up of these cases is necessary. It must also be mentioned that the follow-up values of iron parameters in P/SVA and TVA patients were not systemically collected from all patients who had normal values at the time of celiac disease diagnosis. Finally, the number of control children was rather small, even though we believe that they were a representative of the Finnish population in respect of iron parameter levels (43). Further, as these children belong to families where celiac disease is present they also have an increased risk for the condition and, on the other hand, possibly reduced daily gluten consumption.

In conclusion, we showed that the development of anemia and subclinical iron deficiency in celiac disease is a continuum and may already appear in seropositive children with morphologically normal mucosa. These findings support early diagnosis and either careful follow-up or alternatively active dietary treatment of children with potential celiac disease.

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Supplemental Table 1. Supplemental digital content. Baseline characteristics in 19 children with potential celiac disease, 83 with villous atrophy celiac disease and in 23 non-celiac controls.

Captions in the text:

“There were no significant differences in age or gender between the groups (Supplemental Table 1, supplemental digital content).”

“By definition, all 102 patients with celiac disease suspicion and none of the controls had positive TG2-ab and/or EmA. Also, HLA DQ2/8 was present in all seropositive children and 59% of the controls (Supplemental Table 1, supplemental digital content).”

Table 1. Baseline characteristics in 19 children with potential celiac disease, 83 with villous atrophy celiac disease and in 23 non-celiac controls.

	Potential celiac disease, n=19	Celiac disease n=83		Non-celiac controls, n=23
		P/SVA, n=67	TVA, n=16	
	%	%	%	%
Age, median (range), years	6.3 (3.5-16.9)	7.5 (1.6-15.2)	6.1 (3.7-15.6)	6.0 (2.1-11.4)
Girls	74	73	69	44
Clinical presentation				
Gastrointestinal[1]	72	75	94	ND
Extra-intestinal[2]	17	22	6	ND
Screen-detected[3]	11	3	0	ND
Celiac disease in family	72	47	33	ND
Associated disease[4]	10	1	6	ND
Other chronic disease[5]	26	37	50	ND
Positive EmA/TG2ab	100	100	100	0
HLA DQ2/8	100	100	100	59

[1]Diarrhea, stomach pains, constipation, bloating
[2]Rash, dizziness, retarded growth, anemia, iron deficiency, leg pains, tiredness, arthralgia
[3]Family risk of celiac disease, previous type 1 diabetes
[4]Type 1 diabetes, autoimmune thyroidal disease
[5] Rheumatoid arthritis, allergies, congenital glaucoma, asthma, atopic dermatitis, anorexia nervosa
ND, no data; HLA DQ2/8, celiac disease-associated human leucocyte antigen

Table 2. Baseline laboratory parameters, anthropometric data and small-bowel mucosal histology in 19 children with potential celiac disease and 83 with villous atrophy celiac disease. Values except transglutaminase 2 targeted IgA deposits are shown as medians with lower and upper quartiles.

	Potential celiac disease n=19	Celiac disease n=83		P value
		P/SVA n=67	TVA n=16	
EmA, titer	1:50 (1:5, 1:100)	1:500 (1:100, 1:2000)	1:1500 (1:500, 1:4000)	**<0.001**
TG2 antibodies, U/l	24.5 (10.3, 33.0)	70.0 (21.0, 120.0)	120.0 (120.0, 120.0)	**<0.001**
Albumin, g/l	38.0 (36.5, 39.5)	38.0 (35.0, 40.0)	37.0 (36.0, 39.0)	0.517
ALP, U/l	225 (182, 265)	206 (135, 222)	214 (128, 237)	0.429
ALT, U/l	14.0 (12.0, 17.5)	17.0 (14.0, 24.3)	21.5 (16.5, 34.5)	**0.032**
TSH, mU/l	3.2 (2.7, 3.9)	2.1 (1.6, 3.2)	3.7 (2.95, 7.2)	0.323
Height, SD	-0.4 (-1.6, 0.3)	0.0 (-1.1, 0.8)	-0.4 (-0.7, 0.9)	0.178
BMI, kg/m^2	16.0 (15.3, 21.2)	11.2 (14.6, 17.7)	17.3 (15.9, 18.5)	0.768
CD3+ IELs, cells/mm	46 (24, 61)	92 (75, 111)	105 (95, 151)	**<0.001**
γδ+ IELs, cells/mm	22.0 (9.4, 35.1)	39.5 (25.9, 54.6)	34.4 (21.9, 52.5)	**0.002**
Positive IgA deposits, %	94	97	100	0.576

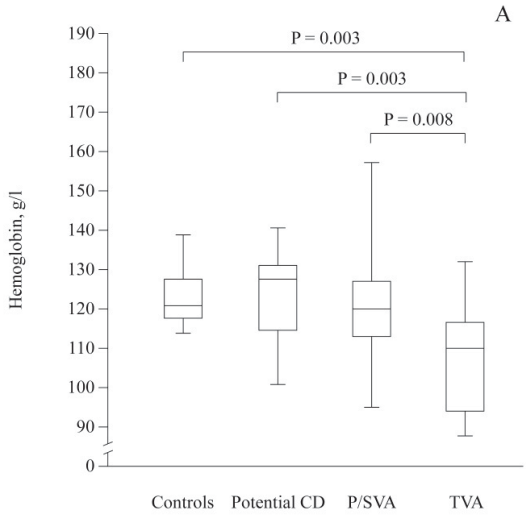
EmA, endomysial antibodies; TG2, transglutaminase 2; ALP alkaline phosphatase; ALT, alanine aminotransferase; TSH, thyroid-stimulating hormone; BMI, body mass index; IEL, intraepithelial lymphocyte

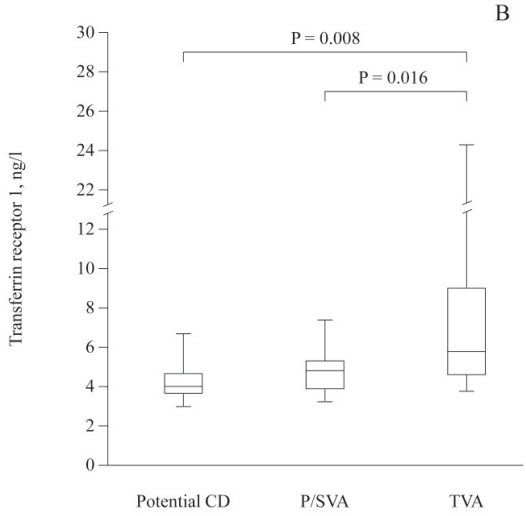
Table 3. Percentages of anemia and abnormal iron parameters in 23 non-celiac controls, 19 children with potential celiac disease and 83 with villous atrophy celiac disease.

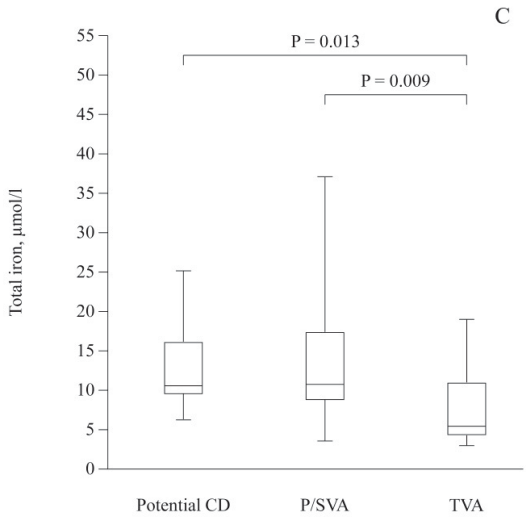
	Non-celiac controls n=23	Potential celiac disease n=19	Celiac disease n=57		P value
			P/SVA n=67	TVA n=16	
	%	%	%	%	
Anemia	0.0	15.3	22.4	62.5	**<0.001**
Low total iron	4.8	0.0	13.6	50.0	**<0.001**
High TfR1	4.8	15.8	20.4	46.7	**0.002**
Low ferritin	0.0	21.1	35.2	86.7	**<0.001**
Low transferrin saturation	9.5	11.1	40.5	71.4	**<0.001**
Low vitamin B12	0.0	0.0	0.0	0.0	1.000
Low erythrocyte folate	0.0	0.0	2.3	0.0	1.000

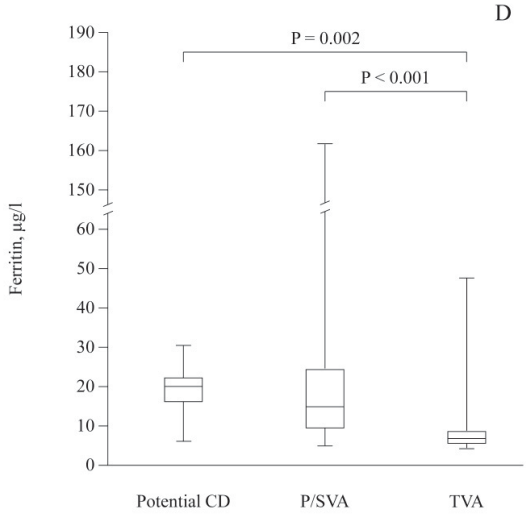
TfR1, transferrin receptor 1
Low denotes values below reference values. High denotes values above reference values.

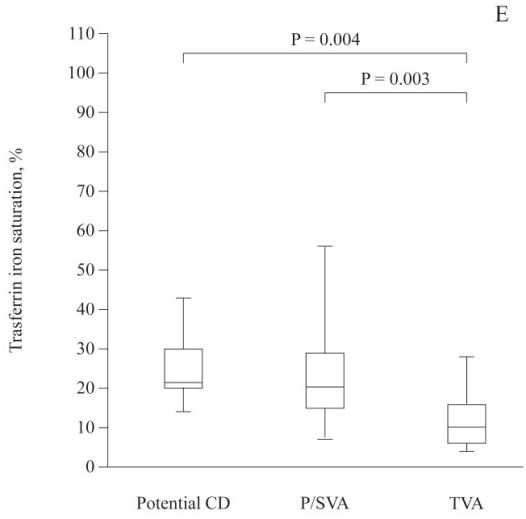
Figure1. Hemoglobin (A), transferrin receptor 1 (B), total iron (C), ferritin (D), transferrin iron saturation (E) and hepcidin (F) values in 23 healthy children, in 19 children with potential celiac disease (potential CD), in 67 children with partial or subtotal villus atrophy (P/SVA) and in 16 children with total villus atrophy (TVA). The values are presented as medians with quartiles (boxes) and range (whiskers). Only significant differences between the groups are shown (brackets).



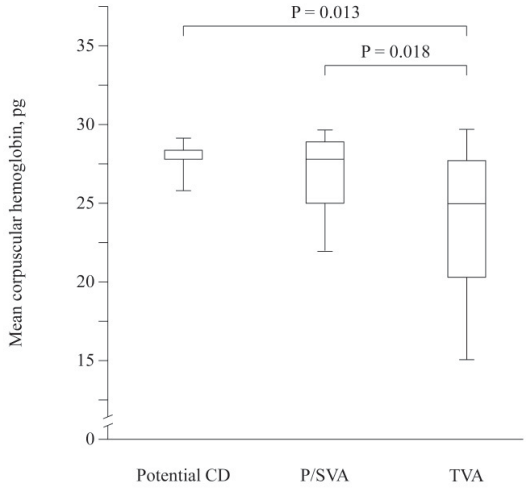

B
P = 0.008
P = 0.016
Transferrin receptor 1, ng/l
30
28
26
24
22
12
10
8
6
4
2
0
Potential CD
P/SVA
TVA


C
P = 0.013
P = 0.009
Total iron, µmol/l
55
50
45
40
35
30
25
20
15
10
5
0
Potential CD
P/SVA
TVA

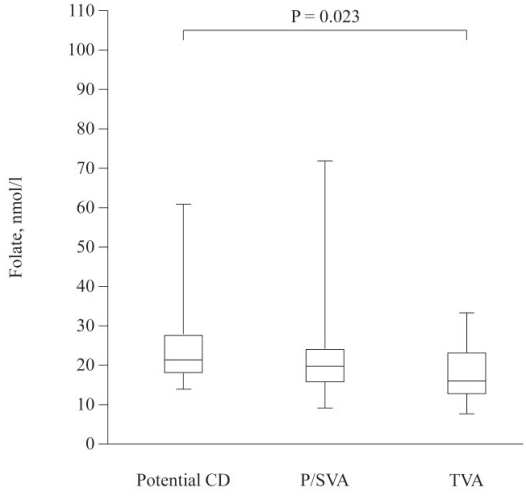

D
P = 0.002
P < 0.001
Ferritin, µg/l
190
180
170
160
150
60
50
40
30
20
10
0
Potential CD
P/SVA
TVA


E
P = 0.004
P = 0.003
Trasferrin iron saturation, %
110
100
90
80
70
60
50
40
30
20
10
0
Potential CD
P/SVA
TVA

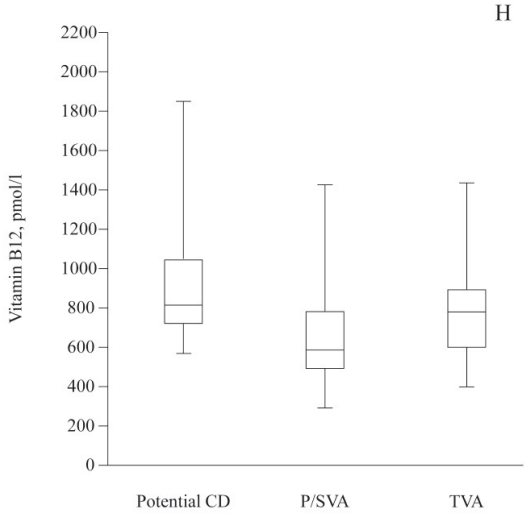
F


P = 0.013
P = 0.018
Mean corpuscular hemoglobin, pg
35
30
25
20
15
0
Potential CD
P/SVA
TVA

G


P = 0.023
Folate, nmol/l
110
100
90
80
70
60
50
40
30
20
10
0
Potential CD
P/SVA
TVA

H


Vitamin B12, pmol/l
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Potential CD
P/SVA
TVA

I

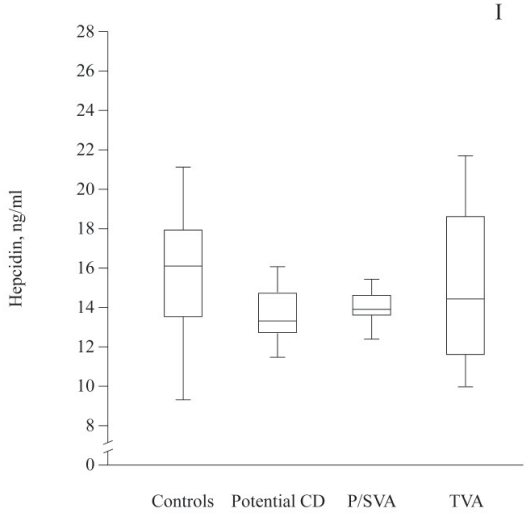
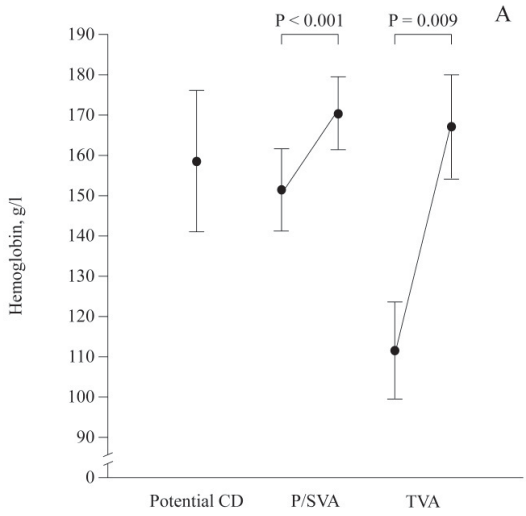
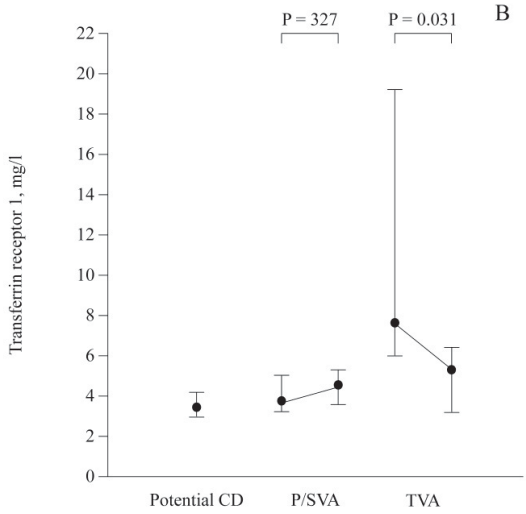
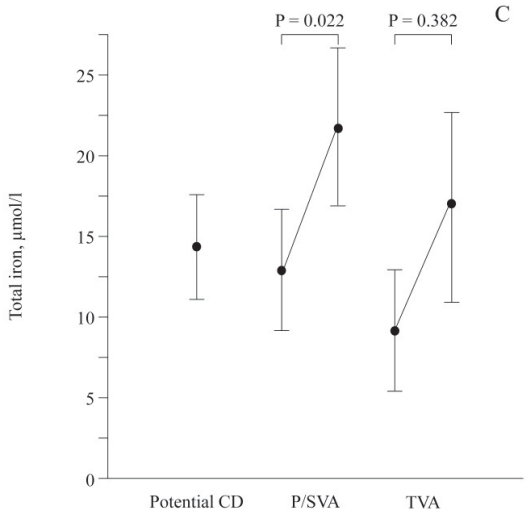
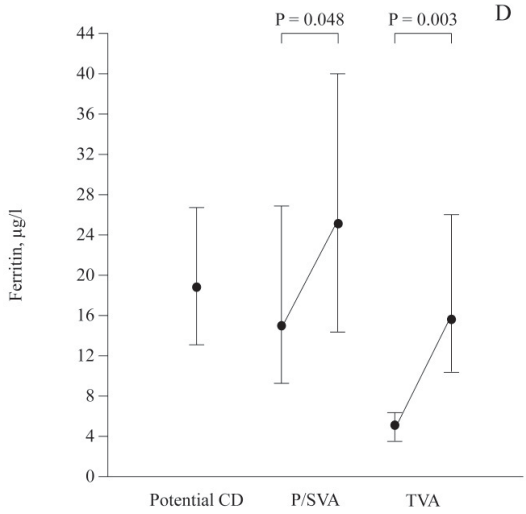

Hepcidin, ng/ml
28
26
24
22
20
18
16
14
12
10
8
0
Controls
Potential CD
P/SVA
TVA

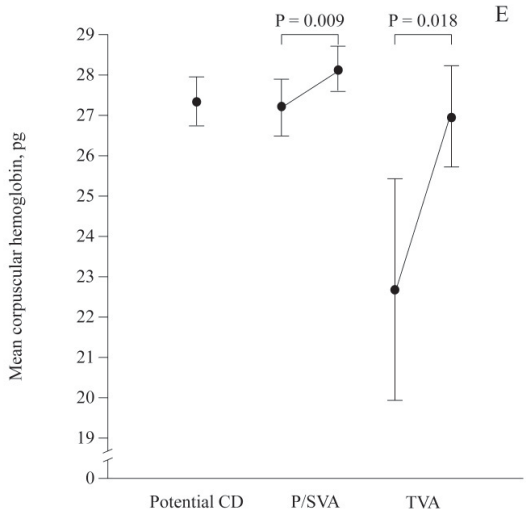
Figure 2. Hemoglobin (A), transferrin receptor 1 (B), total iron (C), ferritin (D) and albumin (E) in 19 children with potential celiac disease (potential CD), 67 children with partial or subtotal (P/SVA) and 16 children with total villus atrophy (TVA) at the time of endoscopy and after an average of seven months on a gluten-free diet (only P/SVA and TVA). The values are shown as medians and quartiles. Only significant changes within the groups are shown (brackets).

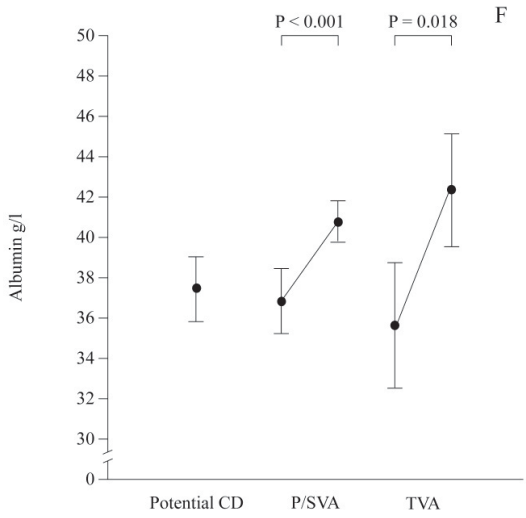



B
P = 327
P = 0.031
Transferrin receptor 1, mg/l
22
20
18
16
14
12
10
8
6
4
2
0
Potential CD
P/SVA
TVA


C
P = 0.022
P = 0.382
Total iron, µmol/l
25
20
15
10
5
0
Potential CD
P/SVA
TVA


D
P = 0.048
P = 0.003
Ferritin, µg/l
44
40
36
32
28
24
20
16
12
8
4
0
Potential CD
P/SVA
TVA


E
P = 0.009
P = 0.018
Mean corpuscular hemoglobin, pg
29
28
27
26
25
24
23
22
21
20
19
0
Potential CD
P/SVA
TVA


F
P < 0.001
P = 0.018
Albumin g/l
50
48
46
44
42
40
38
36
34
32
30
0
Potential CD
P/SVA
TVA

PUBLICATION IV

Iron transporter protein expressions in children with celiac disease

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Article

Iron Transporter Protein Expressions in Children with Celiac Disease

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Abstract: Anemia is a frequent finding in children with celiac disease but the detailed pathophysiological mechanisms in the intestine remain obscure. One possible explanation could be an abnormal expression of duodenal iron transport proteins. However, the results have so far been inconsistent. We investigated this issue by comparing immunohistochemical stainings of duodenal cytochrome B (DCYTB), divalent metal transporter 1 (DMT1), ferroportin, hephaestin and transferrin receptor 1 (TfR1) in duodenal biopsies between 27 children with celiac disease and duodenal atrophy, 10 celiac autoantibody-positive children with potential celiac disease and six autoantibody-negative control children. Twenty out of these 43 subjects had anemia. The expressions of the iron proteins were investigated with regard to saturation and the percentage of the stained area or stained membrane length of the enterocytes. The results showed the stained area of ferroportin to be increased and the saturation of hephaestin to be decreased in celiac disease patients compared with controls. There were no differences in the transporter protein expressions between anemic and non-anemic patients. The present results suggest an iron status-independent alteration of ferroportin and hephaestin proteins in children with histologically confirmed celiac disease.

Keywords: celiac disease; anemia; iron transporter

1. Introduction

Celiac disease is an immune-mediated disorder driven by ingested gluten [1]. A frequent and sometimes the only clinical finding in untreated patients is anemia, generally considered to be caused by damaged duodenal mucosa and the resulting malabsorption of iron [2,3]. Nevertheless, there is a poor correlation between the presence of anemia and the severity of histological damage [2,4,5]. Moreover, duodenal absorption of only about 10% of the dietary iron fulfills the daily needs [6], indicating that the reduced mucosal surface area is not the sole explanation for anemia. In fact, it may be present in so-called potential celiac disease, referring to subjects with endomysial (EmA) or transglutaminase 2 (TGA) celiac autoantibodies but with a normal small bowel morphology [5,7–9], suggesting that

the pathophysiologic mechanisms behind iron deficiency and anemia are more complex than previously thought.

In healthy conditions, iron is absorbed from the gut by a sophisticated and tightly regulated process [6,10]. In the apical membrane of enterocytes, the duodenal cytochrome B (DCYTB) reduces iron to a ferrous form. A divalent metal transporter (DMT1) transfers ferrous iron into the enterocyte where it is either utilized in mitochondria, stored as ferritin or transported to the circulation via basolateral ferroportin. Before being able to bind to the plasma iron carrier transferrin, iron must be reconverted into a ferric form by basolateral hephaestin. The enterocytes may also reuptake iron for their own metabolic functions through transferrin receptor 1 (TfR1). A key regulator of iron absorption and metabolism is hepcidin, which reduces the iron uptake in enterocytes and its release from body storages [11,12]. The details of this regulation, however, are not fully understood [13–16].

It has been suggested that the abnormal expression of the iron transporter proteins could provide an explanation for anemia in celiac disease. So far only a few studies have tested this hypothesis with inconsistent findings [17–20]. We therefore aimed to investigate possible altered transporter protein expression by staining the DMT, DCYTB, ferroportin, hephaestin and TfR1 in duodenal biopsies of children with histologically confirmed or potential celiac disease and autoantibody-negative controls.

2. Materials and Methods

2.1. Patients and the Study Design

The study was conducted at Tampere University Hospital, Tampere, Finland and the National Institute for Mother and Child Health, Bucharest, Romania. Twenty-seven children (age < 17 years) with EmA and/or TGA and a duodenal lesion comprised the celiac disease group. Ten children with positive EmA and TGA but a non-diagnostic histology comprised the potential celiac disease group. Six children who were endoscopied due to unexplained gastrointestinal symptoms but who had normal duodenal villi and negative EmA/TGA were used as controls. All 43 children were further divided into those with or without anemia.

The study was conducted according to the Helsinki Declaration. The study protocol and patient recruitment were approved by the Ethics Committee of the Pirkanmaa Hospital District, Finland and the Ethics Committees of the University of Medicine and Pharmacy "Carol Davila" and the National Institute for Mother and Child Health "Alessandrescu-Rusescu", Romania. Written informed consent was obtained from all study participants and their guardians.

2.2. Celiac Disease Serology and Small Bowel Mucosal Morphology

EmA titers were measured by an indirect immunofluorescence method using a human umbilical cord as a substrate [21]. A dilution of 1:5 was considered positive and positive sera were further diluted 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:4000. The EliA Celikey test (Phadia, Uppsala, Sweden) was used to determine TGA. The cut-off for seropositivity was set at >7.0 U/L according to the manufacturer's instructions.

A minimum of four representative forceps biopsies were taken from the duodenum. The paraffin-embedded specimens were cut, stained with hematoxylin and eosin and evaluated for celiac disease diagnosis by an experienced pathologist. Only correctly oriented histological sections were accepted for the histological analyses [22]. Subjects with crypt hyperplasia and a villous atrophy in the duodenal mucosa (Corazza–Villanacci B1-B2) were diagnosed with celiac disease whereas children with a non-diagnostic histology (Corazza–Villanacci A) formed the potential celiac disease and control groups [23,24].

2.3. Laboratory Parameters and Hepcidin

The following associated laboratory parameters were measured by standard methods: hemoglobin (reference value (Rf) from 100–141 to 130–160 g/L depending on age and sex [25]), plasma soluble transferrin receptor (sTfR, Rf from 1.6–5.2 mg/L to 2.0–6.8 mg/L),

mean corpuscular volume (MCV; Rf from 72–88 to 87–146 fl [25]), serum total iron (Fe, Rf 6–25 mmol/L), plasma ferritin (Rf > 10 mg/L), transferrin iron saturation (Rf 15–50%), serum folate (Rf 10.4–42.4 nmol/L) and serum vitamin B12 (Rf 140–490 pmol/L). In addition, serum bioactive hepcidin (hepcidin-25) levels were measured using a commercial solid-phase enzyme-linked immunosorbent assay (EIA-5258, DRG Diagnostics, Marburg, Germany) according to the manufacturer's instructions [5].

2.4. Immunohistochemistry

For the immunohistochemistry, 5 μm-thick sections were cut from the formalin-fixed, paraffin-embedded duodenal specimens. After deparaffination and rehydration antigens were exposed by heat-induced epitope retrieval. Thereafter, a non-specific staining was blocked followed by overnight incubation with primary antibodies (Supplementary Table S1). After washing the primary antibodies, the specimens were incubated overnight with a secondary antibody prior to the blocking of the endogenic peroxidase and a visualization of the staining with either ImmPRESS or VECTASTAIN Elite ABC reagent (Vector Laboratories Inc, Peterborough, UK). Finally, sections were counterstained with hematoxylin.

2.5. Digital Analysis of the Stained Sections

All slides were scanned as whole-slide images using a SlideStrider scanner at a resolution of 0.16 μm per pixel (Jilab Inc., Tampere, Finland). The images were stored as JPX files and viewed with a JVSview program from where they were exported to a Fiji Image J program for further analysis [26]. Of the DCYTB sections, both the entire visible epithelial apical membrane and the DCYTB stained membrane were drawn and measured. The stained membrane length was divided by the whole membrane length to assess the percentage of the apical membrane covered with the protein. Thereafter, from DMT1, ferroportin, hephaestin and TfR1 stained sections of the epithelium were selected, other parts cut out and the images consisting of only the epithelium were stored as TIF files (Supplementary Figures S1 and S2). Subsequently, the files were transferred to a Matlab program (The MathWorks Inc. Natick, Massachusetts) where they were transformed from RGB to HSV images to access the color saturation independently of the lightness. To measure only the primary antibody staining, a red color was chosen from the hue channel within values 0–0.1 and 0.9–1. The saturation channel was then thresholded according to all sections in each stained protein series using Otsu's method [27]. Finally, the value of the mean saturation of each section divided by the maximum saturation of the protein series and percentage of the stained area were measured for each section.

2.6. Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics version 26.0 (IBM Corp. Armonk, NY). The clinical characteristics and prevalence of anemia are presented as percentage distributions. The skewness of the quantitative data was assessed by the Shapiro–Wilk method and most of the variables were not normally distributed. For simplicity, all data are thus expressed as medians with quartiles except for age, which is given with a median and a range. Staining results as mean/maximum saturation and the stained area were compared between groups using a non-parametric Mann–Whitney U test. Correlations between hepcidin, plasma transferrin receptor 1, serum ferritin and the DCYTB stained apical border percent and in other proteins' mean/maximum saturations and stained areas were calculated using Spearman's rank (rS) correlation. p values < 0.05 were considered significant.

3. Results

There was no significant difference between children with celiac disease and potential celiac disease in age, gender or median hepcidin values or, despite a non-significant trend, in the frequency of anemia or low MCV (Table 1). The former group nevertheless had a higher frequency of increased sTfR values and lower ferritin (Table 1) as well as a higher

median EmA (1:1000 vs 1:50, $p < 0.001$) and TGA (120 U/l vs. 17 U/l, $p = 0.001$). The controls (two boys, two girls, 50% anemia) were slightly older (median 10.6 (range 3.3, 15.3) years) than the celiac and potential celiac patients.

Table 1. Clinical characteristics and laboratory values of 37 children with celiac disease (CD) and potential CD.

Variable	CD, *n* = 27		Potential CD, *n*=10		*p* Value
	n	%	*n*	%	
Girls	18	67	8	80	0.431
Anemia	14	52	3	30	0.236
High sTfR	12 [1]	46	1	10	0.043
Low MCV	10 [1]	35	1	10	0.140
	Median	Q_1, Q_3	Median	Q_1, Q_3	
Age, yrs (range)	6.8	2.7, 14.4	6.1	4.1, 16.9	0.555
Ferritin, mg/L	7.0 [1]	4.8, 15.5	20.5	11.3, 29.8	0.017
Hepcidin, ng/mL	13.7 [2]	12.6, 15.2	15.4	13.2, 18.2	0.286

MCV, mean corpuscular volume; Q1 and Q3, lower and upper quartiles; sTfR, soluble transferrin receptor. Data was available from all cases except 126 and 217.

The stained area of ferroportin was increased in the celiac disease patients compared with the controls and a similar although non-significant trend was observed in the saturation of the staining (Table 2). In hephaestin the saturation was significantly decreased in celiac disease compared with the controls with a similar trend in the stained area. No significant differences between the study groups were observed in either saturation or the stained area of the other iron transporters (Table 2), nor were there any differences in either the saturation or the stained area of any of the iron transporters between children with or without anemia (Table 3).

Table 2. Iron transporter protein saturations and the stained areas of enterocytes in the duodenal biopsies of the study subjects.

Iron Transporter Protein	CD *N* = 27		Potential CD *N* = 10		Controls *N* = 6		CD vs. Potential CD	CD vs. Controls	Potential CD vs. Controls
	Median	Q_1, Q_3	Median	Q_1, Q_3	Median	Q_1, Q_3	*p* Value	*p* Value	*p* Value
DCYTB									
Stained apical border, %	54	36, 76	50	24, 79	50	33, 73	0.679	0.751	0.662
DMT1									
Mean/max saturation, %	42	36, 51	43	35, 52	37	33, 50	0.999	0.342	0.828
Stained area, %	59	56, 62	60	49, 67	57	48, 65	0.827	0.653	0.745
Ferroportin									
Mean/max saturation, %	64	62, 66	64	59, 69	61	59, 63	0.827	0.072	0.329
Stained area, %	66	54, 75	68	40, 78	45	22, 57	0.999	0.024	0.129
Hephaestin									
Mean/max saturation, %	27	25, 29	28	26, 31	31	27, 37	0.234	0.028	0.195
Stained area, %	1	0, 22	4	1, 21	16	8, 38	0.266	0.080	0.195
TfR1									
Mean/max saturation, %	52	48, 54	50	49, 55	53	51, 62	0.821	0.325	0.233
Stained area, %	59	49, 69	42	33, 68	64	47, 73	0.257	0.437	0.233

CD, celiac disease; DCYTB, duodenal cytochrome B; DMT1, divalent metal transporter 1; TfR1, transferrin receptor 1. Data available in each analysis were from at least 90% of the patients.

There was a positive correlation between ferritin values and TfR1 saturations (r_S 0.594, $p = 0.015$) and the stained area (r_S 0.761, $p = 0.001$) in children with celiac disease. A moderate negative correlation was also found between sTfR values and hephaestin saturation (r_S –0.349, $p = 0.046$) when evaluated in all study subjects whereas this was not observed when evaluated separately in celiac disease patients. No other correlations between the hepcidin, ferritin or sTfR values and the stainings of the iron transporter were detected (data not shown).

Table 3. Iron transporter protein saturations and the stained areas of enterocytes in the duodenal biopsies of children with and without anemia.

Iron Transporter Protein	All Study Children, $n = 43$					Children With CD, $n = 27$				
	Anemia, $n = 20$		No Anemia, $n = 23$		p Value	Anemia, $n = 14$		No Anemia, $n = 13$		p Value
	Median	Q_1, Q_3	Median	Q_1, Q_3		Median	Q_1, Q_3	Median	Q_1, Q_3	
DCYTB										
Stained apical border, %	54	13, 78	56	37, 73	0.999	53	10, 79	63	42, 70	0.689
DMT1										
Mean/max saturation, %	43	37, 51	39	36, 54	0.582	43	39, 51	39	37, 53	0.446
Stained area, %	59	56, 62	59	54, 66	0.388	59	56, 61	59	57, 63	0.744
Ferroportin										
Mean/max saturation, %	64	59, 65	64	60, 68	0.372	64	62, 65	65	62, 69	0.128
Stained area, %	65	46, 74	65	44, 77	0.875	65	55, 74	66	51, 77	0.624
Hephaestin										
Mean/max saturation, %	27 [1]	26, 29	28	25, 32	0.594	27 [2]	25, 29	27	25, 31	0.663
Stained area, %	5 [1]	1, 22	3	0, 23	0.795	3 [2]	0, 19	1	0, 25	0.744
TfR1										
Mean/max saturation, %	50 [1]	49, 54	52	49, 55	0.452	50 [3]	49, 54	53	48, 55	0.750
Stained area, %	55 [1]	42, 62	61	43, 70	0.292	55 [3]	55, 64	61	50, 70	0.469

CD, celiac disease; DCYTB, duodenal cytochrome B; DMT1, divalent metal transporter 1; TfR1, transferrin receptor 1. Data available in each analysis were from at least 90% of the patients except [1] 17, [2] 12 and [3] 11 patients.

4. Discussion

The main finding of the present study was an increased expression of ferroportin and a decreased expression of hephaestin in children with histologically confirmed celiac disease compared with the non-celiac controls. There were no other significant differences between the study groups in the expression of iron transporter proteins. In addition, no differences in any of these proteins were detected when anemic and non-anemic children were evaluated separately.

The expression of the iron transporter proteins and/or their coding mRNAs in celiac disease have previously been reported in three studies comprising adult patients and in one pediatric study [17–20]. In line with our results, Sharma et al. showed an iron status-independent increase in protein levels of ferroportin but also of DMT1 in untreated adult celiac disease [17]. Additionally, they found increased DMT1 and ferroportin mRNAs in iron deficient celiac disease patients and also in anemic non-celiac controls. Tolone et al. later reported that DMT1 mRNA was increased in celiac disease children with mild but not with severe atrophy compared with controls with normal duodenal mucosa [20]. However, they included both potential celiac disease patients and suspected gastroesophageal reflux disease patients in the control group. Additionally, Matysiak-Budnik reported an upregulation of TfR1 protein levels in adults with untreated celiac disease [19]. Barisani et al. reported increased mRNAs and protein levels of DMT1, ferroportin, hephaestin and TfR1 in adult celiac disease patients but, in contrast to the protein levels in ours and Sharma's studies, these findings were iron status-dependent [18]. However, unlike others, Barisani et al. included both untreated patients and patients on a gluten-free diet in the celiac disease group. No earlier studies have reported the decreased hephaestin expression observed here.

These partially inconsistent results between the studies may be attributable to the differences in the number and clinical characteristics of the participants and/or by the variable use of primary antibodies and staining protocols. On the other hand, there may in fact be significant differences between children and adults in intestinal iron transporter protein expression [28]. As our results lacked major outliers and were also consistent within and between the study groups, we believe the present findings to reflect the true state of iron transporter protein expression in the duodenal mucosa of children with untreated celiac disease.

Our findings would suggest that changes in ferroportin and hephaestin expression do not explain the intestinal pathophysiology of anemia in celiac disease but may rather reflect the immaturity of the epithelium [29] of the atrophic duodenal mucosa. Interestingly, Tolone et al. found a distinct polymorphism in the DMT1 gene to be significantly more frequent in anemic than in non-anemic children with celiac disease; in fact, the polymorphism conferred a four-fold risk for the development of anemia [20]. Furthermore, a polymorphism in the transmembrane serine protease 6 gene can be overrepresented in celiac disease patients and its presence predicts an inadequate response to iron supplementation [30,31] whereas polymorphisms in the human hemochromatosis protein gene may provide protection against anemia in celiac disease [31–33]. Thus, genetic variants affecting iron metabolism may at least partially determine a predisposition to anemia in celiac disease.

As an additional novel finding of the present study, we observed a moderately positive correlation between the TfR1 saturation and stained area and the serum ferritin levels in children with celiac disease. Additionally, a negative correlation between the saturation of hephaestin and sTfR levels was shown among all of the children although this was not seen in celiac disease patients when evaluated separately. As sTfR usually increases and ferritin decreases in subjects with iron deficiency, an opposite correlation pointing towards a compensatory increase of intestinal iron absorption would have been expected [34]. However, both the origin and function of circulating ferritin and sTfR are currently unknown [10] and thus their connection with the duodenal iron transporters needs to be further studied.

5. Conclusions

To conclude, the iron status-independent changes observed here in ferroportin and hephaestin in children with histologically confirmed celiac disease likely reflect the immature nature of the epithelium in the atrophic disease state and do not explain the intestinal pathophysiology of anemia in children with celiac disease. Further investigations with a larger number of study subjects and in both children and adults are needed to understand the complex mechanisms of abnormal iron metabolism leading to anemia in celiac disease.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6643/13/3/776/s1. Supplementary Table S1. Specific characteristics of the staining procedures, Supplementary Figure S1. The process for measuring the saturation and stained area of iron transporter proteins in enterocytes as exemplified by ferroportin staining in a patient with a subtotal villous atrophy and anemia. Supplementary Figure S2. The process for measuring the saturation and stained area of iron transporter proteins in enterocytes as exemplified by ferroportin staining in a control child with anemia.

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