Tampere University

LIISA VIITASALO

Microbial Seromarkers in Coeliac Disease

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine and Health Technology
of Tampere University,
for public discussion in the F114
of the Arvo Building, Arvo Ylpön katu 34, Tampere,
on 22 September 2023, at 12 o'clock.

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

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Cover design: Roihu Inc.

ISBN 978-952-03-2985-3 (print)
ISBN 978-952-03-2986-0 (pdf)
ISSN 2489-9860 (print)
ISSN 2490-0028 (pdf)
http://urn.fi/URN:ISBN:978-952-03-2986-0

Carbon dioxide emissions from printing Tampere University dissertations have been compensated.

PunaMusta Oy – Yliopistopaino
Joensuu 2023

ACKNOWLEDGEMENTS

The research for this dissertation was carried out at the Tampere Center for Child, Adolescent and Maternal Health Research and the Celiac Disease Research Center, Tampere University and Tampere University Hospital, and at the Departments of Paediatrics and Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital. I profoundly thank all the patients and their family members for participating in the study.

I owe my sincere gratitude to my supervisors, Sari Iltanen, MD, PhD, and Professor Kalle Kurppa. I thank Sari for introducing me to the field of microbial markers and for offering me the great opportunity to carry out this dissertation project. I thank her for her enthusiastic and encouraging attitude and for her trust and support during the years. I thank Kalle for teaching me scientific writing and for his continuous support and guidance for this project. His ambitious way of doing research and the ability to understand large entities without forgetting the details is admirable indeed.

I wish to thank all my co-authors, Merja Ashorn, Sara Ashorn, Jonathan Braun, Pekka Collin, Heini Huhtala, Tuire Ilus, Katri Kaukinen, Katri Lindfors, Markku Mäki, Laura Niemi and Päivi Saavalainen for great collaboration. I especially want to thank Heini Huhtala for her consistently patient attitude and invaluable help with statistical analyses. Special thanks to Professor Jonathan Braun for enabling the microbial antibody measurements. I also want to thank Eini Eskola and Marja-Leena Koskinen for teaching me to carry out the laboratory analyses. I warmly thank Heidi Kontro for all her help, especially with practical matters. I sincerely thank all the members of the Celiac Disease Research Group for great company in meetings and conferences and inspiring conversations.

I wish to thank the members of my follow-up group, Professor Katri Kaukinen and Pilvi Laurikka, MD, PhD, for their support. My sincere thanks go to my pre-examiners Marko Kalliomäki and Mikael Kuitunen for their invaluable comments on my thesis.

I am grateful for the financial support I have received from the Finnish Coeliac Disease Society, the Finnish Medical Foundation, the Finnish Society of Gastroenterology, the Foundation for Pediatric Research, and the University of Helsinki special funding for university-level health research in Finland.

My heartfelt thanks go to all my colleagues in the HUSLAB Laboratory of Genetics and the Department of Clinical Genetics, many of whom I am privileged also to call my friends in addition to great co-workers. Your constant support and encouraging comments have been invaluable.

I owe my deepest gratitude to all my friends and family. I especially want to thank Elina, Iida, Lotta, Maria, Paula, Pekka, Ville, and Tea for being there in the most enthusiastic and not so easy moments during this project and for understanding my sometimes very busy schedule while working and doing research at the same time. I am beyond grateful for having you in my life. My loving gratitude goes to my parents Maija and Hannu for always believing in me and for their endless support for whatever I have decided to pursue in life. I thank my brilliant brother Olli for always having the energy to comment my research projects in a humorous but still very supportive way. I also want to thank my lovely sister-in-law Noora and my adorable nieces Nelli and Mona for all the joy they have brought into my life.

Helsinki, July 2023

Liisa Viitasalo

ABSTRACT

Coeliac disease is an enteropathy caused by an immune response to ingested gluten in genetically predisposed individuals. It is characterized by the presence of histological damage in the small-bowel mucosa and serum antibodies targeted against transglutaminase 2 (TG2-abs) and endomysium (EmA). The disease develops gradually from an early stage involving only mucosal inflammation and/or coeliac disease-associated autoantibodies to crypt hyperplasia and villous atrophy. Currently the only scientifically accepted treatment is a lifelong gluten-free diet. The normal mucosal architecture is usually restored upon treatment, but a subgroup of patients continues to have persistent clinical symptoms and histological damage; i.e., nonresponsive coeliac disease (NRCD).

Coeliac disease is considered to have a multifactorial background. Nearly all patients carry the human leukocyte antigen (HLA) haplotypes DQ2 and/or DQ8, and over 40 loci outside the HLA region have also been associated with the disease. However, only a fraction of predisposed individuals develops the disease, indicating the presence of environmental modifiers. Intestinal microbiota has an essential role in the development and function of the immune system, and alterations in the microbial composition compared with non-coeliac controls have been associated with coeliac disease. Furthermore, increased serological responses towards commensal microbiota, including yeast *Saccharomyces cerevisiae* and *Pseudomonas fluorescens* and *Bacteroides caccae* bacteria have been reported in patients with active coeliac disease, as well as a decrease in the antibody levels during gluten-free diet. However, the time of the appearance of these microbial antibodies and whether they are associated with the clinical picture of coeliac disease remains unclear. There are also very limited data on the prevalence of microbial antibodies in the at-risk relatives of coeliac disease patients.

The aim of this dissertation was to assess seroreactivity to IgA and IgG class anti-*Saccharomyces cerevisiae* antibodies (ASCA) and IgA class antibodies against the I2 protein of *Pseudomonas fluorescens* (anti-I2 antibodies) and the TonB-linked outer membrane protein of *Bacteroides caccae* (anti-OmpW) in patients with different types

and stages of coeliac disease, the first-degree relatives of coeliac disease patients and non-coeliac controls. Further, association of microbial antibody positivity with HLA and distribution of seropositivity among families were evaluated. The dissertation consists of three individual studies.

In Study **I**, the microbial antibodies were measured in 44 coeliac disease patients with early-stage disease and normal mucosal morphology, at the time when they developed diagnostic atrophy (n=16), and after one year on a gluten-free diet (n=33). It was found that five out of six patients seronegative to coeliac autoantibodies at an early stage already showed seropositivity to at least one of the microbial antibodies, and that the frequency of ASCA positivity decreased significantly on a gluten-free diet. Moreover, ASCA and anti-OmpW antibody levels were higher at the early stage than on a gluten-free diet, whereas the anti-I2 antibody levels were elevated at the time of coeliac disease diagnosis.

In Study **II**, the microbial antibodies of 20 NRCD patients on a gluten-free diet for a median of 3.5 years were compared with those of 58 newly diagnosed coeliac patients before the initiation of dietary treatment, 55 of whom also gave serum samples after one year showing a beneficial response to gluten-free diet, and with those of 80 non-coeliac blood donors. The results showed that seropositivity to ASCA was most frequent in the NRCD group, and that the ASCA IgA and IgG levels were significantly higher in NRCD patients than in the treated diet-responsive patients and controls. Neither seropositivity rate to anti-I2 or anti-OmpW antibodies nor antibody levels distinguished NRCD from diet-responsive coeliac disease, but the anti-I2 levels were higher in NRCD patients than in the controls.

In Study **III**, ASCA, anti-I2 and anti-OmpW antibodies were measured in 463 first-degree relatives of coeliac disease patients, 49 of whom were seropositive to TG2-ab and/or EmA. The results were compared with the 58 diet-responsive coeliac disease patients and the 80 controls also included in Study **II**. The microbial antibody levels were observed to be higher in the TG2-ab/EmA positive than the autoantibody negative relatives. Additionally, seropositivity was more common among the autoantibody negative relatives than among the controls but less common than among the coeliac disease patients. ASCA and anti-I2 antibody levels were also higher in all relatives than in the controls, whereas with anti-OmpW this was seen only in autoantibody positive relatives. Seropositivity to microbial antibodies was not significantly associated with HLA DQ haplotype.

This dissertation demonstrates that adaptive immune response to microbial antibodies may already be present at the early stage of coeliac disease before the

development of small-bowel mucosal atrophy, and, in some patients, even before the emergence of disease-associated autoantibodies. The autoantibody negative first-degree relatives also had increased seroreactivity to microbial antibodies. Therefore, measuring these markers might help to identify those at the highest risk among genetically predisposed individuals, in some cases prior to the emergence of coeliac disease-associated autoantibodies. Combining different microbial antibodies increases the detection rate of at-risk individuals. Elevated ASCA levels are associated with NRCD, while the antibody levels decrease along with the mucosal healing. ASCA could thus serve as an additional noninvasive marker in the follow-up of coeliac disease. Whether the antibody response and the targeted microbes have a causal role in the coeliac disease pathogenesis remains a subject for further research.

TIIVISTELMÄ

Keliakia on krooninen immuunivälitteinen tauti, jossa ravinnon gluteeni aiheuttaa ohutsuolen limakalvovaurion geneettisesti alttiilla henkilöillä. Taudinkuvaan liittyy usein myös vasta-ainemuodostus gluteenia sekä elimistön omia antigeenejä, kuten tyypin 2 transglutaminaasia ja endomysiumia, kohtaan. Keliakia on asteittain kehittyvä sairaus, jonka varhaiseen vaiheeseen liittyy vain suolen limakalvon tulehdus ja / tai vasta-ainemuodostus, joita seuraa kryptahyperplasian sekä villusatrofian kehittyminen. Nykyään keliakian ainoa tiedeyhteisön hyväksymä hoitomuoto on elinikäinen gluteeniton ruokavalio. Vaikka gluteenin poistaminen ruokavaliosta yleensä johtaa ohutsuolen limakalvon parantumiseen, osalla potilaista vaurio ei korjaudu, ja keliakiaan liittyvät oireet jatkuvat, kuten hoitoon reagoimattomassa keliakiassa.

Keliakialla ajatellaan olevan monitekijäinen tausta, jossa sekä geneettiset että ympäristötekijät vaikuttavat taudin puhkeamiseen. Lähes kaikilla keliakiapotilailla on HLA-DQ2- ja/tai HLA-DQ8-haplotyypit, ja lisäksi tunnetaan yli 40 HLA-alueen ulkopuolista kohonneeseen sairastumisriskiin liitettyä lokusta. Kuitenkin vain pieni osa geneettisessä riskissä olevista sairastuu keliakiaan, mikä viittaa ympäristötekijöiden merkitykseen. Suolistomikrobeilla tiedetään olevan keskeinen rooli suolen immuunijärjestelmän kehityksessä ja toiminnassa. Keliakiapotilailla on kuvattu muutoksia suolistomikrobistossa verrattuna ei keliakiaa sairastaviin kontrolleihin. Aktiivisessa keliakiassa on myös kuvattu lisääntynyttä vasta-ainemuodostusta suolistossa esiintyviä mikrobeja, kuten *Saccharomyces cerevisiae* -hiivaa sekä *Pseudomonas fluorescens* -ja *Bacteroides caccae* -bakteereja kohtaan. Vasta-ainetasot laskevat gluteenittoman dieetin ja suolen limakalvon parantumisen myötä. On hyvin vähän tutkimustietoa siitä, missä vaiheessa tautiprosessia kyseisiä mikrobi-vasta-aineita alkaa muodostua, ja onko vasta-ainepositiivisuudella yhteyttä keliakian taudinkuvaan. Kyseisiä vasta-aineita ei juurikaan ole tutkittu keliakiapotilaiden ensimmäisen asteen sukulaisilla, joilla on kohonnut riski sairastua keliakiaan sairastuneen perheenjäsenen kanssa jaettujen geneettisten ja usein myös ympäristön riskitekijöiden kanssa.

Tämän väitöskirjan tavoitteena oli tutkia seerumin vasta-aineita *Saccharomyces cerevisiae* -hiivaa sekä *Pseudomonas fluorescens* -ja *Bacteroides caccae* -bakteereja kohtaan keliakiapotilailla taudin eri vaiheissa ja erilaisissa taudinkuvissa, keliakiapotilaiden ensimmäisen asteen sukulaisilla sekä verenluovuttajilla, jotka eivät sairasta keliakiaa. Lisäksi tutkittiin vasta-ainepositiivisuuden yhteyttä keliakialle altistaviin HLA-DQ2- ja HLA-DQ8-haplotyyppeihin sekä vasta-ainepositiivisuuden jakautumista perheiden sisällä ja perheiden välillä. Väitöskirja koostuu kolmesta eri osatyöstä.

Osatyössä **I** mikrobivasta-aineita tutkittiin 44 potilaalla, joilla oli alkava keliakia. Määritykset toistettiin 16:lla potilaalla, kun heillä oli tähystyksessä todettu ohutsuolen limakalvovaurio, ja 33:lla vuosi gluteenittoman dieetin aloittamisen jälkeen. Viisi kuudesta keliakiavasta-ainenegatiivisesta alkavaa keliakiaa sairastavasta potilaasta oli jo seropositiivinen vähintään yhdelle mikrobivasta-aineelle. ASCA-positiivisten osuus laski merkitsevästi verrattaessa alkavan keliakian ja gluteenittoman dieetin ajanhetkiä. Sekä ASCA– että anti-OmpW-vasta-ainetasot myös laskivat merkitsevästi, kun taas anti-I2-vasta-ainepitoisuudet olivat korkeammat ainoastaan 16 potilaalla limakalvovaurion kehittymisen aikaan verrattuna ajankohtaan vuosi gluteenittoman dieetin aloittamisen jälkeen.

Osatyössä **II** mikrobivasta-aineita mitattiin 20:lta ruokavaliohoitoon reagoimattomalta keliakiapotilaalta, jotka olivat olleet tarkalla gluteenittomalla dieetillä keskimäärin 3.5 vuotta. Tuloksia verrattiin 58 keliakiapotilaaseen diagnoosihetkellä ennen ruokavaliohoidon aloittamista sekä 55:een heistä vuosi tautia lieventäneen ruokavaliohoidon aloittamisen jälkeen sekä 80:een ei keliakiaa sairastavaan verenluovuttajaan. ASCA-positiivisuus oli kaikista yleisintä hoitoon reagoimattomilla keliakiapotilailla, ja sekä IgA- että IgG-luokan vasta-ainetasot olivat korkeammat kuin ruokavaliohoitoon suotuisasti reagoineilla potilailla ja kontrolleilla. Anti-I2- ja anti-OmpW-vasta-aineet eivät erottaneet hoitoon reagoimattomia keliakiapotilaita muista keliaakikoista, vaikka anti-I2-vasta-ainetasot olivat korkeammat kuin kontrolleilla.

Osatyössä **III** mikrobivasta-aineita mitattiin 463:lta keliakiapotilaiden ensimmäisen asteen sukulaiselta, joista 49 oli keliakiavasta-ainepositiivisia. Tuloksia verrattiin osatyön **II** 58 hoitoon reagoineen keliakiapotilaan sekä 80 kontrollin vasta-ainepitoisuuksiin. Mikrobivasta-ainetasot olivat korkeammat keliakiavasta-ainepositiivisilla kuin vasta-ainenegatiivisilla. Mikrobivasta-ainepositiivisuus oli yleisempää myös seronegatiivisilla sukulaisilla kuin kontrolleilla, mutta harvinaisempaa kuin keliakiapotilailla. ASCA- ja anti-I2-vasta-ainetasot olivat kaikilla sukulaisilla korkeammat kuin kontrolleilla, mutta anti-OmpW-tasot olivat

korkeammat ainoastaan keliakiavasta-ainepositiivisilla sukulaisilla. Seropositiivisuus mikrobivasta-aineille ei ollut yhteydessä tiettyyn HLA-haplotyyppiin.

Tämä väitöskirjatutkimus osoitti, että mikrobivasta-ainemuodostusta voi esiintyä jo alkavassa keliakiassa ennen suolen limakalvovaurion syntymistä, osalla jo ennen keliakiavasta-aineita. Myös keliakiavasta-ainenegatiivisilla sukulaisilla esiintyi lisääntynyttä mikrobivasta-ainemuodostusta. Mikrobivasta-aineet voisivat siten mahdollisesti auttaa tunnistamaan ne geneettisesti alttiit henkilöt, joilla on erityisen korkea riski sairastua keliakiaan, mahdollisesti jo ennen keliakiavasta-aineiden muodostumista. Eri mikrobivasta-aineiden yhdistäminen lisää riskissä olevien tunnistamista. ASCA-positiivisuus oli kaikista yleisintä hoitoon reagoimattomassa keliakiassa, kun taas vasta-ainetasot laskevat suolen limakalvon paranemisen myöstä. ASCA voisi siten olla mahdollinen ei-invasiivinen markkeri keliakian hoitovasteen seurannassa. Se, onko mikrobivasta-aineilla tai niiden kohdemikrobeilla rooli keliakian patogeenisissä vaatii lisätutkimuksia.

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ABBREVIATIONS

ACCA	Anti-chitobioside antibodies
AGA	Anti-gliadin antibodies
ALCA	Anti-laminaribioside antibodies
AMCA	Anti-mannobioside antibodies
Anti-I2 antibodies	Antibodies against the I2 protein of *Pseudomonas fluorescens*
Anti-OmpW antibodies	Antibodies against the *Bacteroides caccae* TonB-linked outer membrane protein
ANCA	Anti-neutrophil cytoplasmic antibodies
ARA	Anti-reticulin antibodies
ASCA	Anti-*Saccharomyces cerevisiae* antibodies
APC	Antigen presenting cell
DGP-abs	Antibodies against deamidated gliadin peptides
DH	Dermatitis herpetiformis
EATL	Enteropathy-associated T cell lymphoma
EmA	Endomysial antibodies
ESPGHAN	European Society for Paediatric Gastroenterology, Hepatology, and Nutrition
GALT	Gut-associated lymphoid tissue
GFD	Gluten-free diet
HLA	Human leukocyte antigen
HWP1	*Candida albicans*-hyphal wall protein 1
I2-GST	I2 glutathione-S-transferase fusion protein
IFN	Interferon
IFN-Ɣ	Interferon gamma
IL-10	Interleukin 10
IL-15	Interleukin 15
IL-18	Interleukin 18
IL-21	Interleukin 21

MHC	Major histocompatibility complex
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
NK	Natural killer
NRCD	Nonresponsive coeliac disease
pANCA	Perinuclear anti-neutrophil cytoplasmic antibodies
SCFA	Short chain fatty acid
SILT	Solitary isolated lymphoid tissue
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
RCD	Refractory coeliac disease
TG2	Transglutaminase 2
TG3	Transglutaminase 3
TG6	Transglutaminase 6
TG2-ab	Transglutaminase 2 antibody
TG3-ab	Transglutaminase 3 antibody
TCR	T cell receptor
TLR	Toll-like receptor
ULN	Upper limit of normal
USA	United States of America
Vh/CrD	Villous height/crypt depth ratio

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred in the text by the Roman numerals I-III.

I Viitasalo L, Niemi L, Ashorn M, Ashorn S, Braun J, Huhtala H, Collin P, Mäki M, Kaukinen K, Kurppa K and Iltanen S (2014). Early Microbial Markers of Celiac Disease. J. Clin. Gastroenterol. 48:620–4.

II Viitasalo L., Kurppa K., Ashorn M., Saavalainen P, Huhtala H., Ashorn S., Mäki M., Ilus T., Kaukinen K. and Iltanen S (2018). Microbial Biomarkers in Patients with Nonresponsive Celiac Disease. Dig. Dis. Sci. 63:3434–41.

III Viitasalo L, Iltanen S, Huhtala H, Saavalainen P, Kaukinen K, Lindfors K and Kurppa K (2020). First-degree Relatives of Celiac Disease Patients Have Increased Seroreactivity to Serum Microbial Markers. Nutrients 12:1073.

Author's contribution

I: Statistical analysis, interpretation of the results, writing of the manuscript

II: Laboratory analyses, processing of data, statistical analysis, interpretation of the results, writing of the manuscript

III: Laboratory analyses, processing of data, statistical analysis, interpretation of the results, writing of the manuscript

REVIEW OF THE LITERATURE

1 INTRODUCTION

Coeliac disease is a chronic immune-mediated enteropathy driven by dietary gluten in genetically predisposed individuals (Green & Cellier 2007). Gluten digestion triggers an inflammation in the small-bowel mucosa leading to lymphocyte infiltration and morphological damage of various stages. In addition to histological changes, antibodies targeted against gluten and self-antigens are usually detected in the serum and the mucosal wall. Antibodies and mucosal inflammation often appear before morphological changes (Kurppa et al. 2009, 2010; Mäki et al. 1990), a condition that can be defined as early-stage coeliac disease. Currently the only scientifically accepted treatment for coeliac disease is a lifelong gluten-free diet, initiation of which usually restores the normal mucosal architecture and alleviates possible symptoms. However, a marked proportion of patients suffer from so-called nonresponsive coeliac disease (NRCD), characterized by persistent clinical symptoms and histological damage despite the dietary treatment (Abdulkarim et al. 2002; Leffler et al. 2007; Stasi et al. 2016). When gluten exposure or possible complicating/co-existing conditions have been ruled out, NRCD is re-named refractory coeliac disease (RCD).

Almost all coeliac disease patients carry human leukocyte antigen (HLA) type DQ2 and/or DQ8 haplotypes (Karell et al. 2003). However, the occurrence of these haplotypes is much more common in the population than the prevalence of coeliac disease, suggesting that other factors also contribute to disease development. Accordingly, genome-wide association studies have found over 40 loci associated with coeliac disease, indicating a polygenetic background (Hunt et al. 2008; Trynka et al. 2011). Partly due to shared genetics, close relatives of coeliac patients are at increased risk of being affected (Paavola et al. 2021; Singh et al. 2018).

Besides genetics, environmental factors also modify disease predisposition. The most studied environmental exposures are infections, which are usually suspected of triggering the onset of the disease, although they may also have a protective effect depending on the circumstances (Mårild et al. 2015). Moreover, since the commensal intestinal microbiota is known to play a significant role in the maturation and function of the intestinal immune system (Sommer & Bäckhed 2013), its role has

been studied in several immune-mediated diseases, including coeliac disease (Chapter 3.5). In fact, alterations in the intestinal microbiota have been observed in coeliac patients compared with controls (Di Cagno et al. 2009; Nadal et al. 2007; Chapter 3.5) and dysbiotic microbiota has been associated with persistent symptoms despite a strict gluten-free diet (Wacklin et al. 2014).

The intestinal immune system constantly encounters foreign antigens and must differentiate between harmful and innocuous molecules. In coeliac disease, this self-tolerance to gluten is disrupted. Interestingly, increased reactivity to intestinal microbiota has also been observed in coeliac disease and other immune-mediated diseases. For instance, elevated levels of serum antibodies to baker's yeast *Saccharomyces cerevisiae, Pseudomonas fluorescens* and *Bacteroides caccae* bacteria have been observed in active coeliac disease, followed by a decrease of seroreactivity upon initiation of a gluten-free diet (Ashorn et al. 2008, 2009b). However, these antibodies have mainly been studied in patients with inflammatory bowel disease (IBD), and data on coeliac disease are scarce. There is actually an almost complete absence of research on the time of their appearance and association with the clinical picture of coeliac disease, as well as their presence in the relatives of coeliac disease patients (Gross et al. 2013; da Silva Kotze et al. 2010).

2 COELIAC DISEASE

2.1 Definition and general characteristics of coeliac disease

Coeliac disease is an immune-mediated systemic disease characterized by gluten-induced inflammation in the small-bowel mucosa (Green & Cellier 2007). The pathogenesis involves both adaptive and innate immune reactions and is characterized by the presence of serum antibodies targeted against ingested gluten and self-antigens. The disease has a multifactorial background, including gluten as a known environmental trigger/driver and a polygenetic predisposition. The fact that only a minority of genetically susceptible individuals who eat gluten actually develop the disease suggests a role of other risk-modifying factors. Due to shared genetics, and possibly because of similar environmental conditions, such as the frequency of infections and microbial exposure, relatives of coeliac patients are at increased risk of being affected (Kondrashova et al. 2008; Mårild et al. 2015; Paavola et al. 2021; Singh et al. 2015). Coeliac disease develops gradually from mucosal inflammation and antibody formation to crypt hyperplasia and eventually to subtotal or total villous atrophy. The symptoms and clinical signs are variable and some patients consider themselves completely asymptomatic. Even though initiation of a strict gluten-free diet usually leads to clinical and histological remission, a fraction of patients continues to have mucosal changes and symptoms.

2.2 Epidemiology

Coeliac disease is estimated to affect approximately 1% of the population (Mäki et al. 2003; Mustalahti et al. 2010; Rubio-Tapia et al. 2012). However, there is significant geographic and ethnic variation, reported prevalences ranging from 0.1% to 5.6% (Catassi et al. 1999; Fukunaga et al. 2018). These differences are at least partly due to alterations in genetic predisposition and gluten consumption (Catassi et al. 1999; Fukunaga et al. 2018; Myléus et al. 2009). However, some of the variations remain unexplained. For example, the prevalence of coeliac disease varies markedly between

European countries (Mustalahti et al. 2010; Myléus et al. 2009). Coeliac disease is also more common in Finland than in Russian Karelia despite comparable gluten consumption and genetic background (Kondrashova et al. 2008), further supporting the role of other environmental factors besides gluten in disease development. Likely due to shared genetics and often also similar environment, relatives of coeliac patients are at greater risk of developing the disease than the general population, the pooled prevalence among first-degree relatives being approximately 8% (Singh et al. 2015). Coeliac disease is also more prevalent in women than men, possibly at least partly due to hormonal influence on the immunoregulation (Jansson-Knodell et al. 2019).

In contrast to past decades, when coeliac disease was considered a rare childhood condition (Davidson & Fountain 1950), it is now known that it can develop at any age and that *de novo* cases can be also found in the elderly population (Vilppula et al. 2009). In Finland, the prevalence seems to increase with age from 1.0% in children to 2.7% in adults (Mäki et al. 2003; Mustalahti et al. 2010; Vilppula et al. 2009). Due to atypical, mild or even non-existent symptoms in many patients, coeliac disease remains markedly underdiagnosed, and the clinical prevalence is significantly lower than that observed in population-based screening studies (Singh et al. 2018).

Interestingly, in addition to a better detection rate, the true prevalence of coeliac disease may also have increased in recent decades. In Finland, the prevalence was reported to have doubled from 1.05 % to 1.99% in only two decades (Lohi et al. 2007), and similar observations have also been made in the United States (Catassi et al. 2010). These findings again imply that environmental factors play a significant role in the pathogenesis.

2.3 Risk-moderating factors

2.3.1 Genetic susceptibility

The association between coeliac disease and HLA was first recognized 50 years ago (Stokes et al. 1972). Later emerging HLA-typing methods revealed that the disease is primarily associated with genes encoding HLA class II molecules located on the short arm of chromosome 6. HLA II molecules are receptors on the surface of

antigen presenting cells (APC) that present peptides to CD4+ T cells. These molecules are further divided into HLA-DR, HLA-DP, and HLA-DQ isotypes, each of which consists of an α and a β chain (Dai et al. 2008). HLA-DQ2 and DQ8 molecules have the highest affinity to gluten metabolites, which play a crucial role in coeliac disease pathogenesis (Chapter 2.4.3).

The α and β chains of DQ receptors are encoded by HLA-DQA1 and HLA-DQB1 genes respectively. Patients with HLA DQ2 carry HLA-DQA1*05 and DQB1*02 alleles on the same chromosome (in cis) in a DRB1*03(DR3) haplotype or on the opposite chromosomes (in trans) when the DQ2 molecule is encoded by HLA-DQA1*05 located commonly on DRB1*11, DRB1*12 or DRB1*13 haplotypes and the DQB1*02 allele usually on a DRB1*07(DR7) haplotype (Sollid & Thorsby 1993). The DQB1*02 alleles differ between DR3 and DR7 haplotypes by one amino acid (Sollid et al. 1989) and are thus marked DQB1*0201 and DQB1*0202 respectively. DQ8 heterodimer is inherited in cis position with DQA1*03 and DQB1*0302 alleles inherited on a DRB1*04(DR4) haplotype (Spurkland et al. 1992). More than 90% of all coeliac patients have at least one DQA1 and DQB1 allele encoding the DQ2 molecule and almost all the others have the DQ8 encoding haplotype (Karell et al. 2003). Some patients carry only one allele encoding either the α or β chain of the DQ2 molecule and very few have none of the coeliac disease associated DQA1 and DQB1 alleles (Polvi et al. 1998).

A dose effect of HLA DQ molecules in coeliac disease susceptibility has been observed in several studies. Particularly, homozygosity to DQB1*02 confers the greatest risk of being affected (Pietzak et al. 2009) and possibly also predisposes to an earlier onset and more severe clinical and/or histological presentation (Karinen et al. 2006). However, not all studies have reported such an association with disease severity (Murray et al. 2007; Thomas et al. 2009).

HLA DQ2 and DQ8 encoding alleles are particularly common in European and North American populations, in which as many as 40% present with these heterodimers. Nevertheless, only a minority of susceptible individuals will eventually develop coeliac disease, and it has been estimated that HLA genes account for approximately 40% of genetic predisposition (Petronzelli et al. 1997). Genome-wide association studies have enabled the detection of more than 40 coeliac disease associated non-HLA loci involved in immunological pathways (Hunt et al. 2008), and dense genotyping of candidate loci have revealed numerous additional common and rare variants modifying the disease risk (Trynka et al. 2011). Notably, many of the risk loci identified overlap with other immune-mediated diseases such as type 1

diabetes, rheumatoid arthritis and Crohn's disease (Festen et al. 2011; Smyth et al. 2008; Zhernakova et al. 2011).

2.3.2 Environmental factors

While gluten is the best-known exogenous driver in the development of coeliac disease, as already mentioned, other environmental factors have also been suggested to modify the risk. The more detailed reasons for this assumption are that 1) only a fraction of individuals with predisposing haplotypes develops coeliac disease; 2) there are significant differences in the prevalence of coeliac disease and other immune-mediated conditions between adjacent geographical areas despite similar genetic background and gluten consumption (Kondrashova et al. 2008); 3) there appears to be a rapid rise in true prevalence, at least in some countries (Catassi et al. 2010; Lohi et al. 2007). In fact, disease concordance varies even between monozygous twins (Kuja-Halkola et al. 2016). Further, variability in the risk of developing coeliac disease according to season of birth indicates the role of environmental modifiers (Assa et al. 2018; Daniel et al. 2019).

The most studied of the plausible additional environmental exposures in coeliac disease are infections. For example, Mårild et al. (2015) reported that children who had more than ten infections during the first 18 months of life had a higher risk of coeliac disease than those with less than four infections. In addition, enterovirus infections in childhood have been associated with increased risk (Kahrs et al. 2019; Lindfors et al. 2020), the amount of dietary gluten possibly modifying the effect (Lindfors et al. 2020). Studies on rotavirus have yielded inconsistent results (Stene et al. 2006; Ziberna et al. 2016), although rotavirus vaccination may have a protective effect (Hemming-Harlo et al. 2019). Furthermore, respiratory infections during infancy (Kårhus et al. 2018; Tjernberg & Ludvigsson 2014) and campylobacter infections later in life (Riddle et al. 2013) have been associated with coeliac disease, but additional studies are needed. According to recent evidence, adenovirus infections were not overrepresented among individuals who later developed coeliac disease (Kahrs et al. 2019; Lindfors et al. 2020). Of note, children born in winter seem to have the lowest risk (Assa et al. 2018; Daniel et al. 2019), one possible explanation for this phenomenon being alterations in the burden of infections between (different) seasons. On the other hand, it has been proposed that inferior hygienic conditions may decrease autoimmunity and immune reactions towards

dietary antigens due to more frequent microbial exposure (Bach 2018). This may explain the wide variation in the prevalence of coeliac disease between adjacent regions and rapid changes in disease incidence alongside improving socioeconomic conditions (Chapter 2.2). Hence, *Helicobacter pylori* infections have been reported to protect against coeliac disease (Lebwohl et al. 2013), although this was not seen in later studies involving randomly selected controls (Dore et al. 2018; Jozefczuk et al. 2015).

The composition of the intestinal microbiota has been associated with coeliac disease in several studies (Chapter 3.5). Furthermore, proton pump inhibitors and antibiotics may, for example, affect coeliac disease risk at least partially through alterations in the microbiota, although recent studies have yielded inconsistent results (Kemppainen et al. 2017; Lebwohl et al. 2014; Mårild et al. 2013; Sander et al. 2019). Even though formerly considered significant (Ivarsson et al. 2002), breastfeeding has not been proven to have a protective effect in more recent prospective studies (Hummel et al. 2021; Vriezinga et al. 2014). Moreover, notwithstanding their beneficial effects on immunoregulation, use of probiotics during the first year of life does not seem to decrease the risk of coeliac disease (Oscarsson et al. 2021; Savilahti et al. 2018; Uusitalo et al. 2019).

Factors associated with the perinatal period have also been studied. Caesarean section has been suggested as a predisposing factor (Mårild et al. 2012), however, more recent studies have reported no association between mode of delivery and predisposition to coeliac disease (Sander et al. 2018; Koletzko et al. 2018). Yang et al. (2017) found that mothers' use of dietary supplements vitamin D, n-3 fatty acids and iron during pregnancy did not moderate offspring's risk of developing coeliac disease, whereas Størdal and colleagues (2014) reported iron supplementation during pregnancy to be associated with a higher incidence. Furthermore, Aronsson et al. (2021) reported 25(OH)D concentrations under 30 nmol/l and above 75 nmol/l in early infancy to be associated with increased risk of coeliac disease autoimmunity.

2.3.3 Family risk and other risk groups

Relatives of coeliac patients are at increased risk of also being affected. The strongest disease concordance is between monozygous twins with nearly identical genetics (Kuja-Halkola et al. 2016). Siblings and dizygotic twins may also have a stronger disease susceptibility than other first-degree relatives (Singh et al. 2015). This may be

at least partly due to the possibility of inheriting predisposing HLA DQ2/DQ8 alleles from both parents (Megiorni & Pizzuti 2012). If more than one sibling is affected, the other family members have a higher risk, probably due to a stronger genetic predisposition and shared environmental factors (Gudjónsdóttir et al. 2004). Second-degree relatives are also at higher risk than general population of developing coeliac disease (Paavola et al. 2021; Singh et al. 2015). In addition, female relatives are considered to be at greater risk than males (Lohi et al. 2007; Singh et al. 2015); however, this has not been observed in all studies (Fasano et al. 2003; Paavola et al. 2021).

Several autoimmune/immune-mediated diseases are associated with an increased risk of coeliac disease (Table 2), likely due, at least in part, to a shared genetic predisposition. Many common autoimmune diseases have a strong association with specific HLA alleles (Zhernakova et al. 2013). For instance, HLA II DQ2/DQ8 haplotypes, carried by almost all coeliac patients, are also risk factors for type 1 diabetes (Aly et al. 2006). In addition, genome-wide association studies have found a substantial number of non-HLA loci shared by immune-mediated diseases, further explaining the co-occurrence of these conditions (Zhernakova et al. 2013). Environmental factors also interact with the immune system and may influence the tendency to develop immune-mediated diseases. For example, besides coeliac disease, alterations in the intestinal microbiota have been observed in rheumatoid arthritis, type 1 diabetes and psoriasis (Fahlén et al. 2012; Knip & Simell 2012; Ogrendik 2009; Wacklin et al. 2013). It has been speculated whether the duration of gluten intake or maintenance of a strict gluten-free diet is associated with the risk of concomitant immune-mediated diseases in coeliac disease patients, but studies have yielded inconclusive results (Cosnes et al. 2008; Viljamaa et al. 2005).

Individuals with chromosomal abnormalities, particularly those with Down syndrome or Turner syndrome, are also at increased risk of developing coeliac disease (Table 2). An association with Williams syndrome has also been suggested, although there has been wide variation in the prevalence figures reported (Table 2).

Table 1. Prevalence (%) of coeliac disease among the relatives of the patients the respective studies.

Relationship	Cohort (n)	%	Reference
All first-degree relatives	2,943	12.5	Paavola et al. 2021
	10,252	7.5	Singh et al. 2015
	4,508	4.6	Fasano et al. 2003
	360	44.4	Nellikkal et al. 2019
Monozygous twins	15,111 twin pairs	49*	Kuja-Halkola et al. 2016
	23 twin pairs	83*	Nisticò et al. 2006
Siblings	871	16	Paavola et al. 2021
	2,780	8.9	Singh et al. 2015
Offspring	1,212	7.9	Singh et al. 2015
	864	13	Paavola et al. 2021
Parents	1,295	3.0	Singh et al. 2015
	505	12	Paavola et al. 2021
Second-degree relatives	578	10.9	Paavola et al. 2021
	642	2.3	Singh et al. 2015
	1,275	2.6	Fasano et al. 2003
More distant relatives	125	12.8	Paavola et al. 2021

*Prevalence of coeliac disease in co-twins of previously diagnosed index cases.

Table 2. Prevalence of coeliac disease in different risk groups.

	Cohort	%	Reference
Immune-mediated diseases			
Type 1 diabetes	4,322 children	6.8	Cerutti et al. 2004
	26,605 children and adults	6.0	Elfström et al. 2014
Autoimmune thyroid disease	952 adults	10.2	Krishnareddy et al. 2014
	749 adults	19.9	Castro et al. 2020
Sjögren's syndrome	32	14.7	Iltanen et al. 1999
	925 adults	10.5	Krishnareddy et al. 2014
IgA nephropathy	168 adults	3.6	Collin et al. 2002
	827 adults	8.2	Nurmi et al. 2018
Selective IgA-deficiency	72 children	5.6	Nisihara et al. 2005
	126 children	8.7	Lenhardt et al. 2004
Addison's disease	109 children and adults	2.7	Betterle et al. 2006
	925 adults	0.3	Krishnareddy et al. 2014
Psoriasis	92 adults	4.34	Ojetti et al. 2003
	37 adults	2.7	Akbulut et al. 2013
Vitiligo	64 children and adults	3.1	Shahmoradi et al. 2013
	174 children and adults	2.8	Henker et al. 2019
Autoimmune hepatitis	567 children and adults	3.5*	Haggård et al. 2021
Primary biliary cirrhosis	899 children and adults	2.7	Rubio-Tapia et al. 2007
PSC	61 adults	1.6	Rubio-Tapia et al. 2007
Chromosomal abnormalities			
Down syndrome	1110 children	4.3	Bonamico et al. 2001
	301 children and adults	5.4	Szaflarska-Popławska et al. 2016
Turner syndrome	389 children and adults	6.4	Bonamico et al. 2002
	286 children and adults	8.7	Stoklasova et al. 2019
Williams syndrome	46 children and adults	2.2	Stagi et al. 2014
	101 children	6.9	Pangallo et al. 2020

*Pooled prevalence.

IgA, immunoglobulin A; PSC, primary sclerosing cholangitis

2.4 Pathogenesis

2.4.1 The gut lymphoid system and self-tolerance

The lymphoid system of the intestine is a large organism that harbours a great part of the host's lymphocyte population (Pabst et al. 2008). It encompasses gut-associated lymphoid tissue (GALT) which is organized intestinal lymphoid tissue that forms Peyer's patches in the mucosa and submucosa, smaller lymphoid aggregates termed solitary isolated lymphoid tissues (SILTs) and draining mesenteric lymph nodes (Mowat & Agace 2014). In addition to GALT, single lymphoid cells are diffusely scattered throughout the lamina propria and the intestinal epithelium. The great majority of the immune cells residing within the epithelial layer are lymphocytes, referred to as intestinal intraepithelial lymphocytes (IELs) (Cheroutre et al. 2011). Since IELs are located at the interface between the body and the outside environment and thus constantly come into contact with foreign antigens, they play a crucial role in regulating intestinal immunity (Ma et al. 2021). The IEL density is approximately one cell per ten epithelial cells in the small intestine (Olivares-Villagómez & Van Kaer 2018).

IELs can be divided into T cell receptor expressing (TCR+) and TCR negative (TCR-) cells. T cells are further divided into αβ and γδ cells based on the expression of either an αβ or a γδ TCRs. Associated with the TCR are CD3εγ, CD3εδ, and CD3ζζ dimers which together form a TCR-CD3 complex (Kuhns & Badgandi 2012). Numerous subsets of T cells are categorized according to the different receptors present on the cell surface. In the intestinal epithelium, CD8+ cells are the most abundant T cell population followed by CD4+ T cells (Jarry et al. 1990). In addition, IELs typically express both inhibitory and activating natural killer (NK) receptors (Bhagat et al. 2008; Shires et al. 2001). Regulatory T and B cells are subpopulations of lymphoid cells that act to suppress immune response and thus maintain homeostasis and self-tolerance (Izcue et al. 2009).

In the lamina propria, CD8+ and CD4+T cells are also present as well as plasma cells that secrete mainly Ig A class and, to a lesser extent, IgM class antibodies (Brandtzaeg et al. 1999). The most recently discovered lymphocyte population is innate lymphoid cells, which have the same progenitor as T and B lymphocytes, but due to a lack of certain receptors with clonal diversity, they are categorized as part of the innate immune system (Seo et al. 2020). They are mostly present on mucosal

surfaces in the epithelium and lamina propria (Seo et al. 2020). The lamina propria also contains numerous types of non-lymphoid immune cells, such as dendritic cells, macrophages, mast cells and eosinophils (Mowat & Agace 2014).

In addition to the immunological functions, the epithelial layer provides a physical barrier between the host and the intestinal lumen. The most abundant cells are intestinal epithelial cells (IECs) that are specialized in different functions. For instance, enterocytes and Paneth cells secrete antimicrobial peptides, whereas goblet cells produce mucin to protect the epithelial surface and function as APCs (Takiishi et al. 2017). Tight junctions form a barrier between the IECs preventing the paracellular passage of pathogens and regulate intestinal permeability to water, nutrients and ions (Ramanan & Cadwell 2016).

The immune cells of the mucosal barrier constantly encounter foreign antigens such as food and microbes and must differentiate between harmful and innocuous substances to delimit the growth of pathogens and to maintain tolerance towards self-antigens, nutrients and beneficial microbes. Several mechanisms are involved in the induction of tolerance, in which dendritic cells, regulatory T and B cells and innate lymphoid cells play a decisive role (Seo et al. 2020; Weiner et al. 2011). The development of tolerance is influenced by genetics and environmental factors such as antigen dose and intestinal microbial composition (Wawrzyniak et al. 2017).

2.4.2 Dietary gluten and coeliac disease

Gluten is a glutinous storage protein present naturally in wheat, barley and rye that has beneficial effects on the baking properties of dough and is thus used almost universally. Gluten is a mixture of several proline- and glutamine-rich peptides with a high degree of homology (Shewry et al. 1986). It is classically divided into two categories including alcohol-soluble prolamin proteins termed gliadin in wheat, hordein in rye and secalin in barley, and insoluble glutelin proteins, termed glutenin in wheat. Gluten can be also divided into subgroups according to molecular weight and gliadin is further subdivided into α-, γ- and ω-gliadins (Shewry et al. 1986). Gluten is poorly digested in the gastrointestinal tract due to the capacity of proline residues to resist the degradation by the gastric, pancreatic and brush border peptidases (Shan et al. 2002). Gliadin was first considered the only immunogenic component of wheat gluten, but later it has been shown that glutenin may also activate the innate immune response in coeliac disease (Dewar et al. 2006).

Studies on the effect of the timing of gluten introduction as well as the level of its intake in early childhood among at-risk children have yielded somewhat conflicting results (Aronsson et al. 2019; Crespo-Escobar et al. 2017; Vriezinga et al. 2014). While the optimal time frame for introduction remains unclear, according to current evidence the inclusion of gluten in the diet between four and 12 months of age does not seem to increase the absolute risk of coeliac disease (Szajewska et al. 2016). There is nevertheless recent evidence that increasing daily amounts are associated with elevated coeliac disease risk, and thus avoiding large quantities during the first years of life is recommended (Aronsson et al. 2019; Szajewska et al. 2016). However, more studies are needed to confirm this issue and to determine the upper limit for recommended daily gluten dose in at-risk individuals.

2.4.3 Gluten-induced pathogenic mechanisms

Gliadin peptides are transferred into the lamina propria via both transcellular and paracellular mechanisms. The permeability of the epithelial barrier is increased in active coeliac disease due to structural alterations in the tight junctions (Schulzke et al. 1998). Zonulin is one factor suggested to affect the regulation of tight junctions (Wang et al. 2000), and gliadin has been reported to induce zonulin release in coeliac disease patients (Drago et al. 2006). As another mechanism, gliadin peptides could be taken up via a transcytotic pathway by transferrin receptor CD71, which in active coeliac disease is overexpressed in the epithelial cells (Matysiak-Budnik et al. 2008). Regardless of the route, when entering the lamina propria the glutamine residues of gliadin molecules are deamidated by transglutaminase 2 (TG2) to negatively charged glutamate, which considerably enhances the immunogenicity of gliadin (Dieterich et al. 1997).

Deamidated gliadin peptides bind to HLA DQ2 and DQ8 molecules on the cell membrane of APCs, the DQ2.5 subtype having the highest affinity (Fallang et al. 2009). The peptides bound with HLA DQ2 and/or DQ8 are then presented to CD4+ T helper cells with gliadin-specific T cell receptors (TCRs) that have a high affinity with gliadin epitopes (Molberg et al. 1997). Certain B cells have receptors and antibodies on their cell membranes that bind with high affinity to gliadin peptides, gliadin-gluten complexes as well as TG2 molecules (Stamnaes & Sollid 2015). Gliadin-specific CD4+ T cells help B cells become plasma cells that produce antibodies against TG2 and gliadin peptides (Stamnaes & Sollid 2015). Moreover, it

has been suggested that gliadin- and TG2-specific B cells may also present these antigens to T cells (Sollid 2017). The activated T cells secrete proinflammatory cytokines such as IL-21, interferon gamma (IFN-Ɣ) that exacerbate the inflammation in the intestinal mucosa (Bodd et al. 2010).

In addition to the adaptive response, innate immune mechanisms are also believed to be involved in the cascade that leads to changes in the epithelial cells and the transformation of IELs into cytotoxic killer cells. In stressful conditions, such as during exposure to toxic agents or pathogens, the epithelial cells undergo alterations in their protein expression and morphological changes (Setty et al. 2015). The stressed epithelial cells express MHC (major histocompatibility complex) class I receptors such as HLA-E and MIC on their cell surfaces, and also secrete inflammatory cytokines including type I interferons (IFN), IL-15 and IL-18 (Hüe et al. 2004; Mention et al. 2003; Meresse et al. 2006; Salvati et al. 2002). IL-15 in particular has many proinflammatory effects, such as inducing the proliferation and survival of abnormal IELs, enhancing the number of natural killer (NK) receptors on the IEL cell membrane and lowering the threshold for T cell activation (Mention et al. 2003; Meresse et al. 2006). IL-15 might also contribute to the maturation of dendritic cells into APCs (Mention et al. 2003). IELs recognize the MHC class I molecules by their NK receptors, this interaction inducing apoptosis of epithelial cells (Hüe et al. 2004). Intriguingly, gliadin molecules may also directly activate innate immune pathways independently of CD4+ T cell activation and induce secretion of inflammatory cytokines and enterocyte apoptosis (Maiuri et al. 2003). Furthermore, alterations in the intestinal microbiota may induce epithelial stress and thus promote inflammatory responses (Verdu et al. 2015).

Adaptive and innate immune mechanisms overlap in many immunologic pathways. The activated gliadin-specific CD4+ T cells drive the epithelial cells to upregulate MHC class I receptors and IL-15 secretion and stimulate cytotoxic IELs via TCRs (Sollid & Jabri 2013). IL-15 secreted by epithelial and dendritic cells enhances IL-21 secretion by gliadin-specific CD4+ T cells and certain other lymphocytes and suppresses the regulatory CD4+ T cells, thus expediting the loss of tolerance to antigens (Sarra et al. 2013). The immune mechanisms involved in the coeliac disease pathogenesis are illustrated in Figure 1.

It is thought that the epithelial damage is eventually induced by activated intraepithelial cytotoxic lymphocytes; however, it remains unclear which factors are needed in the licensing of these cells to kill the epithelial cells (Abadie et al. 2012). It is suggested that interplay between IELs, stressed epithelial cells and adaptive

immunity eventually leads to the mucosal damage. However the mechanisms remain at least partly unclear (Abadie et al. 2012). In any case, the gluten-triggered immune reaction eventually leads to enhanced apoptosis of enterocytes and to small bowel mucosal atrophy. After the lymphocyte infiltration, elongation of the crypts is the earliest found structural alteration (Oberhuber et al. 1999). Following crypt hyperplasia, the villous cells experience disturbed maturation and the villi become shorter and may eventually disappear completely (Oberhuber et al. 1999).

2.4.4 Generation of antibodies

Anti-reticulin antibodies (ARA) targeted against the reticular fibres of connective tissue surrounding vessels and muscle fibres (endomysium) were the first autoantibodies to be discovered in coeliac disease (Seah et al. 1971). A few years later Stern and associates introduced anti-gliadin antibodies (AGA) (Stern et al. 1979). These, however, were subsequently observed also to be present in other conditions than coeliac disease (O'Farrelly et al. 1988) and even in healthy individuals (Uibo et al. 1993). Endomysial antibodies (EmA) are autoantibodies targeted against endomysium and associated with coeliac disease in the 1980s (Chorzelski et al. 1984). TG2, an enzyme which catalyses many transamidating and deamidating reactions in addition to gliadin modification, was eventually identified as an autoantigen recognized by both EmA and ARA in 1997 (Dieterich et al. 1997).

With the help of gliadin-specific CD4+ T cells, the B cells differentiate into plasma cells that produce antibodies against gluten-derived deamidated gliadin peptides and TG2 (TG2-abs) that can be detected in the sera of coeliac patients when exposed to dietary gluten (Leffler et al. 2013, Figure 1). Circulating antibodies were formerly thought to be secreted solely by plasma cells located in the small intestine. However, recent evidence shows that extra-intestinal plasma cells may also participate in this process (Iversen et al. 2017). Furthermore, immunoglobulin A (IgA) deposits of TG2-abs have been observed in the duodenal epithelium and lamina propria and in extraintestinal tissues, even in the absence of circulating antibodies (Korponay-Szabó et al. 2004). These deposits may in fact appear even before the development of manifest mucosal lesions and have proven to be accurate predictors of future coeliac disease (Salmi et al. 2006). Although the mechanisms are not completely understood, there is evidence that coeliac antibodies may also actively participate in the disease pathogenesis e.g. by increasing the epithelial transcytosis of

gliadin peptides (Matysiak-Budnik et al. 2008). Levels of TG2-abs have also been shown to correlate significantly with the degree of villous atrophy (Agardh et al. 2015; Taavela et al. 2013b). In addition to TG2-abs, antibodies targeted at other transglutaminases have been found particularly in coeliac patients with extraintestinal manifestations. Antibodies to transglutaminase type 3 (TG3-abs) are deposited in the dermis of patients with dermatitis herpetiformis (Sárdy et al. 2002) and transglutaminase 6 (TG6) antibodies have been detected in coeliac patients with neurological symptoms (Hadjivassiliou et al. 2008). The role of these additional autoantibodies in the diagnosis of coeliac disease remains to be elucidated.

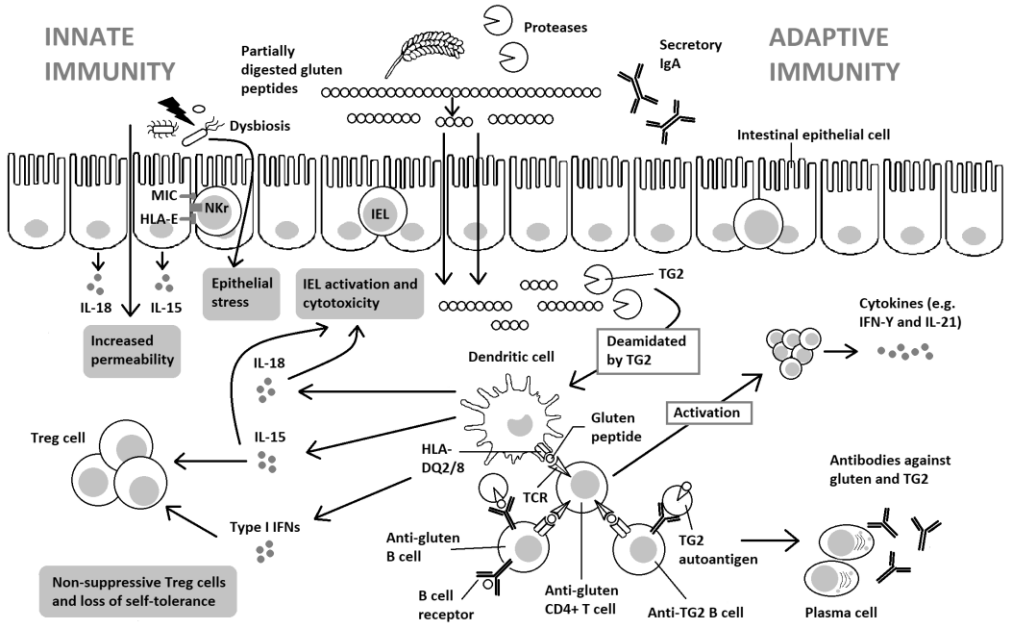

Figure 1. Gluten-induced innate and adaptive immune responses in coeliac disease (adapted from Lindfors et al. 2019). HLA, human leukocyte antigen; IEL, intraepithelial lymphocyte; IFN, interferon; IFN-ϒ, interferon gamma; NKr, natural killer receptor; TG2, transglutaminase 2; Treg cell, regulatory T cell

2.5 Diagnosis

2.5.1 Duodenal biopsy

Before the emergence of endoscopy, other methodology such as the laborious measurement of faecal fat was used in the diagnosis of coeliac disease (Sheldon & MacMahon 1949). Histologic evaluation of the intestinal mucosa was first conducted on autopsy specimens (Manson-Bahr 1924) and later on laparotomy samples until the development of peroral biopsy methods in the 1950s (Shiner 1956). The first biopsies were taken by rigid endoscopy and then by suction capsules that were later replaced by flexible endoscopes (Demling & Hagel 1985; Greene et al. 1974). Normal mucosal villi were discovered to be long finger- or leaflet-like structures lined with short crypts, whereas shortened or disappearing villi (villous atrophy) and crypt hyperplasia were detected in coeliac patients (Doniach & Shiner 1957). The development of endoscopic techniques enabled multiple biopsies to be taken from different sites, thereby giving a more detailed view of the coeliac disease-associated histological findings. The first classification of morphological changes was presented by Doniach and Shiner, who divided the histology into normal, partial and subtotal villous atrophy (Doniach & Shiner 1957). This classification was later established by the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) (Meeuwisse 1970). Later Marsh introduced a classification encompassing a wider spectrum of different stages of mucosal damage ranging from normal (type 0) to lymphocyte infiltration (1), to crypt hyperplasia (2) and to villous atrophy (3) (Marsh 1992). Oberhuber and colleagues further divided villous atrophy into mild, moderate and severe subtypes (3a-c respectively) (Oberhuber et al. 1999). Corazza and Villanacci made their own modifications to the Marsh-Oberhuber classification by dividing the morphology into Grades A, B1 and B2, corresponding to Marsh-Oberhuber classes 1-2, 3a-b and 3c respectively (Corazza & Villanacci 2005).

In addition to the aforementioned categorical classifications, a continuous villous height/crypt depth ratio (Vh/CrD) has also been used to estimate the state of mucosal damage (Kuitunen et al. 1982). This is more laborious than the Marsh-Oberhuber grading but has its own advantages, such as better repeatability and correlation with clinical and laboratory presentations (Taavela et al. 2013a). There are differing views on the optimal cut-off value for Vh/CrD, the thresholds used ranging from 1.0 to 3.0 in different studies (Chang et al. 2005; Corazza & Villanacci

2005; Wahab et al. 2002). In Finland, a cut-off of 2.0 is used based on the normal values observed in healthy controls (Kuitunen et al. 1982). This cut-off has also been shown to correlate with coeliac disease-associated antibodies and mucosal inflammation (Koskinen et al. 2010).

Another quantitative parameter in histological evaluations is the density of CD3+ IELs. In categorical classifications morphology and inflammation are evaluated consecutively. However, since these are not necessarily correlated, separate determination of IEL density improves the diagnostic accuracy (Lähdeaho et al. 2011). IEL density was first evaluated from haematoxylin-eosin-stained paraffin-embedded specimens and later by immunohistochemical CD3-staining from either paraffin embedded or frozen samples, of which the latter also enables evaluation of αβ and γδ IELs (Järvinen et al. 2003; Kuitunen et al. 1982). Increased density of γδ IELs is fairly specific to coeliac disease, although also related to some other conditions (Järvinen et al. 2003; Kokkonen et al. 2000). In active coeliac disease, the absolute and relative amounts of αβ and γδ T cell IELs increase (Halstensen & Brandtzaeg 1993). While the numbers of αβ T cells gradually normalize after initiation of gluten-free diet, the elevated levels of γδ T cells persist (Kutlu et al. 1993), suggesting possible regulatory or anti-inflammatory properties (Bhagat et al. 2008).

There are several technical factors affecting the diagnostic accuracy of the histology. The biopsy samples must be cut and oriented properly in order to achieve reliable results and prevent false negative interpretation (Taavela et al. 2013a). Moreover, due to possible patchiness of the coeliac lesion, at least four representative biopsies from different sites on the duodenum are recommended (Lebwohl et al. 2011). Adding so-called bulb biopsies may improve the diagnostic yield, but may simultaneously impair specificity and even lead to false-positive diagnoses (Taavela et al. 2016). It must also be realized that even if the biopsy sampling and processing are correctly performed, the mucosal alterations typical for coeliac disease are not pathognomic as they can also be seen in connection with certain infections, immune disorders, nutritional deficiencies and as effects of certain medications (Aziz et al. 2017).

2.5.2 Serological tests

Serological tests are the preferred method in the first-line case-finding and screening of coeliac disease. Nowadays the most widely used tests are those measuring EmA and TG2-abs. Since the introduction of EmA, they have been regarded as the gold standard test for coeliac disease (Chorzelski et al. 1984), determined by indirect immunofluorescence using either monkey oesophagus or human umbilical cord as a substrate (Chorzelski et al. 1984; Ladinser et al. 1994). In several studies EmA have demonstrated almost 100% specificity for untreated coeliac disease (Table 3). As a disadvantage, the test protocol is subjective and rather expensive and the sensitivity may be impaired, for example, in patients with mild histological damage (Abrams et al. 2004).

TG2-abs are measured by several methods, of which the most widely used is an enzyme-linked immunosorbent assay (ELISA) -based test using human recombinant TG2 as an antigen. Other methods include radiobinding assays and more recently developed electrochemiluminescence assays (Habtamu et al. 2015). Multiplex assays have also been developed (Abdukhakimova et al. 2021; He et al. 2020). Since TG2-abs testing is more cost-effective, objective and easier to perform than EmA (Dieterich et al. 1998), it has become the preferred serologic assay. In general, TG2-abs are a more sensitive but less specific marker than EmA (Tables 3 and 4). There is also some variation in the accuracy of TG2-ab test depending on the quality of the TG2 antigen (Giersiepen et al. 2012). Therefore, only the use of well-validated TG2-ab assays with a calibration curve is recommended (Husby et al. 2012). Furthermore, mildly elevated TG2-ab levels in non-coeliac subjects have been reported e.g. during certain infections (Ferrara et al. 2010). In patients with IgA deficiency, determination of corresponding immunoglobulin G (IgG) class EmA and TG2-ab is recommended in order to avoid false negative results (Korponay-Szabó et al. 2003).

On some occasions, coeliac disease cannot be detected by serology. Seronegativity has been observed particularly among elderly patients and in RCD (Ilus et al. 2014a; Volta et al. 2016a). In these cases, the diagnosis is usually based on the detection of gluten-sensitive small bowel mucosal damage (Aziz et al. 2017). In addition, as mentioned, even in the absence of circulating autoantibodies, TG2-specific deposits may be detected in the duodenal biopsies as a specific marker for coeliac disease (Korponay-Szabó et al. 2004). Altogether, seronegative coeliac disease is a rare condition and, therefore, other possible causes of villous atrophy should be carefully ruled out (Aziz et al. 2017).

AGAs are no longer recommended as a primary serologic method in coeliac disease mainly due to their poor specificity (Leffler & Schuppan 2010). As an exception, they may be useful in diagnosing gluten ataxia (Hadjivassiliou et al. 1998). Interestingly, antibodies directed at deamidated gliadin peptides (DGP-abs) have proven more accurate than those against naïve gliadin peptides (Kaukinen et al. 2007a). DGP-abs seem to be a sensitive marker, especially for identifying patients with early-stage coeliac disease (Kurppa et al. 2011). However, the specificity may be inadequate, especially in the absence of positive TG2-abs (Gould et al. 2019). Combination assays measuring TG2-abs and DGP-abs mostly show a high accuracy, tests combining IgG class DGP-abs and IgA class TG2-abs being the most specific, whereas measuring both IgA and IgG class TG2-abs and DGP-abs increases the sensitivity but is inferior in specificity (Schyum & Rumessen 2013). However, further studies on the use of combination assays are needed, and at present TG2-abs remain the preferred first-line test for coeliac disease (Lewis & Scott 2010).

So-called point-of-care tests based on the detection of TG2 and/or DGP-abs in a fingertip blood sample have also been developed. In a recent meta-analysis, the respective pooled sensitivity and specificity for these tests were 94.0% (95% CI 89.9-96.5%) and 94.4% (90.9-96.5%) (Singh et al. 2019). However, the majority of these studies included high-risk patients with a clinical suspicion of coeliac disease, and it is not known how these tests perform in screening and among individuals with lower pre-test probability (Singh et al. 2019). Moreover, since these tests are non-quantitative, they cannot be used as a diagnostic assay in non-invasive criteria (Chapter 2.5.3). Altogether, the role of point-of-care tests in the diagnostics of coeliac disease remains to be established.

Table 3. Accuracy of endomysial antibodies in the diagnostics of coeliac disease.

Cohort (n)	Sensitivity %	Specificity %	PPV %	NPV %	Reference
Adults					
39	100	100	100	100	Gülseren et al. 2019
Children					
136	98.6 - 100	61.1 - 71.9	82.7 - 88.6	95.8 - 100	Ho et al. 2020
445	98.3	99.5	99.6	98.1	Roca et al. 2019
183	97	68	85	91	Mubarak et al. 2012
Children and adults					
262	93.7	100	100	94.4	Volta et al. 2008
257	99	100	100	99	Raivio et al. 2008
271	88.9	98.1	ND	ND	Collin et al. 2005
268	98	85	89	97	Bürgin-Wolff et al. 2013

ND, no data; NPV, negative predictive value; PPV, positive predictive value

Table 4. Accuracy of IgA class transglutaminase 2 antibodies in the diagnostics of coeliac disease.

Cohort (n)	Commercial assay(s)	Positivity threshold	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Reference
Adults							
144	Bio-Rad Autoimmune EIA assay	10 x ULN	70	100	100	69	Beig et al. 2021
740	Aeskulisa Diagnostics, EliA Celikey	10 x ULN	54.0	90.0	98.7	12.5	Penny et al. 2021
836	4 separate kits*	1 x ULN	ND	ND	83.6-100	ND	Ylönen et al. 2020
		10 x ULN	ND	ND	100	ND	
39	Immo Diagnostics	1 x ULN	100	98.4	95.5	100	Gülseren et al. 2019
		10 x ULN	81	100	100	93	
310	Delta Biologicals	8.9 x ULN	69	100	100	31	Tortora et al. 2014
945	Celikey, Eurospital	5 x ULN	10.1-59.1	100	100	42-53.1	Zanini et al. 2012
Children							
542	BioPlex 2200	1 x ULN	92.8	92.3	69.0	98.6	Pacheco et al. 2022
		10 x ULN	50	99.5	95.4	91.5	
136	Celikey, Quanta Lite	1 x ULN	96.1–96.3	58.3-64.8	74.5-80.6	92.1	Ho et al. 2020
		10 x ULN	31.7–32.9	98.3-100	96.2-100	49.1-53.6	
696	QUANTA Flash, EliA Celikey	1 x ULN	89-90	98-99	89-95	98-99	Bogaert et al. 2020
		10 x ULN	57-77	100	100	94-97	
445	EliA Celikey	10 x ULN	98.7	93.0	93.9	98.5	Roca et al. 2019
898	Euroimmun	10 x ULN	97.1	89.3	98.8	93.4	Wolf et al. 2017
1,974	7 separate kits**	10 x ULN	99.3	93	96.1	ND	Smarrazzo et al. 2017
707	8 separate kits***	10 x ULN	71.0	93.5	99.1	ND	Werkstetter et al. 2017
17,505	Euroimmun	10 x ULN	ND	ND	95.9	99.4	Gidrewicz et al. 2015
183	ELiA Celikey	1 x ULN	97	78	89	92	Mubarak et al. 2012
		10 x ULN	73	100	100	66	

*Celikey, Eurospital, Inova and Orgentec; **Aesku, Celikey (Pharmacia & Upjohn), EliA Celikey, Euroimmun, Eurospital, Inova, Orgentec; ***EliA Celikey, Varelisa Celikey, Inova Quanta Lite, Inova Quanta Flash, Eurospital, Euroimmun, 2 different tests from R-Biopharm / Zedira

IgA, immunoglobulin A; ND, no data; NPV, negative predictive value; PPV, positive predictive value; ULN, upper limit of normal

2.5.3 Changing diagnostic criteria

The diagnosis of coeliac disease has traditionally been based on the detection of the above-described histological damage, positive serology providing further support (Walker-Smith et al. 1990). However, less invasive but accurate diagnostic methods have been eagerly sought. A remarkable step was taken in 2012 when the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) suggested that the diagnosis could be set without biopsy for symptomatic children who have TG2-abs > 10× the upper limit of normal (ULN), positive EmA and coeliac disease-associated HLA (Husby et al. 2012). The serology-based approach has subsequently been proven accurate for coeliac disease in the largest studies on this subgroup of paediatric patients (Werkstetter et al. 2017; Wolf et al. 2017). In a recent update by ESPGHAN, the criteria were extended to asymptomatic children and genetic testing was no longer considered mandatory (Husby et al. 2020). In Finland, a serology-based diagnosis is also accepted for adult patients with the aforementioned serologic findings (Coeliac disease, Current Care Guidelines 2018). This diagnostic approach has proven accurate with different TG2-ab assays and pre-test probabilities (Penny et al. 2021; Ylönen et al. 2020). According to a recent study, the TG2-ab cut-off for serology-based diagnosis might be set lower than 10x ULN, but additional evidence is needed (Ylönen et al. 2020).

The diagnostic criteria for coeliac disease are not, however, uniform across different countries. Biopsy remains the diagnostic standard for adults in almost all other countries than Finland (Al-Toma et al. 2019) and also for children in some countries such as the USA (Hill et al. 2005). In case of only mildly elevated TG2-abs or negative EmA and in subjects with IgA deficiency, the diagnosis in all guidelines still relies on the characteristic histopathologic findings. Despite the benefits of serology-based diagnostics, some challenges remain, such as the incomparability between different TG2-ab assays due to the lack of standardization (Beltran et al. 2014). Further, serology-based diagnostics has been criticized e.g. for the lack of an option for histologic follow-up (Kurien et al. 2015).

Early-stage coeliac disease

Cases with positive coeliac disease serology without obvious morphological damage in the duodenal mucosa have become more common with increasing screening.

Even though low positive serum titers are not 100% specific, usually positive autoantibodies are early signs of a gradually developing disease. In addition to positive serology, mucosal leukocyte infiltration, mild epithelial changes and the above-mentioned antibody deposits often precede crypt hyperplasia and villous atrophy (Kurppa et al. 2009, 2010; Mäki et al. 1990). Of note, patients with this so-called early-stage coeliac disease may already have symptoms and other clinical signs and benefit from an experimental gluten-free diet even before the development of diagnostic mucosal lesions (Repo et al. 2017; Volta et al. 2016b).

2.6 Clinical picture

Historically, coeliac disease has been considered to be a childhood disease with severe gastrointestinal symptoms, weight loss and failure to thrive (Visakorpi & Mäki 1994). In recent decades, the clinical picture has shifted from this so-called classical phenotype to a wide range of gastrointestinal and extraintestinal manifestations that may emerge at any age (Vilppula et al. 2008; Volta et al. 2014). The broadened spectrum of phenotype is probably to a large extent caused by improved diagnostic yield due to increased knowledge among medical practitioners and the introduction of the sensitive serological tests (Kivelä et al. 2015). However, a true change towards milder clinical and histological presentation may also have occurred (Kivelä et al. 2015; Volta et al. 2014). Altogether, the reported symptoms of coeliac disease are unspecific and some patients have a clinically silent disease with no recognizable symptoms at all (Chapter 2.6.3). Some of the manifestations may lead to persistent health problems, especially when the diagnosis is delayed or adherence to gluten-free diet is poor (Chapter 2.6.4). Distinguishing between a complication and a manifestation of coeliac disease is challenging, but complications are often defined as irreversible outcomes of the disease whereas manifestations can be treated by gluten-free diet (Laurikka et al. 2018).

2.6.1 Gastrointestinal symptoms

The classical intestinal phenotype was characterized by chronic diarrhoea and steatorrhoea, often accompanied by deficiencies of essential vitamins and trace elements. Currently, less severe gastrointestinal symptoms, such as abdominal

discomfort, loose stools and constipation are more frequently reported in all age groups. The prevalence of different gastrointestinal symptoms varies significantly between studies both in children and adults, probably due to largely to variable study designs and diagnostic definitions, although there may also be true country-related differences in the clinical presentation (Agardh et al. 2015; Rashid et al. 2005; Savilahti et al. 2010; Volta et al. 2014).

2.6.2 Extraintestinal manifestations

Extraintestinal manifestations of coeliac disease may derive from many organs, including the skin and liver and the neurological, musculoskeletal and reproductive systems (Äärelä et al. 2016; Hadjivassiliou et al. 2002; Salmi et al. 2011; Tersigni et al. 2014). A marked proportion of patients is estimated to suffer from these, even though the scientific evidence remains scant (Laurikka et al. 2018; Volta et al. 2014). Of note, extraintestinal manifestations may accompany gastrointestinal symptoms or be the sole clinical presentation. Some of these are specific to certain age groups, such as poor growth and delayed puberty in children (Jericho et al. 2017; Nurminen et al. 2019; Rashid et al. 2005) and osteoporosis and infertility in adults (Bottaro et al. 1999; Jericho et al. 2017), whereas e.g. anaemia and elevated liver enzymes are present in all ages (Äärelä et al. 2016; Bottaro et al. 1999; Jericho et al. 2017).

The pathogenesis of extraintestinal manifestations remains mostly obscure. In theory, prolonged inflammation and malabsorption may cause nutritional deficiencies and consequently lead, for example, to anaemia, reduced bone accrual, enamel defects, poor growth and delayed puberty (Bona et al. 2002; Cheng et al. 2010; Harper et al. 2007; Kemppainen et al. 1999). These manifestations have been reported to be more common in patients with a severe clinical and histological presentation of coeliac disease (Nurminen et al. 2019), but this remains unresolved (Laurikka et al. 2018). Another proposed yet unproven mechanism in the pathogenesis of extraintestinal manifestations is adaptive humoral autoimmunity towards e.g. different transglutaminases (Hadjivassiliou et al. 2006, 2008; Sárdy et al. 2002).

Dermatitis herpetiformis (DH) is a well-known organ-specific manifestation of coeliac disease, affecting up to 10% of adult coeliac disease patients. It is characterized by itchy blisters and papules most typically on the elbows, knees and buttocks (Collin & Reunala 2003). A specific finding is the presence of IgA class

TG3-ab deposits that can be detected by direct immunofluorescence in perilesional skin biopsies (Zone et al. 1996). Most DH patients also have signs of gluten-induced enteropathy (Salmi et al. 2014). As an additional skin manifestation, an elevated risk of psoriasis has been reported in coeliac disease patients (Ludvigsson et al. 2011).

The most common neurological manifestations of coeliac disease are gluten ataxia and peripheral neuropathy (Hadjivassiliou et al. 2002). Cross-reactivity of anti-gliadin antibodies with Purkinje cell epitopes, perivascular TG2 antibody deposits in brain tissue and circulating TG6 antibodies have been suggested to contribute to the pathogenesis of gluten ataxia (Hadjivassiliou et al. 2008), but the evidence remains inconclusive (Leo et al. 2018).

Untreated coeliac patients often have mildly elevated liver enzymes that normalize on a gluten-free diet (Äärelä et al. 2016) but more severe hepatic complications may also occur (Kaukinen et al. 2002).

Various other common complaints such as headache, fatigue, diverse psychiatric and cardiovascular conditions, arthritis, uveitis, eczema and aphthous ulcers have also been suggested to be extraintestinal manifestations of coeliac disease; however, the actual causality remains to be established (Jericho et al. 2017; Wijarnpreecha et al. 2018).

2.6.3 Screen-detected and asymptomatic coeliac disease

Besides through active case-finding, coeliac patients can be found by screening at-risk groups (Chapter 2.7). Although these patients are often considered to be asymptomatic at the time of diagnosis, they may have unrecognized symptoms and clinical signs (Kivelä et al. 2017; Mahadev et al. 2016). Altogether, distinguishing between asymptomatic and symptomatic patients is complicated, and the clinical phenotype does not necessarily correlate with the serological or histological findings at diagnosis (Björck et al. 2017; van der Pals et al. 2014).

2.6.4 Complications of coeliac disease

Long-term untreated coeliac disease may lead to persistent consequences no longer responsive to gluten-free diet (Laurikka et al. 2022). The risk for complications is partially dependent on the age at disease onset (Kivelä & Kurppa 2018). For example,

abnormalities in bone formation may cause permanent enamel defects and suboptimal bone accrual leading to osteoporosis and fractures later in life if not treated early enough (Majorana et al. 2010; Tau et al. 2006). Furthermore, impaired growth due to untreated coeliac disease in childhood may affect adult height (Bosio et al. 1990). Neurological manifestations are present at all ages but more commonly in adults (Hadjivassiliou et al. 2002, 2003). Untreated coeliac disease has also been associated with various reproductive problems, such as infertility, an increased risk for spontaneous abortions and intrauterine growth retardation (Jericho et al. 2017; Tersigni et al. 2014), possibly due to deficiencies in nutrients crucial to normal foetal development (Tersigni et al. 2014).

According to population-based studies, overall cancer risk seems not to be elevated in coeliac patients (Card et al. 2004; Ilus et al. 2014b). However, an increased risk for lymphoma and certain gastrointestinal malignancies has been reported (Gao et al. 2009; Lohi et al. 2009). Continuing gluten intake has been suggested to be a predisposing factor (Viljamaa et al. 2006). Additionally, delayed diagnosis or recurring dietary lapses may eventually lead to RCD, which may in turn progress to intestinal lymphoma (Chapter 2.9).

2.7 Screening for coeliac disease

As described above, the clinical picture of coeliac disease ranges from severe gastrointestinal or extraintestinal symptoms and signs to a complete absence of symptoms (Chapter 2.6). The diverse and unspecific presentation makes clinical case-finding challenging, particularly as patients with mild and/or vague symptoms may not seek medical care. Coeliac disease thus remains a markedly underdiagnosed condition (Popp et al. 2019). To increase the suboptimal yield, screening of subjects at increased risk for coeliac disease, especially family members of previously diagnosed patients and individuals with other autoimmune diseases and chromosomal abnormalities (Chapter 2.3.3 and Tables 1-2), is usually recommended (Husby et al. 2020; Ludvigsson et al. 2014; Rubio-Tapia et al. 2013). However, such targeted screening still leaves a major proportion of patients undiagnosed (Kivelä & Kurppa 2018).

To further increase the patient detection rate, wide-scale screening of the population or at least all individuals with a known genetic predisposition has been proposed (Catassi & Fasano 2014). Of note, since coeliac disease can develop at any

age (Myléus et al. 2009; Vilppula et al. 2009), to obtain the optimal yield, screening of seronegative subjects should be repeated regularly, with a currently debated frequency (Paavola et al. 2021). Although screening and early treatment would likely prevent long-term complications, the cost-effectiveness of such an approach as well as the benefits of treatment in relation to the burden of a restrictive gluten-free diet – particularly in asymptomatic patients – remain unclear (Chou et al. 2017). Promisingly, several studies have reported screen-detected patients to achieve similar or even better dietary compliance and quality of life than those found by case-finding (Kinos et al. 2012; Mahadev et al. 2016). However, coeliac disease diagnosis and treatment may also lead to increased anxiety and health concerns in asymptomatic patients (Kivelä et al. 2018; Ukkola et al. 2011). Furthermore, the long-term natural history of seropositive subjects not fulfilling the current diagnostic criteria remains poorly defined (Kurppa et al. 2009; Volta et al. 2016b; Vriezinga et al. 2014). Altogether, further studies are needed to justify population-wide screening (Chou et al. 2017).

2.8 Treatment

2.8.1 Dietary treatment

A lifelong gluten-free diet continues to be the only scientifically accepted treatment for coeliac disease. This means stringent exclusion of wheat, barley and rye and other gluten-containing foods from the diet (See et al. 2015). In contrast, oats that have not been contaminated by gluten during the harvesting or manufacturing process are nowadays considered safe for the great majority of patients (Aaltonen et al. 2017). A daily dose of 10-30 mg of gluten is generally considered safe, whereas 30-100 mg of gluten per day is often enough to induce intestinal inflammation (Catassi et al. 2007; Collin et al. 2004). The definition "gluten-free" can be used of products that contain less than 20 milligrams of gluten per kilogram (Food and Drug Administration 2013; European Commission 2014).

Initiation of gluten-free diet usually alleviates possible symptoms and eventually restores the normal mucosal architecture (Ludvigsson et al. 2014). However, some patients may have incomplete response despite careful diet (Chapter 2.9). Since gluten is widely used in the food industry, maintaining a gluten-free diet is often

challenging and may entail social, psychological and financial burdens. Tolerance of gluten is highly variable (Lähdeaho et al. 2011) and in some patients even minimal amounts can activate the disease (Catassi et al. 2007). Gluten-free products may also contain increased amounts of fat and sugar and reduced amounts of fibre, protein, vitamins and minerals compared to the equivalent gluten-containing foods and thus predispose to obesity and nutritional deficiencies (Martin et al. 2013; Wild et al. 2010).

2.8.2 Other treatment options

The above-mentioned challenges with a gluten-free diet underline the need for complementary treatment options. An increased understanding of the development of coeliac disease has enabled the development of agents that target different steps in the pathogenetic pathway. One approach is to abolish the immunogenicity of gluten by enzymatic degradation or detoxification by gliadin-binding or antigenic molecules (Bethune & Khosla 2012; Sample et al. 2017). Some drugs aim to prevent gluten-induced inflammation by enhancing the epithelial barrier. Larazotide is a synthetic peptide that locally regulates tight junctions (Gopalakrishnan et al. 2012). The current evidence on its benefits is inconclusive (Kelly et al. 2013; Paterson et al. 2007). Therapeutic agents have also been developed to reprogramme the immune system to become nonresponsive to gluten peptides. Nexvax2, a vaccine that contains gluten epitopes recognized by T cells, gave promising first-line results (Daveson et al. 2017) but did not prevent gluten-induced symptoms in a later study (Truitt & Anderson 2019). Additionally, microbes, such as commensal and other bacterial strains, may be utilized e.g. in the degradation of gluten peptides by microbial enzymes, fortifying the intestinal barrier and downregulating the gluten-induced immune responses (Caminero et al. 2019). Probiotics have been shown to decrease the levels of inflammatory markers in serum and stool samples and to have a balancing effect on the intestinal microbiota (Olivares et al. 2014; Primec et al. 2019), but more studies on their efficacy are needed. Promisingly, a recent randomized, double-blind, placebo-controlled trial found a selective oral TG2 inhibitor ZED1227 to significantly impede the development of villous atrophy and diminish symptoms during gluten challenge (Schuppan et al. 2021). However, larger cohorts and a longer surveillance are needed to confirm the efficacy and safety of

these (Schuppan et al. 2021). All in all, despite progress in pharmaceutical agents, gluten-free diet remains at present the treatment of choice in coeliac disease.

2.9 Nonresponsive and refractory coeliac disease

Although most coeliac disease patients show clinical and histological improvement after initiating gluten-free diet, some continue to have persistent symptoms and mucosal inflammation (Stasi et al. 2016); i.e. NRCD. NRCD has been defined as persistence or re-emergence of clinical symptoms and/or histological damage despite dietary treatment (Abdulkarim et al. 2002; Leffler et al. 2007; Stasi et al. 2016). However, the current criteria are not uniform, as the required minimum duration of symptoms and/or mucosal damage varies from six (Leffler et al. 2007) to 12 months (Abdulkarim et al. 2002). Moreover, while some authors regard continuing symptoms as NRCD (Dewar et al. 2012), others state that persistent mucosal damage is also required (Kaukinen et al. 2007b). Here it must be acknowledged that complete histological recovery may take several years despite strict dietary adherence (Kaukinen et al. 2007b; Pekki et al. 2015) and distinguishing between NRCD and slow response can be challenging. Depending on the definition and settings, NRCD has been estimated to affect approximately 10-20% of patients (Leffler et al. 2007; Stasi et al. 2016). To rule out non-coeliac reasons for NRCD, it is essential to confirm the diagnosis by re-evaluating the original serology and histology and response to gluten-free diet and check the possibility of a coexisting associated condition and, if needed, perform additional studies such as HLA determination (Abdulkarim et al. 2002).

The most common reason for NRCD is continuing, intentional or inadvertent, gluten exposure, other frequent causes being irritable bowel syndrome, bacterial overgrowth, lactose intolerance and other inflammatory conditions (Table 5). However, a fraction of patients develops a true RCD, with no other apparent causes for the continuing disease activity. As with NRCD, the exact definition of RCD is challenging, but it has been described as "continuing mucosal villous atrophy and malabsorption despite a minimum of 12 months of gluten-free diet" (Hujoel & Murray 2020). Before setting the diagnosis, other causes for NRCD should again be appropriately excluded and strict adherence to gluten-free diet confirmed by a qualified dietitian (Hujoel & Murray 2020; Penny et al. 2020). Repeated biopsies are recommended in the diagnostic workflow (Penny et al. 2020). Of note, RCD is nearly

nonexistent in children, and possible coexisting conditions should be meticulously sought in this age group (Rowinski & Christensen 2016).

The reported prevalence of RCD has ranged from 0.31% to 4% of coeliac patients (Ilus et al. 2014a; Roshan et al. 2011; Rowinski & Christensen 2016). Ilus et al. (2014a) discovered a low prevalence of 0.31% in Finland, where the overall diagnostic rate of coeliac disease is high. When compared to patients with uncomplicated disease, the patients who later developed RCD were older, more often seronegative and had more severe symptoms at diagnosis (Ilus et al. 2014a). Of note, even patients with a good clinical response but persistent villous atrophy may be at elevated risk for severe complications (Kaukinen et al. 2007b). As regards the clinical presentation, by definition patients present with some stage of malnutrition (Hujoel & Murray 2020), other commonly reported symptoms being diarrhoea, abdominal pain, bloating and weight loss (Malamut et al. 2009). Hypoalbuminemia and anaemia are also frequently seen (Malamut et al. 2009), and even gastrointestinal bleeding, fever, night sweats and bowel obstruction may occur (Al-Toma et al. 2007).

RCD is further divided into two types. Type I is the milder form, which is histologically often indistinguishable from slow-responding coeliac disease, although the proportion of CD4+ T cells may be moderately increased (Malamut et al. 2009). Intestinal ulcerations are small or nonexistent (Malamut et al. 2009). The clinical course of RCD type II is more severe and patients often have large ulcers and severe malnutrition (Malamut et al. 2009; Wierdsma et al. 2016). Type II is characterized by a clonal expansion of T cells with an abnormal immunophenotype (Malamut et al. 2009). These aberrant cells constitute at least 20% of IELs in flow cytometry in type II RCD (Verbeek et al. 2006). It has been shown that somatic JAK1 and STAT3 gain-of-function mutations have a central role in the transition to type II RCD as they make T cells hyperresponsive to IL-15 and other cytokines contributing to their proliferation (Ettersperger et al. 2016). The aberrant T cells have a cytotoxic capability (Ettersperger et al. 2016) which leads to severe villous atrophy and often to ulcerative disease (Malamut et al. 2009). The aberrant T cells are also precursors for lymphoma cells (Cellier et al. 2000) and type II RCD patients have thus a particularly high risk of developing enteropathy-associated T cell lymphoma (EATL). The prevalence of EATL has been reported to be approximately 30-50% five years after diagnosis in type II RCD, as opposed to only 14% in type I (Al-Toma et al. 2007; Malamut et al. 2009).

While the overall factors leading to RCD remain largely speculative (Malamut et al. 2009; Perfetti et al. 2016), some genetic and environmental associations have been observed. Homozygosity to DQ2 and single nucleotide polymorphisms (SNP) rs7259292 in MYO9B gene and rs2041570 on chromosome 7 are associated with type II RCD (Hrdlickova et al. 2018; Wolters et al. 2007). Somatic JAK1 and STAT3 mutations and trisomy of 1q22-q44 are also recurrent findings in clonal IELs (Verkarre et al. 2003).

As there is no curative treatment for RCD, the aim is to remedy the malnutrition and prevent the development of EATL (Hujoel & Murray 2020). In addition, for example, immunosuppressants may be used, open-capsule budesonide being considered a first-line treatment (Hujoel & Murray 2020). Increased knowledge of the pathogenesis has enabled studies of specific treatment options such as antibodies targeting IL-15 and NK receptors (Cheminant et al. 2019; Vicari et al. 2017), but the benefits of these remain to be confirmed. Type II RCD has a significantly poorer prognosis than type I (Malamut et al. 2009). While in type I RCD the five-year survival rate has been reported to be 93-96%, in type II it is between 44 and 58% and only 8% among those with EATL (Malamut et al. 2009; Al-Toma et al. 2007).

Table 5. Reported causes of nonresponsive coeliac disease.

Cause	Frequency, %	References
Ongoing gluten exposure	23-40	Abdulkarim et al. 2002 Dewar et al. 2012; Leffler et al. 2007; Stasi et al. 2016
Irritable bowel disease	7-22	Abdulkarim et al. 2002; Leffler et al. 2007; Stasi et al. 2016
Slow response to gluten-free diet	20	Stasi et al. 2016
Refractory coeliac disease	2-16	Abdulkarim et al. 2002; Leffler et al. 2007; Stasi et al. 2016
Intestinal bacterial overgrowth	6 -13	Abdulkarim et al. 2002; Dewar et al. 2012; Leffler et al. 2007; Rubio-Tapia et al. 2009
Incorrect diagnosis of coeliac disease	5-11	Abdulkarim et al. 2002; Dewar et al. 2012; Stasi et al. 2016
Pancreatic insufficiency	2-11	Abdulkarim et al. 2002; Dewar et al. 2012
Colitis (microscopic, lymphocytic, ulcerative, collagenous)	5-10	Abdulkarim et al. 2002; Dewar et al. 2012; Leffler et al. 2007
Disaccharidase deficiency	2-8	Abdulkarim et al. 2002; Leffler et al. 2007; Stasi et al. 2016
Gastrointestinal reflux disease	4	Stasi et al. 2016
Eating disorder	4	Leffler et al. 2007
T cell receptor gene rearrangement, lymphoma	2	Abdulkarim et al. 2002
Gastrointestinal cancer	1-2	Abdulkarim et al. 2002; Dewar et al. 2012; Leffler et al. 2007
Diverticular disease	2	Dewar et al. 2012
Tropical sprue	2	Abdulkarim et al. 2002
Medication induced diarrhoea	2	Dewar et al. 2012
Crohn's disease	1	Leffler et al. 2007
Peptic ulcer disease	1	Leffler et al. 2007
Food allergy	1	Leffler et al. 2007
Immunodeficiency	1	Leffler et al. 2007

2.10 Follow-up of coeliac disease

Surveillance in coeliac disease aims to help maintain a strict gluten-free diet, improve health-related quality of life, confirm clinical and histological recovery and prevent long-term complications (Al-Toma et al. 2019; Husby et al. 2020). It may entail clinical follow-up, dietary consultations, laboratory surveillance and endoscopy. There is limited data on the optimal implementation of follow-up (Ludvigsson et al. 2014) and the guidelines vary between countries (Lundin et al. 2021). Studies on the impact of long-term surveillance on dietary compliance are also scarce. There is some evidence of reduced adherence in non-followed children (Barnea et al. 2014), whereas studies on adults have reported contradictory results (Hall et al. 2009; Pekki et al. 2018). A significant portion of both paediatric and adult patients are lost to follow-up soon after diagnosis (Hall et al. 2009; Kivelä et al. 2018; Pekki et al. 2018).

Despite varying recommendations, clinical, dietary and serological surveillance are usually recommended (Al-Toma et al. 2019; Bai et al. 2017; Ludvigsson et al. 2014; Rubio-Tapia et al. 2013). Individual guidance by a dietitian after diagnosis has been reported to be effective in improving dietary compliance (Rajpoot et al. 2015). Resolution of possible nutritional deficiencies should be verified by blood tests and many guidelines also recommend screening for associated autoimmune diseases (Al-Toma et al. 2019; Bai et al. 2017; Ludvigsson et al. 2014; Rubio-Tapia et al. 2013). In children, normal growth and development should be ensured (Al-Toma et al. 2019; Bai et al. 2017; Rubio-Tapia et al. 2013). Social support, provided, for example, by an advocacy group, may have a positive influence on the motivation to maintain gluten-free diet (Hall et al. 2009).

Serology is used in clinical practice as a supporting tool to assess dietary adherence. However, assays for TG2-abs are not sufficiently accurate for evaluating mucosal recovery (Leonard et al. 2017), and normalized antibody levels do not exclude gluten exposure (Mehta et al. 2018). Antibody measurements may thus be useful mainly by indicating lack of compliance rather than verifying a strict diet. However, there is also evidence that repeatedly positive TG2-ab levels may in some cases be misleading, particularly if the diet has lasted only a few years and a sensitive assay is used. For example, a recent study reported no significant difference in clinical or histological presentation between treated coeliac patients with repeatedly positive or fluctuating TG2 titers and patients with normalized TG2-abs (Farina et al. 2021). DGP-abs have shown a better correlation with mucosal healing and dietary

compliance in some studies (Agardh 2007; De Chaisemartin et al. 2015), but more evidence is needed.

Assays measuring enzymatic degradation-resistant gluten immunogenic peptides (GIP) from urine and stool samples have been proposed as potential new tools for monitoring dietary adherence in coeliac disease. Although sensitive in detecting ingested gluten (Costa et al. 2019; Porcelli et al. 2020), considerable individual variation has been observed during gluten challenges (Coto et al. 2021; Silvester et al. 2020). A recent randomized trial showed a low accuracy of urinary GIP in assessing ongoing gluten consumption (Monachesi et al. 2021).

Histological follow-up continues to be the most reliable approach to assess mucosal recovery, if performed correctly (Chapter 2.5.1). However, it is an invasive and expensive method for frequent surveillance. Particularly in children, other methods are generally considered sufficient due to the usually excellent dietary response and low malignancy risk (Koletzko et al. 2017). According to recent international and Finnish guidelines, follow-up endoscopy is not essential even for adults with small risk for complications (Al-Toma et al. 2019; Bai et al. 2017; Ludvigsson et al. 2014; Rubio-Tapia et al. 2013). Some guidelines recommend repeat biopsy after one year for patients with continuing symptoms and abnormal laboratory values (Bai et al. 2017; Ludvigsson et al. 2014). However, this approach has been questioned, since mucosal recovery may take longer, and the findings at this time point may not reflect the treatment compliance or the risk of complications (Pekki et al. 2017). Subsequently, a time point of two years after diagnosis has been suggested (Al-Toma et al. 2019; Rubio-Tapia et al. 2013). A more personalized approach considering the patient's age, disease severity and dietary response have been advocated (Pekki et al. 2015, 2017). In line with this, the Finnish current care guidelines recommend follow-up endoscopy mainly for elderly and seronegative patients and those with a severe clinical presentation or NRCD (Duodecim 2018). The surveillance of RCD requires repeat endoscopies and biopsies, combined with imaging in type II (Al-Toma et al. 2019; Hujoel & Murray 2020).

3 INTESTINAL MICROBIOTA

3.1 Development and composition

The gut microbiota has considerable effects on human health (Lynch & Pedersen 2016). The intestinal tract is colonized by more than 10^14 (commensal) bacteria, archaea, viruses and fungi that interact with the host by metabolic and immunomodulating functions (Lynch & Pedersen 2016; Turnbaugh et al. 2007). It is generally thought that the colonization of the intestine begins at birth, however, this assumption has been questioned by the observation of bacteria and bacterial components in the placenta and the amniotic fluid (Aagaard et al. 2014; DiGiulio et al. 2008).

In the first months of life, the microbiota is unstable and fluctuates rapidly (Yatsunenko et al. 2012). The early microbiota is predominated by *Actinobacteria* phyla, especially the *Bifidobacterium* genus, and *Proteobacteria* phyla (Rodríguez et al. 2015). During the first year of life, *Bifidobacteria* are the most abundant genus, however, the proportional representation diminishes with age (Yatsunenko et al. 2012). The microbiota gradually increases in diversity and matures towards an adult-like microbiota, staying relatively stable after the first years of life (Matamoros et al. 2013; Rodríguez et al. 2015; Yatsunenko et al. 2012). In adults, the microbiota is dominated by *Bacteroidetes* and *Firmicutes* phyla (Turnbaugh et al. 2007). Later in life, changes in immunology, digestion and nutrient absorption may lead to a decrease in microbiota diversity (Rinninella et al. 2019; Rodríguez et al. 2015).

Even though *Bacteroidetes* and *Firmicutes* account for > 90% of the microbial composition in adults (Turnbaugh et al. 2007), there is marked variation in their relative proportions and at species and strain levels (Human Microbiome Project Consortium 2012). It has been proposed that intestinal microbiota could be categorized into separate enterotypes characterized by different dominant bacteria genera and means of energy production (Costea et al. 2018). Although this view is controversial, recognizing different compositional patterns might help to understand the relationships between intestinal microbiota and human health (Costea et al. 2018).

In addition to interindividual variation, there is fluctuation in the microbiota composition and abundance between different parts of the gastrointestinal tract. For example, only acid-resistant species, mainly *Lactobacillus* and *Streptococcus* survive in the stomach as opposed to the high diversity in the distal part of the gut (Aron-Wisnewsky et al. 2012; Dicksved et al. 2009). Owing to an increasing pH and transit time along the gastrointestinal tract, the amount of bacteria shifts from <10^3 bacteria / ml in the stomach to as high as 10^12 / ml in the large intestine (Aron-Wisnewsky et al. 2012).

In addition to the prokaryotic bacterial communities, the intestine is also colonized by eukaryotic microbiota, mostly fungi (Nash et al. 2017). The Human Gut Microbiome Project found that the mycobiome was lower in diversity than the bacterial community, and there was high intra- and interindividual variability (Nash et al. 2017). However, many fungal species were present in a majority of samples, the most prevalent being *Saccharomyces, Malassezia,* and *Candida* (Nash et al. 2017). One of the most commonly detected fungi was *Saccharomyces cerevisiae* (Chapter 4.1).

Gut microbiota has several resistance mechanisms to maintain stability. For example, synthesizing crucial metabolites for other bacteria, reducing energy intake and excretion of agents that are toxic for pathogens are means of responding to exogenous and endogenous disturbance (Sommer et al. 2017). High diversity is a significant factor in maintaining a stable state, since in conditions with limited resources, multiple resilient species can restrict the invasion of pathogenic species (Lozupone et al. 2012). However, severe perturbations, such as drugs, considerable dietary changes, oxidative stress and bacterial toxins may destabilize the microbial community (Weiss & Hennet 2017).

One of the greatest advances in microbiome studies has been the development of culture-independent approaches to analyse the human microbiota. Studying DNA and RNA from biological samples has also enabled identification and quantification of non-cultivable microbes. One of the first methods introduced was the sequencing of the prokaryotic 16S ribosome RNA gene, which enabled investigation of the composition of intestinal microbiota, whereas recent RNA-based technologies enable transcriptome analyses also reflecting the microbial function (Gao et al. 2021). Various computational techniques have been developed to analyse the high-throughput sequencing data produced (Knight et al. 2018).

3.2 Factors shaping the intestinal microbiota

3.2.1 Host genetics

Studies of twins indicate that the host genome is also involved in the modulation of the gut microbiota. Monozygotic twins with an identical genotype have more similarities in the microbiota than do dizygotic twins (Goodrich et al. 2014). Interestingly, the contribution of the host genotype to the microbiota composition seems to vary between different phyla, as genetics strongly influence the makeup of *Firmicutes* communities *Christensenellaceae, Ruminococcaceae* and *Lachnospiraceae*, whereas environmental factors, such as diet, are more determinative on the *Bacteroidete*s composition (David et al. 2014; Goodrich et al. 2014).

Different approaches have detected polymorphisms and loci associated with the interindividual variations in the intestinal microbiota, the majority of them being involved in immune pathways (Khachatryan et al. 2008). For instance, polymorphisms in HLA complex encoding the MHC molecules have been shown to influence the microbiota composition in animal models (Khan et al. 2019). In humans, the HLA DQ2/8 haplotypes predisposing to coeliac disease have been associated with reduced abundance of *Bifidobacteria* (De Palma et al. 2010b; Olivares et al. 2015). Even though the underlying mechanisms remain unelucidated, given that the principal function of MHC II molecules is T cell activation, it has been suggested that they influence microbiota populations by inducing adaptive immune reactions (Cenit et al. 2015). Outside HLA, genes known to be associated with IBD susceptibility, such as *NOD2* and *FUT2*, have been shown to be involved in the structuring of gut microbiota (Knights et al. 2014; Tong et al. 2014). Although certain variants may have a considerable effect on microbiota composition (Khachatryan et al. 2008), it is likely that numerous loci have a slight effect on the variability of intestinal bacteria (Cenit et al. 2015).

Studies on epigenetics have shed new light on the interaction between host and intestinal microbiota. The host immune cells respond to environmental triggers by modulating their transcription activity without altering the underlying DNA sequence, this mechanism enabling dynamic communication between the microbiota and host cells (Woo & Alenghat 2017). Although the detailed mechanisms of the epigenetic regulation remain to be elucidated, it has been reported that short chain fatty acids (SCFAs) produced by intestinal bacteria (Chapter 3.3) promote anti-

inflammatory conditions (Arpaia et al. 2013). Interestingly, it has been suggested that dysbiosis and loss of self-tolerance may be associated with disturbance of epigenetic regulation (Woo & Alenghat 2017), and a more profound understanding of these mechanisms might provide new insights on the pathogenesis of inflammatory intestinal diseases.

3.2.2 Environmental modifiers

From birth, the intestinal microbiota responds to the environment. The mode of delivery has a major influence on the bacterial phyla translocated from the mother. Babies born by cesarean section have less *Bifidobacteria* and lower microbial diversity than vaginally delivered babies (Arboleya et al. 2012). However, these differences may be milder later in childhood (Salminen et al. 2004). The microbiota of babies born pre-term often has lower diversity and higher proportions of pathogenic bacteria than in their full-term counterparts (Arboleya et al. 2012; Hiltunen et al. 2022). The mode of feeding also has a significant influence on the infant's microbiota. Breastmilk favours the colonization of beneficial genera such as *Bifidobacteria* (Martín et al. 2012).

Besides infancy, diet modulates the microbiota throughout life. For instance, a marked difference has been observed between individuals on a Western-type diet rich in protein and lipids and a more vegetable-based diet (Yatsunenko et al. 2012). Western diet is also associated with lower bacterial diversity (Yatsunenko et al. 2012). Obese individuals have been shown to have decreased microbial diversity, lowered relative proportion of *Bacteroidetes* and higher levels of SCFAs in faecal samples (Riva et al. 2017). Besides diet, other extrinsic factors such as lifestyle, geographic location, and the amount of exercise may shape the gut microbial community (Figure 2). Further, overall state of health and treatments may affect the microbial diversity and relative bacterial proportions. Antibiotic treatments favour certain bacteria to the detriment of others and often lead to a decrease in bacterial diversity and abundance (Pérez-Cobas et al. 2013).

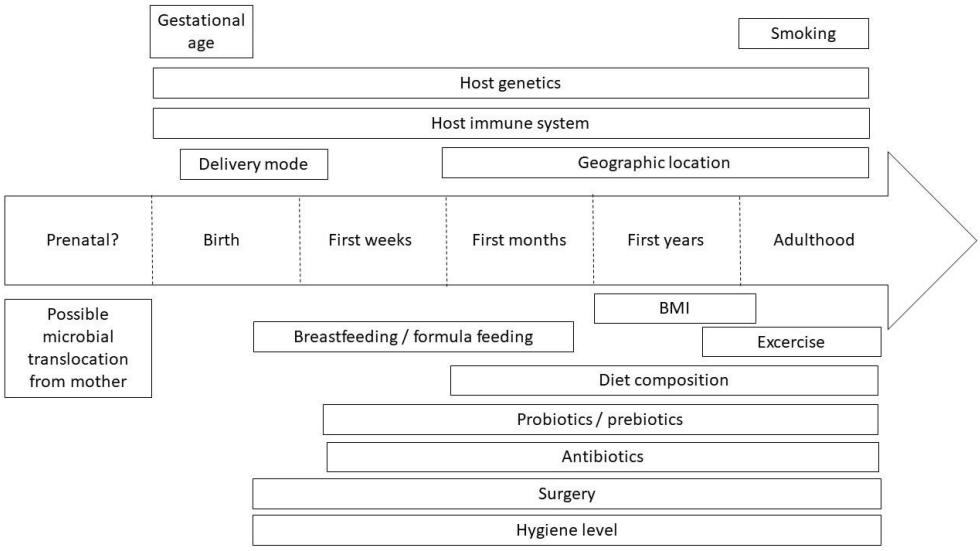

Figure 2. Factors that influence the composition of intestinal microbiota throughout the life course (adapted from Matamoros et al. 2013, Rinninella et al. 2019 and Rodríguez et al. 2015). BMI, body mass index

3.3 Host-microbiota interactions

It is believed that communication between the host and the microbiota is crucial for normal intestinal function. Commensal bacteria participate in human metabolism by mediating the digestion, synthesis and extraction of many nutrients and metabolites (Rinninella et al. 2019). Further, commensal bacteria delimit the invasion of pathogens by several mechanisms, such as consuming the surrounding nutrients, pH modification and toxin extraction (Rinninella et al. 2019).

Intestinal bacteria produce SCFAs by anaerobic fermentation of carbohydrates and fibre. SCFAs are not only an energy source for bacteria and IECs but also important signal molecules that have many anti-inflammatory effects that promote immune homeostasis (Arpaia et al. 2013). SCFAs contribute to tissue repair by driving cellular proliferation, inducing the differentiation of regulatory T cells, stimulating mucus secretion from goblet cells and regulating tight junction permeability (Rooks & Garrett 2016, Figure 3).

Animal studies have shown that interaction with commensal microbes is mandatory for proper maturation and function of the immune system (Sommer & Bäckhed 2013). In addition to directly restricting the growth of pathogens, the microbiota enhances the immune system's capability to delimit pathogen invasion. For instance, the microbiota induces epithelial cells to produce antimicrobial peptides and strengthens the intestinal barrier (Kamada et al. 2013, Figure 3). Additionally, immune cells express pattern-recognizing receptors (PRRs), such as toll-like and NOD-like receptors, which are activated by microbial components and are thus important signal molecules in the host-microbiota crosstalk (Bach 2018; Kamada et al. 2013, Figure 3). PRRs have an essential role in the induction of inflammatory response but also in the development of tolerance towards microbial antigens (Bach 2018, Kamada et al. 2013).

The resident microbiota influences differentiation and function of both innate and adaptive immunity cells. For instance, certain bacteria drive the differentiation of T cells to anti-inflammatory Treg cells and others to proinflammatory T cell subgroups (Atarashi et al. 2011; Ivanov et al. 2009). Innate lymphoid cells also participate in maintaining homeostasis in the intestinal mucosa by secreting a wide range of cytokines in response to microbial stimuli and also participate actively in protection against acute infections (Seo et al. 2020). Commensal microbiota regulates IgA production from B cells and plasma cells and, in turn, secreted IgA molecules bind to bacterial surfaces preventing them from entering the lamina propria and thus enhancing the epithelial barrier function (Lathrop et al. 2011, Figure 3). Thereby, IgA deficiency, often coexisting with autoimmune diseases (Singh et al. 2014), influences the microbial composition and function (Fadlallah et al. 2018).

Studies on mice indicate that the intestinal immune system also influences the composition and function of the intestinal microbiota (Qiu et al. 2013; Zenewicz et al. 2013). The microbiota also has an ability to adapt to changing inflammatory conditions. In a recent study, intestinal microbes reacted to activation of the immune system seen as alterations in the transcriptomic and metabolomic profiles, such as a decrease in SCFA concentration and increased production of immunomodulatory peptides (Becattini et al. 2021). These adaptations occurred within hours before detectable alterations in the microbiota composition and were speculated to affect the human immune response (Becattini et al. 2021).

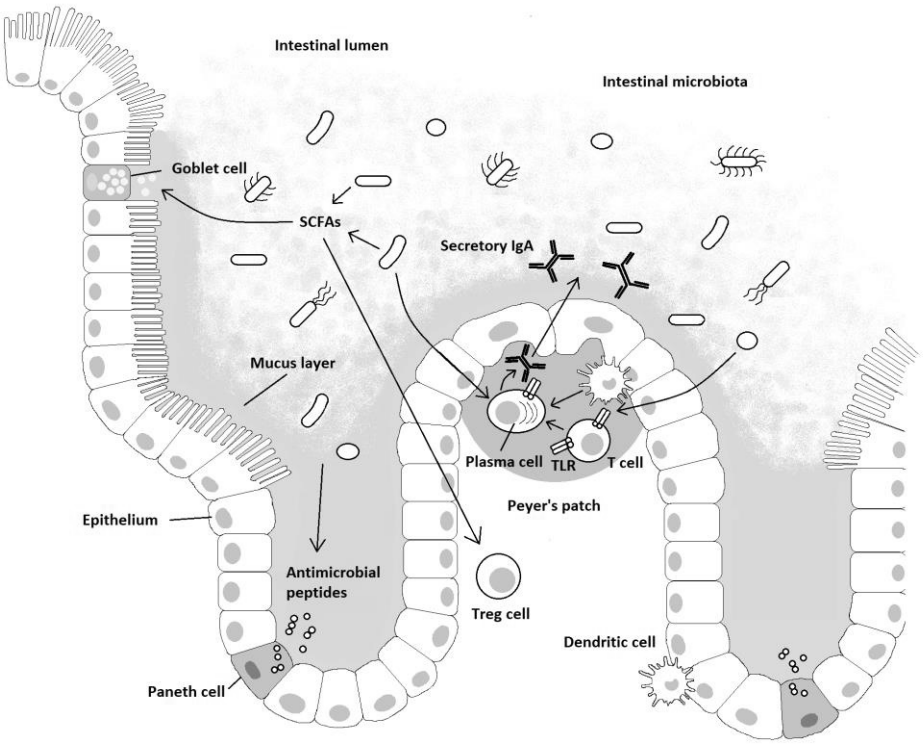

Figure 3. Crosstalk between the intestinal microbiota and the host immune system (adapted from Caruso et al. 2020). SCFAs; short-chain fatty acids; TLR, toll-like receptor

3.4 Microbiota and autoimmune diseases

The immune system has multiple mechanism to maintain tolerance towards self-antigens. Breakdown of one or more of these mechanisms may lead to loss of self-tolerance and eventually to an autoimmune reaction. The manifestations of autoreactivity range from subclinical findings, such as circulating autoantibodies, to a severe autoimmune disease with immune-mediated tissue destruction (Theofilopoulos et al. 2017). The development of autoimmunity involves both innate and adaptive immune pathways and is characterized by activation of helper T cells that induce generation of autoantibodies or cytotoxic T cells (Theofilopoulos et al. 2017).

Most autoimmune diseases are considered to have multifactorial background with a contribution of both genetic and environmental factors (Selmi et al. 2012).

Hundreds of loci have been found to be associated, certain MHC haplotypes having the strongest correlation (Gutierrez-Arcelus et al. 2016). In addition, many environmental factors, such as dietary components and chemicals, have been associated with autoimmune diseases. Infectious agents including viruses, bacteria, fungi and parasites can induce or trigger autoimmune responses by a variety of mechanisms. After infection, molecular similarity between the infectious agent and host epitopes, termed antigen mimicry, may maintain the inflammatory conditions by stimulating the microbe-targeted T cells (Rojas et al. 2018). This cross-reactivity may also be caused by epitope spreading, meaning modification of host proteins to structurally resemble microbial antigens (Gershwin 2008). Stimulation of PRRs may also initiate autoimmunity (Duke 1989). Activated APCs release proinflammatory cytokines that cause tissue destruction, which in turn exposes hidden autoantigens that activate autoreactive lymphocytes (Kivity et al. 2009). T cells recognize and kill virally infected cells but also give rise to a proinflammatory milieu also leading to apoptosis of uninfected cells (Vojdani 2014). In addition, a viral infection may induce autoreactivity via polyclonal activation and proliferation of B cells seen as both monoclonal and polyclonal autoantibodies (Agmon-Levin et al. 2009).

On the other hand, infections may also have a protective role. The 'hygiene hypothesis' suggests that frequencies of infections and autoimmune diseases are negatively correlated (Bach 2002). Accordingly, epidemiological studies have shown significant differences in the incidences of allergic and autoimmune diseases between industrialized and developing countries. Studies in particular on adjacent populations with similar genetic background and living environments but with different socioeconomic conditions support the suppressive effect of infections on autoimmunity (Kondrashova et al. 2005, 2008; Laatikainen et al. 2011). In addition, mouse models have yielded evidence on the inhibitory effect of infections on the development of autoreactivity (Greenwood et al. 1970; Wilberz et al. 1991).

There is evidence that intestinal microbiota also modulates autoreactivity. Mouse models suggest that early microbial colonization in particular strongly influences the immune development and the proportions of different immune cell populations (An et al. 2014; Olszak et al. 2012), thus imbalances in the early microbiota composition may lead to disturbances in immunoregulation. Vatanen et al. (2016) followed-up Finnish, Estonian and Russian children with similar HLA risk class until three years of age and found significant functional and structural differences in the early gut microbiota. They suggested that this finding might reflect the differences in the

prevalence of autoimmune diseases between the studied populations (Vatanen et al. 2016).

Dysbiosis describes a state of the microbiota that leads to an imbalance with the host immune system. Alterations in the proportion of anti-inflammatory and proinflammatory bacteria may contribute to the onset or continuation of certain diseases (Levy et al. 2017). Reduced diversity is often one marker of microbial imbalance (Levy et al. 2017). Dysbiosis has been associated with many diseases such as IBD, type 1 diabetes, multiple sclerosis and coeliac disease (Kostic et al. 2015; Miyake et al. 2015; Walker et al. 2011; Chapter 3.5). In IBD, an enrichment of facultative anaerobic bacteria and decreased counts of SCFAs-producing obligate anaerobes are often observed (Lloyd-Price et al. 2019). A depletion of *Bifidobacteria* has also been reported (Joossens et al. 2011), although this remains debatable (Takahashi et al. 2016). Despite certain characteristic findings, there is wide interindividual variation in the alterations of gut microbiota in IBD patients (Schirmer et al. 2019). Therefore, identifying a causal association between specific microbes and IBD remains a future challenge.

Another phenomenon related to dysbiosis is small intestinal bacterial overgrowth (SIBO), defined as an increased total count of bacteria and a predominance of colonic-type, Gram-negative bacteria in the small intestine (Bures et al. 2010; Pimentel et al. 2020). A recent study discovered that SIBO is associated with a relative abundance of specific microbial taxa, such as *Klebsiella, Escherichia, Enterococcus, and Clostridium* at the expense of strict anaerobes usually common in the duodenum (Barlow et al. 2021). SIBO may be associated with a wide range of digestive and extraintestinal diseases (Bures et al. 2010). Typical findings and symptoms are diarrhoea, abdominal pain and bloating, and nutritional deficiencies (Bures et al. 2010). The causality is still somewhat debatable, however, SIBO seems to be significantly more frequent in IBD compared with healthy and non-IBD controls, as well as being associated with a complicated disease behaviour in IBD (Shah et al. 2019).

Although microbial imbalance has been associated with many immune-mediated diseases, the order of appearance of the microbial and immunological changes remains largely unclear. A longitudinal study in patients who developed type 1 diabetes during surveillance reported that anti-islet β-cell autoantibodies appeared before the microbial changes (Kostic et al. 2015). However, reduced microbiota diversity was present before the development of overt diabetes, suggesting that loss of the balanced microbiota may still contribute to the pathogenesis (Kostic et al.

2015). Altogether, causality between dysbiosis and autoimmunity remains to be elucidated.

3.5 Intestinal microbiota and coeliac disease

Several cross-sectional studies on duodenal and faecal microbiota in coeliac disease have been performed, most of them showing differences between patients and controls. The most common observations are an increase in *Bacteroides* and *Proteobacteria* and a reduction in *Lactobacilli* and *Bifidobacteria* genera (Collado et al. 2009; De Palma et al. 2010a; Nadal et al. 2007; Sánchez et al. 2013). Some studies have found the ratio of beneficial bacteria, such as *Lactobacilli* and *Bifidobacteria* and potentially harmful bacteria, e.g., certain *Bacteroides* strains, *Escherichia coli* and *Enterobacteriaceae*, to be lower in coeliac patients than in controls (Nadal et al. 2007; Di Cagno et al. 2009). However, there has been wide variation in the findings, at least partly due to small study groups and different methodologies, and not every study has found significant differences between coeliac patients and controls (Kalliomäki et al. 2012; De Meij et al. 2013). SIBO could be overrepresented in coeliac disease, although this remains to be proven (Losurdo et al. 2017).

Microbiota may affect the presentation of coeliac disease. Wacklin and colleagues reported coeliac patients with gastrointestinal symptoms or anaemia to have lower duodenal microbiota diversity than those with DH, and the two groups also had differing bacterial compositions (Wacklin et al. 2013). Moreover, in their subsequent study, persistent gastrointestinal symptoms were associated with dysbiotic microbiota in patients on a long-term gluten-free diet (Wacklin et al. 2014). Interestingly, SIBO tends to be more common in symptomatic than asymptomatic coeliac patients and hence the condition should be considered in the diagnostics of NRCD (Losurdo et al. 2017, Chapter 2.9).

Since diet has a considerable effect on the composition of intestinal microbiota, gluten-free diet also causes certain alterations. For example, healthy individuals had decreased counts of *Bifidobacteria* and *Lactobacillae* and increased amounts of *E. coli* and *Enterobacteriaceae* in faecal samples after one month on a gluten-free diet (De Palma et al. 2009). Gluten-free diet may also diminish bacterial diversity in coeliac patients (Nistal et al. 2012). Therefore, distinguishing between the changes in the microbiome caused by the diet and healing of the intestinal mucosa is challenging. Furthermore, in studies of duodenal samples, controls and coeliac patients on a

gluten-free diet often have gastrointestinal symptoms as an indication for endoscopy, which also adds bias to the comparisons. However, a recent study by Zafeiropoulou and colleagues aimed to identify independently coeliac disease-associated changes in faecal microbiota (Zafeiropoulou et al. 2020). They found 11 taxa associated with coeliac disease irrespective of treatment status while certain microbial alterations vanished on a gluten-free diet (Zafeiropoulou et al. 2020).

Interestingly, there is evidence that the coeliac disease-associated HLA DQ haplotypes influence the early microbial colonization. For example, studies have shown decreased amounts of *Actinobacteria* and increased counts of *Firmicutes* and *Proteobacteria* in the stool samples of infants at genetic risk of coeliac disease compared to HLA DQ2/8 negative controls (Olivares et al. 2015; Sellitto et al. 2012). At genus level, increased quantities of *Bacteroides* and *Enterococcus*, an increased *Bacteroides-Prevotella* ratio and alterations in the *Bacteroides* genus have been observed (De Palma et al. 2010; Leonard et al. 2020; Sánchez et al. 2011). In the future, prospective multi-omics approaches with large study cohorts will hopefully advance our understanding of the role of intestinal microbiota in coeliac disease.

4 MICROBIAL ANTIBODIES

4.1 Anti-*Saccharomyces cerevisiae* antibodies

Saccharomyces cerevisiae, more commonly known as baker's or brewer's yeast, is widely utilized in baking and fermentation processes and is thus a permanent ingredient in a grain-containing diet (Sicard & Legras 2011). This yeast strain may trigger immune responses in certain individuals manifested as circulating anti- *Saccharomyces cerevisiae* antibodies (ASCA). These antibodies are targeted against the carbohydrate epitopes of the phosphopeptidomannan of the *Saccharomyces cerevisiae* cell wall. Interestingly, similar oligomannose structures in other yeast species, such as the opportunistic pathogen *Candida Albicans*, are able to mimic *Saccharomyces Cerevisiae* epitopes and induce the production of ASCA (McKenzie et al. 1992; Standaert-Vitse et al. 2006).

ASCA were first detected in Crohn's disease patients (Main et al. 1988; McKenzie et al. 1990). Seropositivity to ASCA was observed in approximately 60% of patients and was less frequent in those with ulcerative colitis (UC) (Main et al. 1988; Quinton et al. 1998). The corresponding figures among healthy controls have been 0-16% (Bernstein et al. 2011; Prideaux et al. 2012). ASCA positivity was later found to be associated with earlier age of onset, complicating disease behaviour and increased risk for needing surgery in Crohn's disease (Arnott et al. 2004; Mow et al. 2004; Papp et al. 2008). At present, ASCA are the most accurate microbial marker in distinguishing IBD from non-IBD and also in distinguishing Crohn's disease from UC (Prideaux et al. 2012), and several commercial assays are available (Papp et al. 2008; Vermeire et al. 2001a). More recently studied glycan ASCA targeted against covalently immobilized mannan have proven comparable with the traditional ASCA (Papp et al. 2008).

Even though ASCA were first thought to be a specific marker for Crohn's disease (McKenzie et al. 1990), elevated levels have later been detected in other autoimmune diseases (Tables 6 and 7). ASCA were first studied in coeliac disease patients by Giaffer, who observed higher ASCA IgG levels than in UC patients and healthy controls (Giaffer et al. 1992). Other studies have also detected higher prevalence of ASCA positivity in coeliac disease than in UC and even similar antibody levels among

coeliac and Crohn's disease patients (Giaffer et al. 1992; Granito et al. 2005). Prevalence of ASCA positivity among coeliac patients has been summarized in Table 7. ASCA levels have also been shown to decrease significantly on a gluten-free diet (Ashorn et al. 2009b; Granito et al. 2005; Mallant-Hent et al. 2006a). ASCA positivity tends to be more common in adults than children (Table 7) and the antibody levels also tend to normalize more rapidly on diet in paediatric patients (Mallant-Hent et al. 2006a).

Studies have also shown healthy relatives of Crohn's disease patients to have increased frequency (17 - 35%) of ASCA positivity (Seibold et al. 2001; Vermeire et al. 2001b). Elevated ASCA levels have also been observed in the relatives of coeliac disease patients positive to TG2-abs and/or EmA, but not among seronegative relatives (da Silva Kotze et al. 2010).

Table 6. Prevalence of seropositivity to ASCA in immune-mediated diseases other than coeliac disease.

	ASCA IgA, %	ASCA IgG, %	ASCA IgA and IgG, %	ASCA IgA or IgG, %	Reference
Antiphospholipid syndrome	ND	ND	ND	20.0	Krause et al. 2007
Autoimmune hepatitis	11.9	16.4	10.4 – 15.2	17.9 – 27.5	Czaja et al. 2004; Muratori et al. 2003
Graves' disease	0.8 - 16.6	5.7 - 12.5	ND	ND	Mankaï et al. 2013b;
Hashimoto's thyroiditis	2.6 - 13.6	3.8 - 7.9	ND	ND	Shor et al. 2012; Yazıcı et al. 2010
Ankylosing spondylitis	19.2 – 20.6	7.7 – 10.9	4.6	26.9 - 30	Aydin et al. 2008; De Vries et al 2010
Behcet's disease	18.9	5.9	3.5	21.2	Fresko et al. 2005
Crohn's disease	10-46	10-52	6-28	10-69	*
Primary biliary cirrhosis	11.6 - 24.2	10.6 - 18.6	5.7 - 6.3	22.7 - 29.8	Hu et al. 2013; Muratori et al. 2003; Sakly et al. 2008
PSC	32.0	28.0	16.0	44.0	Muratori et al. 2003
Scleroderma	16.2	43.2	ND	ND	Fedrigo et al. 2019
Sjögren's syndrome	ND	ND	ND	4.8	Alunno et al. 2018
SLE	12.1	4.5 - 29.3	ND	31.9	Mankaï et al. 2013a; Shor et al. 2012
Rheumatoid arthritis	9.6 - 40	20 - 20.5	7.2	22.9	Melayah et al. 2022; Dai et al. 2009
Type 1 diabetes	9.8	21	6.2	24.5	Sakly et al. 2010
Ulcerative colitis	2-10	0-12	2-4	0-27	**
Vasculitides	ND	6.5	ND	ND	Shor et al. 2012

*Arnott et al. 2004; Ashorn et al. 2009a; Damoiseaux et al. 2002; Iltanen et al. 2006; Mallant-Hent et al. 2006b; Mow et al. 2004b; Ruemmele et al. 1998; Sendid et al. 1998 **Ashorn et al. 2009a; Damoiseaux et al. 2002; Iltanen et al. 2006; Mallant-Hent et al. 2006b; Ruemmele et al. 1998

ASCA, Anti–*Saccharomyces cerevisiae* antibodies; ND, no data; PSC, primary sclerosing cholangitis; SLE, systemic lupus erythematosus

Table 7. Prevalence of seropositivity to ASCA in children and adults with treated and untreated coeliac disease.

Patients (n)	ASCA IgA, %	ASCA IgG, %	ASCA IgA or IgG, %	ASCA IgA and IgG, %	Reference
Untreated coeliac disease					
Children					
14	21.4	14.3	28.6	ND	Damoiseaux et al. 2002
83	ND	ND	18.1	ND	Mallant-Hent et al. 2006a
41	14.6	43.9	51.2	7.3	Granito et al. 2005
75	9.3	20.0	22.6	6.6	Toumi et al. 2007
48	ND	ND	25.0	ND	Ashorn et al. 2008
Adults					
23	34.8	43.5	52.2		Damoiseaux et al. 2002
28	ND	ND	60.7	ND	Mallant-Hent et al. 2006a
64	23.4	60.9	64.1	20.3	Granito et al. 2005
50	12.0	34.0	34.0	12.0	Toumi et al. 2007
86	ND	ND	58.1	ND	Ashorn et al. 2008
Treated coeliac disease					
Children					
83	ND	ND	1.2	ND	Mallant-Hent et al. 2006a
21	4.7	0.0	4.7	0	Toumi et al. 2007
Adults					
40	27.5	40	ND	ND	Candelli et al. 2003
28	ND	ND	28.6	ND	Mallant-Hent et al. 2006a
21	4.7	9.5	9.5	4.7	Toumi et al. 2007

ASCA, anti-*Saccharomyces cerevisiae* antibody; IgA, immunoglobulin A; IgG, immunoglobulin G; ND, no data

4.2 Antibodies against the I2 protein of *Pseudomonas fluorescens*

The bacterial DNA sequence I2 was first discovered by Sutton and colleagues in Crohn's disease patients (Sutton et al. 2000a). A nucleic acid homology analysis revealed the *I2* sequence to bear a close resemblance to *Pseudomonadaceae*, and eventually the sequence was shown to originate from the *pfiT* locus of *Pseudomonas fluorescens* (Wei et al. 2002). The *I2* sequence was observed in both healthy and affected ileal specimens, indicating that *Pseudomonas fluorescens* is a low-level commensal of the ileal mucosa that may expand its colonization to colonic mucosa in Crohn's disease (Sutton et al. 2000a). In murine assays, I2 caused CD4+ T cells to proliferate and secrete IL-10, and displayed features indicating that the protein acted as a T cell superantigen (Dalwadi et al. 2001).

Sutton et al. also studied possible seroreactivity to I2 by constructing a recombinant I2-GST fusion protein and measuring antibody binding with ELISA (Chapter 6.2.4). They found that the frequency of seropositivity was significantly increased and the mean absorbance elevated in patients with Crohn's disease compared with UC patients, non-IBD-patients and controls (Sutton et al. 2000a). The controls had 10-100 fold lower levels of IgG antibodies than Crohn's disease patients and no IgA binding. In subsequent studies ~50-60% of Crohn's disease patients have presented with seropositivity to IgA class anti-I2 antibodies (Table 8), while in controls this has varied 0-31% (Ashorn et al. 2008, 2009a; Bernstein et al. 2011; Iltanen et al. 2006; Sutton et al. 2000a). Anti-I2 seropositivity has also been associated with a more severe phenotype, longer disease duration and higher risk of surgery in Crohn's disease (Arnott et al. 2004; Mow et al. 2004b). Moreover, seropositive patients have more often achieved a clinical response to faecal diversion (Spivak et al. 2006) and antibiotic treatment (Mow et al. 2004a).

In addition to Crohn's disease, Ashorn et al. found a majority of untreated coeliac patients to be seropositive to anti-I2 antibodies and to have significantly higher antibody levels than non-coeliac controls (Ashorn et al. 2008, 2009a, Table 8). Furthermore, the frequency of seropositivity and antibody levels decreased significantly during gluten-free diet (Ashorn et al. 2009b). Petersen et al. (2020) also observed cross-reactivity between gluten epitopes and *Pseudomonas fluorescens* peptides, indicating a possible role in the pathogenesis.

Table 8. Frequency of seropositivity to IgA class anti-I2 antibodies in IBD and coeliac disease.

Patients (n)	Seropositivity to anti-I2 antibodies, %	Reference
Crohn's disease		
330	50	Landers et al. 2002
303	59.4	Mow et al. 2004b
142	52	Arnott et al. 2004
18	50	Iltanen et al. 2006
27	59	Spivak et al. 2006
38	60	Melmed et al. 2007
732	58.1	Devlin et al. 2007
18	44.4	Ashorn et al. 2009a
50	14	Bernstein et al. 2011
616	50.3	Murdoch et al. 2012
Ulcerative colitis		
12	42	Iltanen et al. 2006
32	34	Melmed et al. 2007
36	41.7	Ashorn et al. 2009a
50	4	Bernstein et al. 2011
Coeliac disease		
131	70.2	Ashorn et al. 2008

Anti-I2 antibody, antibodies against the I2 protein of *Pseudomonas fluorescens*; IgA, immunoglobulin A

4.3 Antibodies against the *Bacteroides caccae* TonB-linked outer membrane protein

The antibody targeted against *Bacteroides caccae* TonB-linked outer membrane protein (anti-OmpW antibody) was found in the search for bacterial antibodies cross-reactive with UC-related perinuclear anti-neutrophil cytoplasmic antibodies (pANCA, Chapter 4.4). There is evidence that several enteric bacterial antigens may induce pANCA (Seibold et al. 1998). Using monoclonal pANCA, Cohavy et al. identified a previously uncharacterized 100-kDa protein derived from *Bacteroides caccae* (Cohavy et al. 2000). *Bacteroides* are a genus of gram-negative, obligate anaerobic rod-shaped bacteria common in colonic microflora (Turnbaugh et al. 2007). Sequence analysis revealed a high level of homology between TonB-linked outer membrane proteins and the gene was therefore named *OmpW* (Wei et al. 2001).

TonB-linked outer membrane proteins are involved in the transduction of essential substances, such as iron, across the outer membrane of gram-negative bacteria (Noinaj et al. 2010). The *OmpW* sequence is specific for *Bacteroides* caccae and closely related to the outer membrane proteins RagA of *Porphyromonas gingivalis* and SusC of *Bacteroides thetaiotaomicron* (Wei et al. 2001).

Wei et al. detected increased frequency of seropositivity and elevated levels of IgA class anti-OmpW antibodies in Crohn's disease, whereas this was not seen with IgG class antibodies (Wei et al. 2001). Of note, neither the frequency of seropositivity nor the anti-OmpW antibody levels correlated with pANCA. One explanation for this discrepancy may be the use of an individual monoclonal antibody that may not recognize all the predominant pANCA epitopes. The results indicated that several different bacterial epitopes may induce the production of pANCA (Wei et al. 2001).

Seropositivity to anti-OmpW antibodies has been observed in up to 44-61% of Crohn's disease patients, in 61% of coeliac disease patients and in 33-42% of UC patients (Table 9). Additionally, Iltanen et al. (2006) found paediatric IBD patients to have elevated OmpW levels compared to non-IBD controls with gastrointestinal symptoms of other origin, although no significant differences were seen in their later study (Ashorn et al. 2009a). Untreated coeliac disease patients were also reported to present with higher levels of anti-OmpW antibodies than healthy controls (Ashorn et al. 2008) and to show significant decrease in serum levels during gluten-free diet (Ashorn et al. 2009b).

Table 9. Frequency of seropositivity to IgA class anti-OmpW antibodies in IBD and coeliac disease.

Patients (n)	Seropositivity to IgA class anti-OmpW	Reference
Crohn's disease		
18	61	Iltanen et al. 2006
18	44.4	Ashorn et al. 2009a
Ulcerative colitis		
12	42	Iltanen et al. 2006
36	33.3	Ashorn et al. 2009a
Coeliac disease		
131	61	Ashorn et al. 2008

Anti-OmpW antibodies, antibodies against the *Bacteroides caccae* TonB-linked outer membrane protein; IgA, immunoglobulin A

4.4 Other microbial antibodies and autoantibodies

Besides ASCA, anti-I2 and anti-OmpW, other microbial antibodies and autoantibodies have also been associated with immune-mediated diseases. Anti-neutrophil cytoplasmic antibodies (ANCA) are targeted against proteins in the cytoplasmic granules of polymorphonuclear neutrophil granulocytes (Weiner & Segelmark 2016). pANCA are a distinct subset that differs from ANCA detected in vasculitides (Saxon et al. 1990). pANCA are associated especially with UC (Kuna 2013), and pANCA-positive Crohn's disease patients usually have a UC-like phenotype (Mow et al. 2004b; Ruemmele et al. 1998). Damoiseaux et al. (2002) reported pANCA positivity also to be significantly more frequent in patients with coeliac disease (21.6%) than in healthy controls (2.9%).

In addition to anti-OmpW, anti-OmpC antibodies cross-reactive with pANCA have also been identified. Anti-OmpC antibodies are targeted against the outer membrane porin of *Escherichia coli* (Cohavy et al. 2000). Although IgG class anti-OmpC antibodies were first identified in UC (Cohavy et al. 2000), IgA class anti-OmpC antibodies were later shown to be more frequent in Crohn's disease (Landers et al. 2001). The frequency of seropositivity to OmpC is approximately 25-50% in Crohn's disease and 2-25% in UC (Kuna 2013; Mitsuyama et al. 2016). Of note, increased anti-OmpC reactivity has also been reported in healthy relatives of Crohn's disease patients (Mei et al. 2006).

Flagellins are common bacterial proteins that may cause both innate and adaptive immune reactions (Lodes et al. 2004; Winstanley & Morgan 1997). Lodes et al. (2004) discovered serum antibodies targeted against Cbir1 flagellin in spontaneously colitic mice, and observed serum anti-Cbir1 antibodies to be elevated in patients with Crohn's disease as opposed to UC patients and healthy controls. Anti-Cbir1 antibodies are present in 50-55% in Crohn's disease and in <10% in UC (Kuna 2013; Mitsuyama et al. 2016). Clinical data on the presence of anti-Cbir1 antibodies in coeliac disease are lacking.

In addition to ASCA, other IBD-associated anti-glycan antibodies have later been discovered (Dotan et al. 2006). Anti-laminaribioside (ALCA) and anti-mannobioside (AMCA) IgG antibodies and IgA class anti-chitobioside antibodies (ACCA) are mainly targeted against the cell wall components of microorganisms such as fungi and bacteria. The most recently discovered anti-glycan antibodies are IgA class anti-L and anti-C directed respectively against large polysaccharides laminarin and chitin (Seow et al. 2009). The rate of seropositivity to the aforementioned antibodies is approximately 10-30% in Crohn's disease and up to 10% in UC (Mitsuyama et al. 2016; Prideaux et al. 2012).

Due to low sensitivity, pANCA and microbial antibodies are not considered suitable for population screening nor for replacing endoscopy or other traditional tools in the diagnostic work-up for IBD (Prideaux et al. 2012). However, they may help in differentiating between phenotypes, as antibodies directed against OmpC, Cbir1 and glycan structures are more frequent in Crohn's disease and pANCA in UC (Kuna 2013; Mitsuyama et al. 2016). A combination of pANCA and Crohn's disease-associated ASCA has proven especially accurate in distinguishing Crohn's disease from ulcerative colitis (Ruemmele et al. 1998; Papp et al. 2008). The number of positive microbial antibodies and the magnitude of the antimicrobial response is positively correlated with disease severity in IBD (Prideaux et al. 2012).

There is very limited data on anti-OmpC, ASCA, ALCA, ACCA and AMCA in coeliac disease, but Papp et al. (2009) discovered increased frequency of seropositivity and higher antibody levels of AMCA and ACCA, but not OmpC and ALCA, in untreated coeliac patients compared to healthy controls. Additionally, the levels of each antibody decreased significantly during gluten-free diet. Furthermore, as in IBD, the presence and the magnitude of antimicrobial response was associated with more severe clinical picture (Papp et al. 2009).

THE PRESENT STUDY

5 AIMS

The main aim of the dissertation was to investigate seroreactivity to ASCA, anti-I2 and anti-OmpW antibodies at different stages of coeliac disease, in first-degree relatives of coeliac patients and in non-coeliac blood donors.

The specific aims were:

1. To investigate frequency of seropositivity and levels of ASCA, anti-I2 and anti-OmpW antibodies in early-stage coeliac disease before the development of small-bowel mucosal atrophy and to compare the results to those obtained at the time of coeliac disease diagnosis and on a gluten-free diet (**I**).

2. To compare seropositivity rate and levels of ASCA, anti-I2 and anti-OmpW antibodies of coeliac patients with persistent villous atrophy despite a strict gluten-free diet to coeliac patients with a beneficial response to gluten-free diet, and to non-coeliac blood donors (**II**).

3. To study serological responses to ASCA, anti-I2 and anti-OmpW antibodies in first-degree relatives of coeliac patients and to compare the results with those of coeliac patients at the time of diagnosis and on a gluten-free diet, and with non-coeliac blood donors (**III**).

6 MATERIALS AND METHODS

The studies were conducted at the Tampere Center for Child, Adolescent and Maternal Health Research and the Celiac Disease Research Center, Tampere University and Tampere University Hospital, and at the Departments of Paediatrics and Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital.

6.1 Study participants

6.1.1 Early-stage coeliac disease patients (I)

Study **I** comprised 44 adults and adolescents gathered from two separate subcohorts. The first group consisted of 16 patients with normal small-bowel mucosal architecture in their first oesophagogastroduodenoscopy conducted due to suspicion of coeliac disease and continued on a gluten-containing diet. During follow-up, they underwent another endoscopy in which they presented with villous atrophy and crypt hyperplasia (Marsh III) and were diagnosed with coeliac disease. Serum samples taken from them all at the beginning of follow-up and 14 subjects were also resampled at the time of diagnosis. The remaining 28 study participants were coeliac disease autoantibody-positive patients with normal mucosal architecture but an increased density of CD3+ and γδ intraepithelial lymphocytes as markers of mucosal inflammation. Experimental gluten-free diet was initiated and a beneficial clinical and serological response was considered to confirm the diagnosis of coeliac disease (Kurppa et al. 2010). Altogether 33 out of the 44 patients in Study I gave follow-up serum samples after being on a gluten-free diet for one year.

6.1.2 Nonresponsive coeliac disease patients (II)

Study **II** consisted of 20 biopsy-proven adult coeliac disease patients who had been on a strict gluten-free diet confirmed by a qualified dietitian for a median of 3.5 years but who continued to have persistent symptoms and mucosal atrophy (NRCD group). Dietary lapses and other reasons for nonresponsiveness were thoroughly ruled out by interviews, laboratory testing, abdominal imaging and colonoscopy. A repeat oesophagogastroduodenoscopy was carried out after one year for 15 patients and within 2-4 years for the remaining five patients. Two patients developed RCD with abnormal intraepithelial lymphocyte phenotype during the surveillance period.

6.1.3 Diet-responsive coeliac disease patients (II, III)

A cohort of 58 biopsy-proven adults with clinical, serological and histological response to gluten-free diet represented the responsive coeliac disease patients in Studies **II** and **III** (Ashorn et al. 2009b). Oesophagogastroduodenoscopy with duodenal biopsy, clinical assessment and serum sampling for serological analyses were carried out at diagnosis and after one year of dietary treatment. Sera for microbial antibody measurements were available from 55 patients on a gluten-free diet.

6.1.4 Relatives of coeliac disease patients (III)

For Study **III**, a large cohort of subjects with self-reported coeliac disease was first recruited in a nationwide search through patient societies and by utilizing newspaper advertisements (Kurppa et al. 2012). Their medical records were obtained with their permission and the presence of biopsy-proven coeliac disease was confirmed. Next, the relatives of these patients were invited to an interview and screening for TG2-abs and EmA. An additional blood sample was drawn for research purposes. Exclusion criteria for the relatives were previously diagnosed coeliac disease or DH or being on a self-initiated gluten-free diet. Altogether 3,031 relatives participated in the antibody screening. Finally, a subgroup of 463 first-degree relatives was randomly selected for microbial antibody measurements in the present study.

6.1.5 Non-coeliac controls (II, III)

Serum samples of 80 healthy adult blood donors with negative TG2-ab values received from the Finnish Red Cross comprised the non-coeliac control group for Studies **II** and **III** (Ashorn et al. 2008).

6.2 Data collection

6.2.1 Small-bowel mucosal biopsies (I-III)

All participants in Studies **I-III** except the non-coeliac controls and relatives underwent one or more oesophagogastroduodenoscopies, during which multiple duodenal biopsies were taken using standard methodology. The biopsy specimens obtained were either embedded in paraffin or frozen with liquid nitrogen immediately after sampling (see 6.2.2 and 6.2.3).

6.2.2 Mucosal morphology (I-III)

For the morphological evaluations, the duodenal biopsies were fixed in formalin and embedded in paraffin (**I-III**). The specimens were cut 5 μm thick and stained with haematoxylin-eosin. The mucosal VH/CrDs were measured from well-oriented biopsy cutting from at least three separate villus-crypt units (Kuitunen et al. 1982). A ratio of 2.0 or more was considered normal (Kaukinen et al. 2007b; Kuitunen et al. 1982). All specimens were evaluated without prior knowledge of individual disease history or results of other conducted investigations.

6.2.3 Mucosal inflammation (I-III)

For evaluation of mucosal inflammation, IEL density was estimated from separate frozen biopsies by immunohistochemistry (**I-III**). The specimens were embedded in optimal cutting temperature compound (Miles Labs, Elkhart, Indiana, USA) and stored at -70–80 °C until tested. The frozen specimens were cut into 5 μm thick

sections. The CD3+ IELs were stained with monoclonal antibody Leu-4 (Becton Dickinson, San Jose, California, USA) and γδ IELs with T cell receptor-bearing cell γ antibody (Endogen, Woburn, Massachusetts, USA). Positively stained IELs were calculated from at least 30 fields of 1.6 mm epithelial length. The IEL density was expressed as cells/mm (Savilahti et al. 1997). The cut-offs used for increased IEL density were 37 cells/mm for CD3+ cells and 4.3 for γδ cells (Järvinen et al. 2003).

6.2.4 Serological tests

Transglutaminase 2 and endomysial antibodies (I-III)

Serum IgA class TG2-abs were measured by commercial ELISA according to the manufacturer's instructions. The first-degree relatives were tested by commercial INOVA (INOVA diagnostics, San Diego, California, USA, cut-off for seropositivity ≥ 30 U/mL) seroassay (**III**) and the other study groups by commercial Celikey (Phadia, Freiburg, Germany; cut-off ≥5.0 U/ml) seroassay (**I-III**). Measurements of serum IgA class EmA were carried out by indirect immunofluorescence using human umbilical cord as substrate (Ladinser et al. 1994). Titers 1: ≥ 5 were considered positive and further diluted up to 1:4000 or until negative. In case of selective IgA deficiency, the sera were tested for corresponding IgG class antibodies (Korponay-Szabó et al. 2003).

Anti-*Saccharomyces cerevisiae* antibodies (I-III)

A commercial ELISA kit (Quanta Lite ASCA; INOVA Diagnostics Inc., San Diego, California, USA) was used for the determination of both IgA and IgG class ASCA. Quantitative results in arbitrary enzyme immunoassay units were obtained from standard curves defined by the manufacturer. The kit included positive and negative controls. Results ≥25 U/ml for IgA and IgG ASCA were considered positive.

Antibodies against the I2 protein of *Pseudomonas fluorescens* and the *Bacteroides caccae* TonB-linked outer membrane protein (I-III)

The I2 glutathione-S-transferase fusion protein (I2-GST) and OmpW protein were produced in E. coli XL-1 blue and E. coli BL-21 strains (Stratagene, La Jolla, California, USA) in our laboratory (Ashorn et al. 2008).

Serum IgA class antibodies to I2-GST and OmpW were measured by in-house ELISA. For anti-I2 measurements, the microtiter plates were coated with 100 µl per well of I2-GST (7,5 µg/ml in TRIS-buffered saline and 1:10 diluted CaCl2) or GST alone and incubated at 37°C for 2.5 hours. For anti-OmpW detection, plates were coated with 100 µl per well of OmpW recombinant protein at 10 µg/ml in TRIS-buffered saline and 1:10 diluted CaCl2. The plates were incubated at 37°C for 2.5 hours and then washed three times with Tween in TRIS-buffered saline (TBS + 3,772 g/l EDTA + 500 µl Tween). For I2, the plates were then blocked with anti-GST 1:10 (100 µl /10 ml Tween in TBS) for 90 minutes at room temperature. The blocking solution was then discarded and the plates were washed three times as described above.

Sera diluted 1:50 was added 100 µl per well and incubated overnight at 4°C. After three washes, alkaline phosphatase-conjugated goat anti-human IgA at a dilution of 1:4000 Tween in TBS was added for 90 minutes at room temperature. After three washes, substrate solution (4 o-phenylenediamine dihydrochloride tablets / 12 ml aqua + 5 µL hydrogen peroxide) was added at 100 µl per well, and the colour was allowed to develop for 25 minutes. The reaction was blocked with 1M sulphuric acid at 100 µL per well. After 0-5 minutes, the plates were read at 490 nm. The sera were tested in duplicate with mean absorbance calculated from two adjacent wells. Non-specific bindings of sera to GST alone were subtracted from the raw values of I2/GST binding to obtain I2-specific absorbances.

For anti-I2 antibodies, the cutoff level for positivity was set at absorbance 0.5. For anti-OmpW, an absorbance cutoff 0.6 was used for children and 1.0 for adults based on our previous studies evincing age differences in the normal range (Ashorn et al. 2008; Iltanen et al. 2006).

6.2.5 Genetic testing (I-III)

The coeliac disease-associated HLA DQ risk haplotypes were determined with either a commercial assay or a tagging SNP method. The latter is based on detecting certain tag SNPs that are in linkage disequilibrium between HLA-DQA1 and DQB1 alleles encoding haplotypes DQ2.5, DQ2.2 and DQ8 associated with coeliac disease (Monsuur et al. 2008). The use of only six different tag SNPs has been shown to reach high accuracy (Koskinen et al. 2009; Monsuur et al. 2008). The commercial assays used in the HLA genotyping were the Olerup SSP DQ low-resolution kit (Olerup SSP AB, Stockholm, Sweden) and the DELFIA Celiac Disease Hybridization Assay (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland).

6.2.6 Dietary assessment (I-III)

All coeliac disease patients included in Studies **I-III** began a gluten-free diet under the guidance of a professional dietitian. Additionally, a systematic re-evaluation was conducted to ensure strict adherence.

6.3 Statistical analyses (I-III)

Categorical data were expressed as percentages in all studies. Quantitative data were expressed using either range (**I**) or medians with lower and upper quartiles (**II-III**). To present overlapping of seropositivity for different microbial antibodies, the data were cross-tabulated (**I-III**). To compare the frequencies of seropositivity to a certain antibody between paired proportions, McNemar's test was used (**I**). In Study **II**, to test whether differences in the age and female/male ratio between the groups affected the comparisons of seropositivity, the groups were adjusted for age and gender by binary logistic regression.

Medians of antibody levels (**I-III**), Vh/CrD (**II**) and densities of CD3+ and γδ cells (**II**) were compared between the study groups by Wilcoxon (**I**), Mann-Whitney (**II**) and Kruskal-Wallis (**II-III**) tests. Dunn–Bonferroni test was used for post hoc pairwise comparisons (**II-III**). Correlations between age and antibody levels (**II**) and

between TG2-abs, EmA and microbial antibodies (**III**) were tested with Spearman's rank correlation coefficient. The chi-square statistic for the change in the -2 log-likelihood from the constant only model to the model with "family" was used in Study **III** to evaluate associations in seropositivity to microbial antibodies within and between the families.

Statistical analyses were carried out with Predictive Analytics SoftWare version 18 (SPSS Inc., Chicago, Illinois, USA) in Study **I**, IBM Statistical Package for the Social Sciences (SPSS) Statistics, version 23 in Study **II** and SPSS Statistics for Windows (IBM Corp., Armonk, New York, USA) in study **III**. In all studies, P value <0.05 was considered significant.

6.4 Ethical considerations (I-III)

The study protocols of Studies **I-III** were approved by the Ethics Committee of the Pirkanmaa Hospital District. Furthermore, all participants or, in the case of children (**I, III**) their parents/legal guardians, gave written informed consent. The studies were also performed in accordance with the Declaration of Helsinki of 1975.

7 RESULTS

7.1 Demographic data (I-III)

The majority of the early-stage-, NRCD- and responsive coeliac disease patients were females, whereas there were more males in the non-coeliac control group (Table 10). The gender distribution was almost equal among the patients' first-degree relatives (Table 10). The median ages of the study groups varied between 41 and 50 years. Some of the early-stage patients and their relatives were children and adolescents, whereas the other groups comprised only adults (Table 10).

Table 10. Demographic data of participants in each study group (**I-III**).

	ECD n=44	NRCD n=20	Responsive CD at dg n=58	Responsive CD on GFD n=55	Relatives sero +ve n=49	Relatives sero -ve n=414	Non-CD controls n=80
Females, %	68.0	85.0	77.6	76.4	42.9	57.2	32.0
Age, median, y	47	50	45	46	41	42	41
Age, quartiles, y	(13-72)***	(43-67)	(36-59)	(38-60)	(31-54)	(28-59)	(31-56)

* Positive serum EmA (titer 1: ≥ 5) and/or TG2-abs (Celikey, Phadia, Freiburg, Germany; cut-off for seropositivity ≥ 5.0 U/ml); ** Negative serum EmA (titer 1: <5) and TG2-abs (INOVA diagnostics, San Diego, California, USA; cut-off for seropositivity ≥ 30 U/mL); *** Range. CD, coeliac disease; EmA, endomysial antibody; ECD, early-stage coeliac disease; GFD, gluten-free diet; NRCD, nonresponsive coeliac disease; sero +ve, seropositive; sero -ve, seronegative; TG2-ab, transglutaminase 2 antibody

7.2 Small-bowel mucosal morphology (I-III)

By definition, all the 44 early-stage coeliac disease patients had normal VH/CrD in the first evaluation (**I**). A subgroup of 16 patients continued with gluten-containing diet and developed diagnostic lesions during later surveillance, while the rest started a gluten-free diet based on elevated EmA and did not undergo re-biopsy (**I**).

At diagnosis, the NRCD patients had lower VH/CrD ratio than the patients who later responded to gluten-free diet, but the difference was not significant (median 0.2 vs. 0.4 respectively, p=0.065). By definition, the VH/CrD of NRCD patients remained low after a median of 3.5 years on a strict diet as opposed to the diet-responsive group (Table 11). Moreover, while on a gluten-free diet, the NRCD patients continued to have the median CD3+ and γδ IEL densities almost as high as the diet-responsive patients at diagnosis (Table 11). In the diet-responsive group, the mucosal architecture was fully restored in 32 (58%) out of the 55 patients after one year on a gluten-free diet, the rest having so far incomplete recovery (**II-III**).

Table 11. Histological data in nonresponsive and responsive coeliac disease patients (**II-III**).

	NRCD	Responsive coeliac disease at dg	Responsive coeliac disease on GFD
VH/CrD, median (quartiles)	0.3 (0.1-1.0)	0.4 (0.2-0.7)*	2.1 (1.5-2.6)*
CD3+ IELs, median (quartiles), cells/mm	38.0 (26.0-55.0)	51.0 (35.5-69.0)	28.0 (19.5-37.5)
γδ IELs, median (quartiles), cells/mm	11.9 (4.7-16.8)	13.4 (9.0-17.2)	8.7 (4.7-12.7)

*Biopsy was conducted on all but one responsive patient. GFD, gluten-free diet; IEL, intraepithelial lymphocyte; NRCD, nonresponsive coeliac disease; VH/CrD, villous height-crypt depth ratio

7.3 Serology

7.3.1 Coeliac disease autoantibodies (I-III)

Altogether 86.4% of the early-stage coeliac disease patients (**I**) had positive TG2-abs and/or EmA (Table 12) and the rate of seropositivity decreased significantly on a gluten-free diet (Table 1 in the original Study **I**). The TG2-ab levels were immeasurably low in 16 out of 20 NRCD patients and remained marginally elevated in four patients (**II**). One patient had IgA deficiency and negative IgG class TG2-abs (**II**). By definition, the coeliac autoantibody-positive relatives were seropositive to TG2-abs and/or EmA and the coeliac autoantibody-negative relatives were seronegative to both markers (Table 12). None of the controls expressed positive TG2-abs or EmA (Table 12).

Table 12. Antibody levels and frequency of seropositivity to TG2-abs and EmA in each study group (**I-III**).

	ECD n=44	NRCD n=20	Responsive CD at dg n=58	Responsive CD on GFD n=55	Relatives sero +ve n=49	Relatives sero -ve n=414	Non-CD controls n=80
TG2-ab, median (quartiles), U/ml	7.2 (4.0-3.2)*	0 (0–0)*	32 (8.7–72)*	1.9 (1.1–3.6)*	58 (37–128)**	8 (7–11)**	0.3 (0.1-0.5)*
Seropositivity;%							
TG2-ab	61.4	20.0	82.5	13.0	98.0	0	0
EmA	81.8	10.0	ND	ND	69.4	0	0
TG2-ab/EmA	86.4	20.0	ND	ND	100	0	0

*Celikey, Phadia, Freiburg, Germany, cut-off for seropositivity ≥ 5.0 U/ml; **INOVA diagnostics, San Diego, California, USA, cut-off for seropositivity ≥ 30 U/ml
CD, coeliac disease; ECD, early-stage coeliac disease; EmA, endomysial antibody; GFD, gluten-free diet; ND, no data; NRCD, nonresponsive coeliac disease; sero +ve, seropositive; sero -ve, seronegative; TG2-ab, transglutaminase 2 antibody

7.3.2 Microbial antibodies (I-III)

Anti-*Saccharomyces cerevisiae* antibodies (I-III)

The frequency of seropositivity to IgA and/or IgG class ASCA was highest in the NRCD group, followed by the untreated diet-responsive coeliac disease patients, the TG2-ab and/or EmA positive relatives, the early-stage patients, the diet-responsive patients on a gluten-free diet and, finally, the coeliac autoantibody-negative relatives (Table 13). None of the non-coeliac controls were seropositive to ASCA (Table 13).

Similarly, the NRCD patients had the highest median levels of ASCA IgA and IgG and the values were significantly increased compared to the responsive coeliac disease patients on a gluten-free diet and the non-coeliac controls (Figure 4). The values were also higher in the NRCD patients than in the diet-responsive patients before treatment, but the difference was not statistically significant (median ASCA IgA 14.50 vs 10.50 U/ml; ASCA IgG 32.50 vs. 23.50 U/ml respectively, p=1.000).

In Study **I**, the median ASCA IgA level was 9.0 U/ml at the early stage of coeliac disease, 7.0 U/ml at the time the diagnosis (n=14) and 7.0 U/ml after one year on a gluten-free diet (n=33). The corresponding ASCA IgG levels were 15.5 U/ml, 10.5

U/ml and 11.0 U/ml respectively. Both ASCA IgA and IgG levels were increased at the early stage and at the mucosal atrophy stage compared to those on a gluten-free diet, whereas there was no significant difference between the early stage and the diagnosis (Figure 2a-b in the original Study **I**). The frequency of seropositivity to ASCA IgA and/or IgG decreased significantly when the early-stage values were compared to those on a gluten-free diet (Table 1 in the original Study **I**). Three out of six of the TG2-ab and EmA -negative early-stage patients already showed seropositivity to ASCA (**I**).

In Study **III**, the coeliac autoantibody-positive relatives showed higher median ASCA IgA/IgG levels than those with negative autoantibodies (IgA 11.1 vs. 8.90 U/ml, p=0.019 and IgG 12.80 vs 8.37 U/ml respectively, p=0.001). However, the relatives had significantly higher ASCA IgA and IgG levels than the non-coeliac controls even after excluding the coeliac autoantibody-positive subjects (Figure 4). There was no significant difference in ASCA IgA levels between the coeliac autoantibody-negative relatives and the diet-responsive coeliac disease patients, but the ASCA IgG levels were lower in the diet-responsive patients both at diagnosis and on a gluten-free diet (Figure 4). There was a weak positive correlation between TG2-ab and ASCA IgA values among the relatives ($r=0.31$, $p<0.001$).

The ASCA IgA and IgG levels were elevated in the diet-responsive patients both at diagnosis and on a gluten-free diet compared to the non-coeliac controls (Figure 2a-b in the original Study **II**).

Adjusting for age and gender or exclusion of children affected neither the comparisons of the seropositivity rate nor the median ASCA levels in Studies **II** and **III** (data not shown). Nevertheless, the ASCA IgA medians among the first-degree relatives were significantly lower in children than in adults (ASCA IgA 6.30 vs. 9.64 U/ml, $p<0.001$ respectively).

Antibodies against the I2 protein of *Pseudomonas fluorescens* (I-III)

Seropositivity to anti-I2 antibodies was most frequent among the diet-responsive patients followed by the TG2-ab and/or EmA positive relatives, NRCD patients, the coeliac antibody-negative relatives, the early-stage patients, and the controls (Table 13).

In Study **I**, the anti-I2 antibody levels differed neither between early stage and diagnosis (median absorbance 0.61 vs. 0.96, p=0.14, respectively) nor early stage and

gluten-free diet (0.61 vs. 0.73, p=0.48). However, the antibody levels decreased significantly upon treatment among the 14 patients who developed mucosal atrophy (Figure 2d in the original Study **I**). Of the six patients who were seronegative to both TG2-abs and EmA at an early stage, four (66.7%) were already seropositive to anti-I2 antibodies.

In Studies **II** and **III**, the diet-responsive patients presented with the highest anti-I2 antibody levels at diagnosis, these also being significantly higher than in the coeliac autoantibody-negative relatives (Figure 5) and controls (Figure 2c in the original Study **II**).

The first-degree relatives in Study **III** had significantly elevated median anti-I2 antibody levels compared to the non-coeliac controls, also when the coeliac autoantibody-positive subjects were excluded (Figure 5). The antibody levels did not differ from those of the diet-responsive patients on a gluten-free diet (Figure 5).

In Study **II**, the anti-I2 antibody levels were significantly elevated in the NRCD patients compared to the controls, whereas there was no significant difference between the NRCD and the diet-responsive patients (Figure 5).

In Studies **II** and **III**, adjusting for age and gender or exclusion of children affected neither the comparisons of the seropositivity rate nor the median levels of anti-I2 antibodies, even though the antibody levels were lower in children than in adult relatives (absorbance 0.34 vs. 0.79 respectively, $p<0.001$).

Antibodies against the *Bacteroides caccae* TonB-linked outer membrane protein (I-III)

The frequency of seropositivity to anti-OmpW antibodies was the highest among the untreated diet-responsive patients followed by the TG2-ab and/or EmA positive relatives, the diet-responsive patients on a gluten-free diet, the NRCD patients, the coeliac autoantibody-negative relatives, the early-stage coeliac disease patients and the controls (Table 13).

In Study **I**, the patients had the same median anti-OmpW antibody level at the early stage and at diagnosis (absorbance 0.79). There was a borderline significant difference between the antibody levels at the early stage and while on a gluten-free diet (median absorbance 0.79 vs. 0.72 respectively, p=0.053). One of the six TG2-ab and EmA-negative patients was seropositive to anti-OmpW antibodies at the early stage of the disease.

The untreated diet-responsive patients had significantly higher anti-OmpW antibody levels than the coeliac autoantibody-negative relatives (Figure 5) and the controls (Figure 2d in the original Study **II**). The antibody levels were also significantly higher when comparing the diet-responsive patients on a gluten-free diet and the controls (Figure 2d in the original Study **II**). The anti-OmpW antibody levels of the TG2-ab and EmA negative relatives did not differ from those of the treated diet-responsive patients nor the controls (Figure 2d in the original Study **III**). The coeliac autoantibody-positive relatives had higher anti-OmpW levels than those with negative antibodies (median absorbance 1.00 vs. 0.81, $p=0.022$). The antibody levels of the NRCD patients did not significantly differ either from those of the diet-responsive patients nor those of the control group (Figure 2d in the original Study **II**).

Adjusting for age and gender or exclusion of children affected neither the comparisons of the seropositivity rate nor the median levels of anti-OmpW antibodies in Studies **II** and **III**, although the antibody levels were again lower in children than in adults in the first-degree relatives (absorbance 0.54 vs. 0.87, respectively, $p<0.001$).

Table 13. Frequency of seropositivity (%) to the serum microbial markers in each study group (**I-III**).

	ECD n=44	NRCD n=20	Responsive CD at dg n=58	Responsive CD on GFD n=55	Relatives sero +ve n=49	Relatives sero -ve n=414	Non-coeliac controls n=80
ASCA IgA	6.8	35.0	20.7	5.4	16.3	10.6	0
ASCA IgG	25.0	55.0	48.3	20.0	28.6	13.0	0
ASCA IgA or IgG	29.5	65.0	51.7	20.0	32.7	18.8	0
ASCA IgA and IgG	2.3	25.0	17.2	5.4	12.2	4.8	0
Anti-I2	63.6	65.0	86.1	74.5	65.3	60.9	31.3
Anti-OmpW	25.0	45.0	58.6	49.1	55.1	39.1	23.8
Any positivity	77.3	80.0	96.6	87.3	85.7	73.2	43.7

* Positive serum EmA (titer 1: ≥ 5) and/or TG2-abs (Celikey, Phadia, Freiburg, Germany; cut-off for seropositivity ≥ 5.0 U/ml); **Negative serum EmA (titer 1: <5) and TG2-abs (INOVA diagnostics, San Diego, California, USA; cut-off for seropositivity ≥ 30 U/mL). Anti-I2, antibodies against the I2 protein of *Pseudomonas fluorescens*; Anti-OmpW, antibodies against the TonB-linked *Bacteroides caccae* outer membrane protein; ASCA, anti-*Saccharomyces cerevisiae* antibody; CD, coeliac disease; ECD, early-stage coeliac disease; EmA, endomysial antibody; IgA, immunoglobulin A, IgG, immunoglobulin G; NRCD, nonresponsive coeliac disease; sero +ve, seropositive; sero -ve, seronegative; TG2-ab, transglutaminase 2 antibody

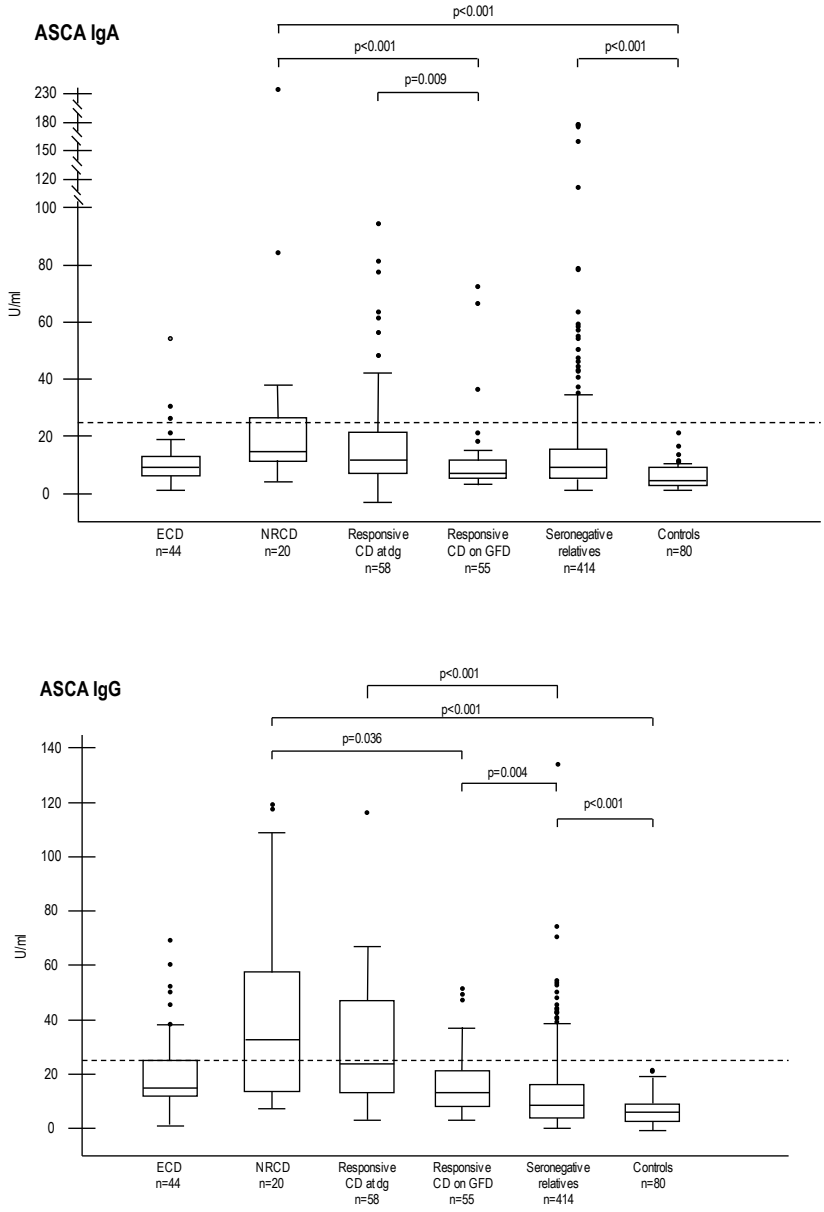

Figure 4. Serum levels of IgA and IgG class anti-*Saccharomyces cerevisiae* antibodies (ASCA) in the study groups. The broken horizontal line denotes the cut-off for seropositivity. The antibody levels of the ECD patients were not compared with the other study groups. CD, coeliac disease; ECD, early-stage coeliac disease; GFD, gluten-free diet; IgA, immunoglobulin A; IgG, immunoglobulin G; NRCD, nonresponsive coeliac disease

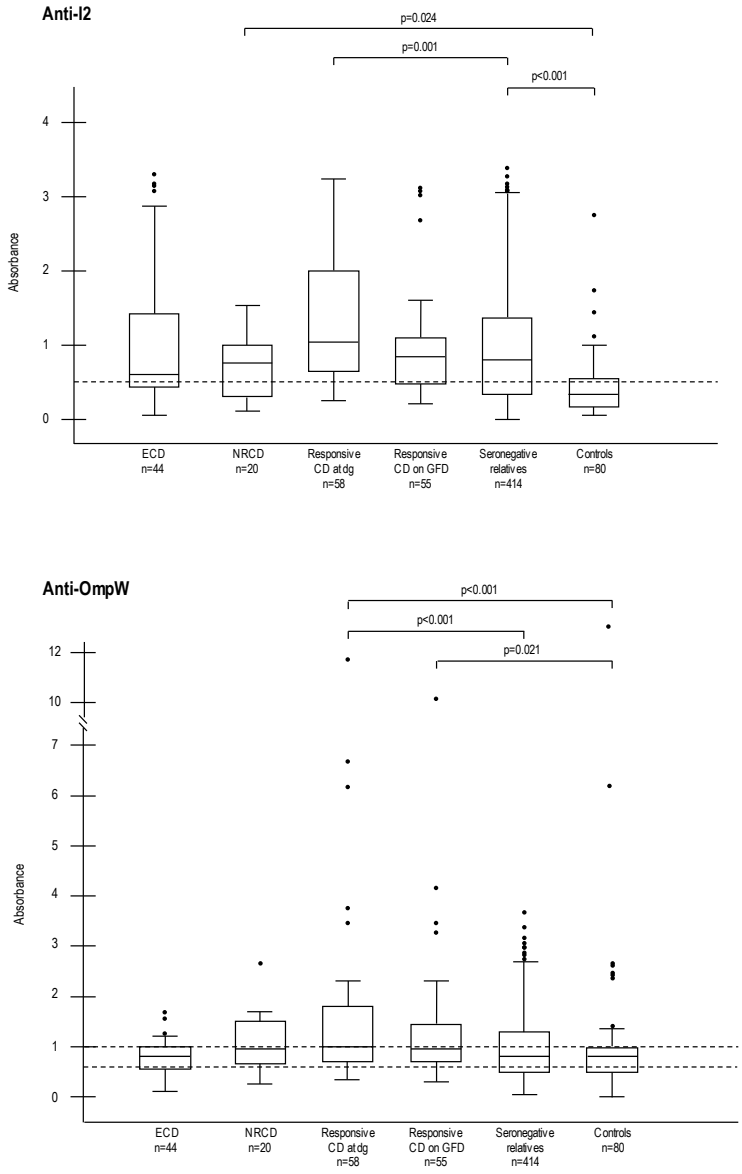

Figure 5. Serum levels of the IgA class antibodies against the I2 protein of *Pseudomonas fluorescens* (anti-I2) and the *Bacteroides caccae* TonB-linked outer membrane protein (anti-OmpW). The broken horizontal line denotes the cut-off for seropositivity. The antibody levels of the ECD patients were not compared with those of the other study groups in the figure. CD, coeliac disease; DG, diagnosis; ECD, early-stage coeliac disease; GFD, gluten-free diet; IgA, immunoglobulin A; NRCD, nonresponsive coeliac disease

Combinations of seropositivity to microbial antibodies (I-III)

Seropositivity to at least one microbial antibody was most frequent in the diet-responsive patients and lowest in the controls (Table 13). Seropositivity to all three microbial antibodies was the most common serotype among the diet-responsive patients at diagnosis (29.3%) and among the NRCD patients on a gluten-free diet (35.0%), whereas seropositivity solely to anti-I2 antibodies was most frequent in untreated early-stage patients (31.8%), diet-responsive patients on a gluten-free diet (30.9%) and coeliac autoantibody-negative relatives (25.1%) (Figure 6). Seropositivity to anti-I2 and anti-OmpW antibodies was most common in 28.6% of coeliac autoantibody-positive relatives. ASCA and anti-OmpW antibody positivity was the rarest combination of seropositivity and was seen in only 1.7% of untreated diet-responsive patients and in 1.2% of the relatives (Figure 6).

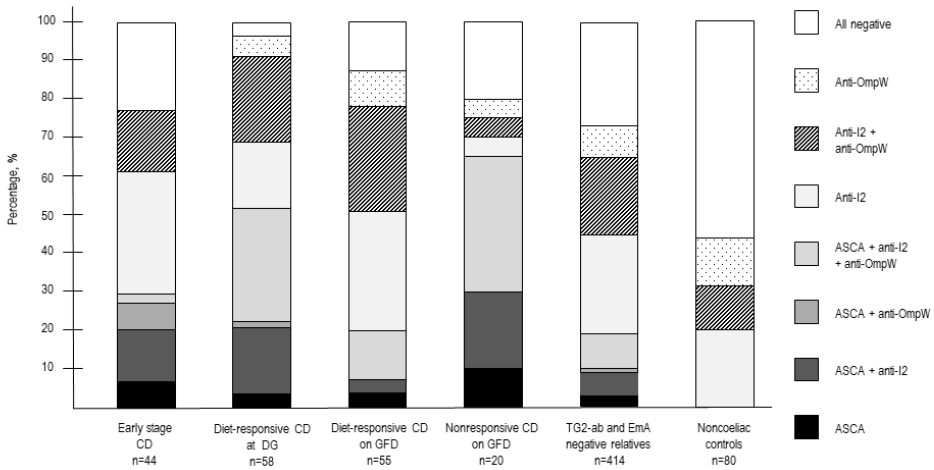

Figure 6. Distribution of seropositivity to ASCA, anti-I2 and anti-OmpW antibodies among the study groups. ASCA positivity signifies seropositivity to either IgA class or IgG class ASCA, or both. ASCA, anti-*Saccharomyces cerevisiae* antibody; Anti-I2, antibodies against the I2 protein of *Pseudomonas fluorescens*; Anti-OmpW, antibodies against the *Bacteroides caccae* TonB-linked outer membrane protein; CD, coeliac disease; GFD, gluten-free diet

Seropositivity to microbial antibodies in the families (III)

Clustering of antibody positivity within and between the families was evaluated in Study **III**. Seropositivity to anti-I2 and anti-OmpW antibodies was significantly more frequent between relatives in the same family than between different families ($p < 0.001$ for anti-I2 and $p = 0.001$ for anti-OmpW respectively). Regarding ASCA, this was only evident when coeliac autoantibody-positive relatives were also included in the analysis ($p = 0.007$).

7.4 Genetics (I-III)

All the early-stage-, NRCD- and diet-responsive coeliac disease patients had the coeliac disease-associated HLA-DQ2 and/or DQ8 haplotypes (**I-III**). The non-coeliac controls were not genotyped. There were no significant differences in the distribution of seropositivity across microbial antibodies in Study **III** when the coeliac autoantibody-negative relatives were divided into subgroups according to their HLA haplotype (Table 2 in the original study **III**).

8 DISCUSSION

8.1 Anti-*Saccharomyces cerevisiae* antibodies

ASCA have formerly been associated mainly with biopsy-proven coeliac disease (Chapter 4.1). In Study **I**, ASCA were already present in early-stage disease at a higher frequency (29.5%) compared to prevalences reported in healthy individuals (0-16%) (Prideaux et al. 2012), and three out of six of the patients with negative TG2-abs and EmA already showed seropositivity to ASCA. There is a lack of comparable studies, but Granito et al. reported that in their study cohort of 105 coeliac disease patients (Granito et al. 2005), five out of 63 patients positive to IgA and/or IgG class ASCA presented with positive TG2-abs and EmA and increased intraepithelial lymphocytes but normal mucosal architecture (Granito et al. 2006). Nevertheless, the prevalence of ASCA positivity among all the early-stage patients was not reported and thus comparisons with Study **I** were not possible. In any case, the present study and their findings indicate that ASCA may emerge early in the disease course, sometimes even before the coeliac autoantibodies.

Of all study groups, the NRCD patients had the highest frequency of ASCA seropositivity. The median ASCA levels did not significantly differ from untreated patients but were higher than in treated diet-responsive patients and controls. Gross and colleagues previously measured IgA class ASCA in nine NRCD patients who also showed higher antibody levels than controls (Gross et al. 2013). In line with the present Study **II**, the ASCA levels were similar to those in untreated coeliac disease patients, probably due to continuing small-bowel mucosal damage. Of note, Gross et al. measured even higher ASCA levels in ten type II RCD patients (Gross et al. 2013). Here, even though the difference was not statistically significant, the NRCD patients had higher ASCA IgA and IgG levels than the untreated diet-responsive patients. Although more research is needed, these findings indicate that ASCA could be related to more complicated disease behaviour in coeliac disease. For comparison, in Crohn's disease several studies have shown an association between ASCA positivity and earlier age of onset, more severe clinical and histological phenotypes

and increased risk of needing surgery (Papp et al. 2008; Arnott et al. 2004; Mow et al. 2004b).

The frequencies of seropositivity to IgA and/or IgG class ASCA observed in the diet-responsive patients at diagnosis (51.7%) and on a gluten-free diet (20.0%) in Studies **II** and **III** respectively are within the range of formerly reported prevalences (Table 7). Ashorn et al. (2009b) have previously shown a significant decrease of ASCA IgA and IgG levels in this diet-responsive patient cohort, and similar findings have also been reported by other groups (Granito et al. 2005; Mallant-Hent et al. 2006a). In line with this, ASCA IgA and IgG levels were also significantly lower in the patients in Study **I** after one year on a gluten-free diet than at the time of diagnosis.

Ashorn et al. (2009b) also observed a negative correlation between VH/CrD and ASCA in the diet-responsive patients. This raises the question whether ASCA could be used as an noninvasive marker in monitoring mucosal recovery, taking into account the suboptimal accuracy of TG2-abs for this purpose (Leonard et al. 2017). The use of ASCA in disease monitoring is further supported by the present observation of high ASCA levels and the highest frequency of ASCA positivity in NRCD patients in Study **II**. However, more studies with longer follow-up periods and several antibody measurement time points would be needed to estimate the accuracy of ASCA in the monitoring of coeliac disease.

In Study **III**, the first-degree relatives of coeliac disease patients had significantly higher ASCA IgA and IgG levels than the controls, even after exclusion of coeliac autoantibody-positive individuals from the comparisons (Figure 4). Da Silva Kotze et al. (2010) have also studied this issue and observed a higher frequency of seropositivity to ASCA IgA or IgG only in TG2-ab and/or EmA-positive relatives compared with healthy controls with no family history of coeliac disease. However, there was also a trend towards overrepresentation of ASCA among the autoantibody-negative relatives. The discrepancy between the present results may thus be, at least partly, explained by their smaller study cohorts and possible methodological differences, as the authors did not report the assays used for the determination of ASCA (da Silva Kotze et al. 2010). Increased seroreactivity to ASCA has also been reported in healthy relatives of Crohn's disease patients (Halfvarson et al. 2005; Seibold et al. 2001; Sutton et al. 2000b; Vermeire et al. 2001).

8.2 Antibodies against the I2 protein of *Pseudomonas fluorescens*

Of all study groups, the anti-I2 antibody levels and the frequency of seropositivity were the highest among the coeliac disease patients in Studies **II** and **III** at the time of diagnosis (86.1%) and the second highest while on a gluten-free diet (74.5%). However, the seropositivity rate was also over 60% among the early-stage and NRCD patients as well as in the first-degree relatives (Table 13). As far as we know, anti-I2 antibodies have not been studied in these groups. For comparison, Ashorn et al. (2008, 2009a) reported seropositivity to anti-I2 antibodies in 70.2% of newly diagnosed coeliac disease patients, in 44.4% of children and adolescents with Crohn's disease and in 41.7% of subjects with UC.

The findings in Study **II** did not support the role of anti-I2 antibodies in detecting NRCD, since there were no significant differences in the antibody levels compared with the diet-responsive patients. However, the antibody levels remained elevated despite a gluten-free diet of a median duration of 3.5 years indicating a sustained immune response towards the I2 antigen. Anti-I2 antibodies have previously been associated with more severe disease phenotype and a higher risk for needing surgery in Crohn's disease (Arnott et al. 2004; Mow et al. 2004b). It would thus be interesting to have a larger cohort of NRCD and especially RCD patients for anti-I2 antibody measurements to determine whether any associations between anti-I2 antibodies and different phenotypes of coeliac disease would be observed.

The first-degree relatives of coeliac disease patients had higher anti-I2 antibody levels than the controls, also after the exclusion of the coeliac autoantibody-positive subjects (Figure 5). Previous data on anti-I2 antibodies among the relatives of coeliac patients are lacking, but in the Crohn's disease study seropositivity to these antibodies increased the risk of diagnosis during the surveillance period of 4.5 years (Joossens et al. 2010). Of note, up to 63.6% of early-stage patients in Study **I**, some of them still negative to TG2-abs and EmA, showed seropositivity to anti-I2 antibodies. In the light of these findings, anti-I2 response may also imply a higher risk of developing coeliac disease. As opposed to ASCA and anti-OmpW, the anti-I2 antibody levels did not decrease significantly during gluten-free diet. One explanation could be the smaller study cohort, since a significant decrease was previously seen in diet-responsive patients (Ashorn et al. 2009b).

Anti-I2 antibodies were rather common (31.3%) in the non-coeliac blood donors who served as controls. For comparison, Sutton et al. (2000) observed anti-I2 antibodies in 18.9% of subjects with intestinal inflammatory diseases other than IBD

and in 3.8% of healthy controls, while in the studies by Ashorn et al. (2009a) and Iltanen et al. (2006) respectively 7.7% and 15% of non-IBD controls with gastrointestinal symptoms were anti-I2 positive. Owing to the negative TG2-ab and EmA values, controls in the present study were unlikely to have coeliac disease and the reason for the higher seropositivity rate compared to those reported in earlier studies remains unclear.

8.3 Antibodies against the *Bacteroides caccae* TonB-linked outer membrane protein

Among all study cohorts, seropositivity to anti-OmpW antibodies was the most frequent in the 58 diet-responsive coeliac disease patients at diagnosis in Studies **II** and **III** and, somewhat surprisingly, the second highest in the TG2-ab and/or EmA positive relatives in Study **III**. The coeliac autoantibody-positive relatives who were not on a gluten-free diet also had significantly higher anti-OmpW antibody levels than the controls, whereas this was not evident in the autoantibody-negative relatives. Due to the high specificity of TG2-abs and EmA (Pacheco et al. 2022; Raivio et al. 2008; Roca et al. 2019), the autoantibody positive relatives were likely to have undiagnosed or incipient coeliac disease, probably explaining the high seropositivity rate. An increased anti-OmpW antibody response has previously been observed in Crohn's disease (Iltanen et al. 2006) and untreated coeliac disease (Ashorn et al. 2008). This dissertation is the first to assess anti-OmpW antibodies in early-stage coeliac disease and NRCD and in the relatives of coeliac disease patients.

As opposed to the coeliac antibody-positive relatives, the anti-OmpW antibody levels of the NRCD patients who had been on a gluten-free diet differed neither from those of the diet-responsive patients nor from those of the controls in Study **II.** These results imply that the anti-OmpW response might be the most pronounced in gluten-consuming patients. The frequency of anti-OmpW seropositivity was markedly lower in the early-stage patients (25.0%) in Study **I**, indicating that the magnitude of the seroresponse may increase along with the development of mucosal damage. Nevertheless, the anti-OmpW levels were already higher at the early stage than when on a gluten-free diet. The levels were also significantly higher at diagnosis than during treatment in the diet-responsive group in Studies **II** and **III**. These findings raise the question whether the gluten intake itself is associated with the anti-OmpW antibody response in genetically predisposed individuals. Of note, in a study

by Zafeiropoulou et al. (2020), the alterations observed in the *Bacteroidetes* population in untreated coeliac disease patients compared with healthy controls disappeared on a gluten-free diet. Moreover, environmental factors, such as diet, are in general more determinative on the *Bacteroidetes* composition than on some other phyla (David et al. 2014). It would thus be interesting to study the association between the abundance of *Bacteroides caccae* and gluten ingestion as well as anti-OmpW levels in coeliac disease patients.

The prevalence of seropositivity to anti-OmpW antibodies in the control group is in line with earlier findings by Ashorn et al. (2009a) and Iltanen et al. (2006). *Bacteroides caccae* is known to be a common commensal in the intestinal mucosa (Snydman et al. 2010), which may explain the anti-OmpW antibody formation in a subset of individuals without known immune-mediated diseases.

8.4 Combinations of seropositivity to microbial antibodies

All combinations of seropositivity to ASCA, anti-I2 and anti-OmpW antibodies were observed in the study groups. In Crohn's disease, rather than a global loss of tolerance to microbial and autoantigens, the seroreactivity differs in magnitude and in antibodies present between patients (Landers et al. 2002), and this dissertation shows this also to be apparent in coeliac disease. Seropositivity to all three microbial antibodies, to anti-I2 and anti-OmpW antibodies and to anti-I2 antibodies only were the most common combinations, whereas seropositivity to ASCA and anti-OmpW antibodies was the rarest finding. In Crohn's disease, Landers et al. categorized the patients into four clusters according to the magnitude of microbial antibody response as follows: high pANCA, high ASCA IgA and IgG, high anti-OmpC and anti-I2 antibodies, and low reactivity to all measured antibodies (Landers et al. 2002). It is interesting that certain antibody combinations, such as ASCA and anti-OmpC in the study by Landers et al. and ASCA and anti-OmpW in this dissertation were rare, implying that different patients are immunoreactive to *Saccharomyces cerevisiae* and the anaerobes *Escherichia coli* or *Bacteroides Caccae.*

Seropositivity to at least one microbial marker was observed in the majority (77.3 – 96.6%) of the coeliac disease patients in the present study. Considering the clustering of antibody responses, testing more than one antibody might increase the sensitivity to identify the genetically predisposed individuals at the highest risk for later disease. Even though neither the NRCD patients nor the coeliac antibody

negative relatives had elevated anti-OmpW antibody levels compared with the controls, adding the anti-OmpW antibodies to the measurements increased the seropositivity rate among the NRCD and diet-responsive coeliac disease patients as well as among the first-degree relatives. As a disadvantage, combining the seroresponses decreases the specificity as some antibodies, like anti-I2 and anti-OmpW antibodies in this dissertation, may also be positive in non-coeliac and non-IBD subjects. Which antibody combination of all the microbial antibodies identified would be the most accurate to distinguish coeliac disease patients, especially those being autoantibody negative, from non-coeliac individuals is a subject for further studies.

8.5 The determinants of microbial antibody response in the relatives of coeliac disease patients

Since most of the coeliac autoantibody-positive relatives likely have undiagnosed coeliac disease, it is logical that they also had higher ASCA, anti-I2 and anti-OmpW antibody levels than the non-coeliac controls. In contrast, the elevated ASCA and anti-I2 antibody levels, also in the coeliac autoantibody-negative relatives, is a novel and a more surprising finding. First-degree relatives share their genetics and often also the living environment, and both factors likely contribute to similar immune responses. However, microbial antibody responses were not associated with HLA DQ2/8, thereby implying other genetic modifiers. Notably, in a study by Setty et al. (2015) relatives of coeliac disease patients with normal small-bowel mucosal morphology and negative TG2-abs had signs of intestinal epithelial stress and proinflammatory markers, such as ultrastructural alterations, increased expression of heat shock proteins and interleukin-15 and activating NK receptors on intraepithelial cytotoxic T cells. This suggests that a proinflammatory state in the mucosa could contribute to the reactivity to microbial antigens observed here.

The first-degree relatives also had higher concordance of anti-I2 and anti-OmpW seropositivity within than between different families, whereas with ASCA this was seen only when autoantibody-positive relatives were included in the analysis. These findings are mostly in line with those of earlier studies among families with Crohn's disease (Amcoff et al. 2016, Halfvarson et al. 2005, Sutton et al. 2000b). Of note, Amcoff et al. (2016) compared anti-I2 levels in Crohn's disease patients and their family members and reported that the differences in anti-I2 antibody levels were

smaller within than between monozygotic twin pairs, even if only one of them had IBD. However, this was not seen in dizygous twin pairs, indicating that genetics plays a marked role in the anti-I2 response. Studies of ASCA have yielded partly contradictory results. Several studies have shown that seropositivity to ASCA is more common among the relatives of IBD patients (Seibold et al. 2001; Vermeire et al. 2001). Sutton et al. (2000) studied families with more than one Crohn's disease patient and found a higher concordance in the seropositivity status and ASCA levels between the family members from the same family than from different families. This similarity was not observed between marital pairs, indicating genetic influence (Sutton et al. 2000b). Additionally, Halfvarson et al. (2005) studied ASCA in monozygotic and dizygotic twin pairs with IBD and observed a higher rate of seropositivity among healthy dizygotic than monozygotic twin siblings. Concordance in the ASCA levels was seen only in monozygotic twin pairs with both siblings affected (Halfvarson et al. 2005). The results in the twin study of Amcoff et al. are in line with those of Halfvarson's group, as ASCA levels correlated only within twin pairs where both had Crohn's disease (Amcoff et al. 2016). Taken together, and based on the aforesaid and present results, the level of ASCA, anti-I2 and anti-OmpW responses seems to be to some extent genetically determined, but environmental factors also have a role, especially for ASCA.

8.6 Does microbial antibody response play a role in coeliac disease pathogenesis?

IBD and coeliac disease are complex diseases considered to have both environmental and genetic origins. A dysregulated immune response to commensal microbiota, reflected by antimicrobial antibody responses, is considered to be part of the aetiopathogenesis of IBD (Nagao-Kitamoto et al. 2016), and several IBD-associated microbial antibodies have been discovered (Cohavy et al. 2000; Lodes et al. 2004; McKenzie et al. 1992; Dotan et al. 2006; Seow et al. 2009; Sutton et al. 2000a; Wei et al. 2001). There is emerging evidence of similar immune dysregulation in coeliac disease, in which especially increased ASCA response has been reported (Giaffer et al. 1992; Damoiseaux et al. 2002). Nevertheless, the exact role of the microbial antibody formation in the pathogenesis of these diseases remains obscure. One theory for the cause of overexpression of microbial antibodies is increased

intestinal permeability (Vanuytsel et al. 2021). The loss of mucosal integrity increases the immune system's exposure to microbial antigens and hence could promote immune responses. Accordingly, the decrease in the antibody response during treatment would be a direct consequence of the restoration of normal mucosal structure. However, no significant correlation between ASCA levels and permeability have been found in IBD studies (Harrer et al. 2003; Vermeire et al. 2001b). Moreover, ASCA positivity may emerge even years before the clinical manifestations of Crohn's disease (Torres et al. 2020), indicating that some other cause than mucosal disruption also contributes to the immune response. The fact that here the antibody response against the microbial antibodies studied was already observed in early-stage coeliac disease suggests that it is more strongly associated with mucosal inflammation than increased permeability. However, it is worth noting that increased intestinal permeability has also been reported in relatives of IBD and coeliac disease patients (Peeters et al. 1997; Secondulfo et al. 2001), and more studies on this complex issue are needed.

Another intriguing question is whether there is a correlation between the antibody response and the presence of the targeted microbe in the intestine. In a study by Mallant-Hent et al. (2006b), ASCA positivity was rarely associated with the presence of *Saccharomyces cerevisiae* DNA in the intestinal mucosa, indicating that the serum antibodies are not explained by continuous exposure to *Saccharomyces cerevisiae*. There is a lack of studies regarding the association between the presence of *Pseudomonas fluorescens* or *Bacteroides caccae* and antibody formation.

One plausible explanation for the lacking relation between certain serum antibodies and presence of the antibody-associated microbe or microbial antigen is cross-reactivity with other antigens (Gershwin 2008; Rojas et al. 2018). As for ASCA, it has been shown that similar oligomannose structures in other yeast species can mimic *Saccharomyces cerevisiae* epitopes and induce the production of ASCA in patients with Crohn's disease (McKenzie et al. 1992). For example, *Candida albicans* may trigger ASCA production during infections when the ASCA epitopes are overexpressed (Standaert-Vitse et al. 2006). The discovery of anti-OmpW antibodies was based on the cross-reactivity of the *Bacteroides caccae* TonB-linked outer membrane protein with pANCA epitopes (Wei et al. 2001). However, the pANCA and anti-OmpW responses were not correlated in IBD patients (Wei et al. 2001) and it remains unclear whether antigens other than the TonB-linked outer membrane protein induce the generation of OmpW antibodies.

Although antibody production seems not to be only a result of mucosal damage, whether certain microbes and the triggered antibody response directly contribute to the pathogenesis of coeliac disease is unclear. The antibodies generated are targeted at commensal enteric flora, and therefore their role in the disease pathogenesis is all but evident. *Pseudomonas fluorescens* is generally considered to have a low level of virulence and rarely causes human infections (Benito et al. 2012). However, it has been speculated that in certain circumstances this pathogen may contribute to Crohn's disease pathogenesis (Wei et al. 2002). The *I2* sequence was found to be enriched in the colonic lesions in patients with Crohn's disease and serological response to the I2 protein was also detected (Sutton et al. 2000a). The observed T cell superantigen properties of the I2 protein also indicated a possible role in disease pathogenesis (Dalwadi et al. 2001). However, no causal role of the I2-targeted immune responses could be proven (Sutton et al. 2000a; Dalwadi et al. 2001). As a possible link with coeliac disease, Petersen et al. (2020) reported that *Pseudomonas fluorescens* peptides may cross-react with gluten epitopes and activate gliadin-primed T cells. Therefore, it may have some role in the pathogenesis of coeliac disease, for example in pro-inflammatory and dysbiotic circumstances. Furthermore, at least two studies have shown *Pseudomonas fluorescens* to increase intestinal permeability, indicating that it could affect the homeostasis of the epithelial barrier (Alnabhani et al. 2015; Li et al. 2016).

Even though the causal role of intestinal microbiota in the pathogenesis of coeliac disease remains to be proven, this is supported by the known modulatory effect of the microbiota to the host immune system (Chapter 3.3). Several mechanisms through which the microbiota could contribute to the loss of tolerance to gluten have been proposed, these including gliadin-mimicking epitopes expressed by microbes, secretion of proteolytic enzymes enhancing gluten degradation (Caminero et al. 2019), infections (Chapter 2.3.2) and increasing the intestinal permeability (Alnabhani et al. 2015; Li et al. 2016). Moreover, imbalances in the intestinal microbiota in coeliac disease have been described in many case-control studies (Chapter 3.5). However, the order of appearance of microbial alterations and coeliac disease-associated mucosal atrophy remains largely unknown.

In addition to the imbalances observed in the intestinal bacteria, there is emerging evidence of an association of fungal dysbiosis and the severity of gastrointestinal diseases (Mukherjee et al. 2015). Fungal dysbiosis has been reported in children with Crohn's disease or coeliac disease (El Mouzan et al. 2018, 2022). In particular, *Candida albicans* has been suggested to play a role in the persistence and/or

exacerbation of IBD (Iliev et al. 2012), and Crohn's disease patients and their healthy relatives have been observed to be more heavily colonized with *C. albicans* than non-IBD controls (Standaert-Vitse et al. 2009). Additionally, Harnett et al. (2017) reported increased faecal counts of both *Candida* and *Saccharomyce*s species in adult coeliac disease patients with persistent symptoms compared with non-coeliac patients with gastrointestinal symptoms. In this sense, it is noteworthy that in the present study seroreactivity to ASCA was most frequent among the NRCD group, particularly when considering that *C. albicans* may trigger ASCA production (McKenzie et al. 1992). In the study by Standaert-Vitse et al. (2009), ASCA levels correlated with *C. albicans* colonization in the healthy relatives of Crohn's disease patients, but the correlation disappeared in the affected probands. The authors suggested that the correlation observed might be associated with a proinflammatory state in the intestine (Standaert-Vitse et al. 2009). Studying the association of ASCA and *C. albicans* in different phenotypes of coeliac disease could clarify the role of *C. albicans* in the ASCA response.

In addition to the evidence of *C. albicans* colonization in coeliac disease, a possible serological link between *C. albicans* and coeliac disease pathogenesis was found. The *Candida albicans*-hyphal wall protein 1 (HWP1), which is expressed during *C. albicans* infections, has high sequence homology with gliadin, thus it has been speculated that *C. albicans* may contribute to coeliac disease pathogenesis (Nieuwenhuizen et al. 2003). Supporting this theory, anti-gliadin antibodies may emerge during *C. albicans* infections (Brinkert et al. 2009). Corouge et al. (2015) also showed that coeliac patients had increased seroreactivity to anti-HWP1 antibodies compared to controls and that the antibody levels were similar to those in patients with *C. albicans* infection. This proven cross-reactivity encourages further exploration of *C. albicans* in the context of coeliac disease.

8.7 Strengths and limitations

The main strength of this dissertation was the utilization of well-defined cohorts of patients with early-stage, diet-responsive and nonresponsive coeliac disease. The original diagnosis of coeliac disease was based on both serological and histological confirmation, except for the subgroup of 28 patients in Study **I,** who were diagnosed on the basis of seropositivity to the highly specific coeliac disease-associated autoantibodies, mucosal lymphocyte infiltration and a beneficial clinical and

serological response to dietary treatment (Kurppa et al. 2010). The NRCD patients in Study **II** underwent extensive elimination of possible coexisting causes for nonresponsiveness. In Study **III**, only the relatives of patients with biopsy-proven coeliac disease were included; relatives with a previously diagnosed coeliac disease, DH or gluten-free diet were excluded. All the at-risk relatives were screened for TG2-abs and EmA and categorized into autoantibody positive and negative subgroups with the main focus on the seronegative group, who were unlikely to have coeliac disease. The microbial antibody levels were also measured at three different time points in Study **I** to demonstrate variations in antibody levels along with disease development. In Studies **II** and **III** with only one measurement, the antibody levels were compared with those of controls unlikely to have coeliac disease due to negative TG2-ab levels.

As a major limitation, the microbial markers were not measured in NRCD patients at diagnosis, thus it was not possible to compare the results at that time point. The duration of the gluten-free diet was also only one year in both the early-stage and diet-responsive coeliac disease patients, and it would have been informative to observe the development of the antibody levels during a longer follow-up. Also, we did not have a true RCD group, since only two NRCD patients developed abnormal lymphocyte phenotype, thus responses to anti-I2 and anti-OmpW in RCD in particular remain a subject for further studies.

There were also marked differences between the group sizes which may have affected the results. Moreover, as opposed to NRCD and diet-responsive patients, the group of early-stage coeliac disease patients also included children, which may have affected the results due, for example, to shorter duration of gluten exposure.

Additionally, a more comprehensive dietary evaluation would possibly have found factors other than gluten-free diet which may have influenced the results e.g., via modification of microbiota. Detailed information on the general health of the relatives and the control group as well as their histological status was also lacking, thus it remains possible that a subgroup of them had undiagnosed coeliac disease or some other disease affecting the results.

As regards the generalizability of the results, the study participants were ethnically decidedly homogenous. Since both genetics and environmental factors influence intestinal microbiota and host immunity (3.2), individuals with different ethnicities and living conditions probably also have variable reactivity to microbial antigens. The prevalence of coeliac disease also varies across populations, and some

predisposing factors causing high disease frequency in Finland may also have induced more pronounced microbial antibody responses here.

8.8 Summary and future directions

The present dissertation demonstrates that increased serological response to commensal microbiota may be detectable already at the early stages of coeliac disease before the development of significant histological lesions. In some individuals, this may also precede the emergence of coeliac autoantibodies. Additionally, the at-risk first-degree relatives of the patients, also those with negative coeliac disease-associated autoantibodies, presented with increased seroreactivity to microbial antibodies compared with non-coeliac controls. Therefore, measuring microbial antibodies may help to identify those individuals at the highest risk of developing coeliac disease among genetically predisposed individuals. In the future, a prospective study measuring microbial antibody responses in genetically predisposed individuals and comparing the antibody levels between those who developed coeliac disease and those who did not could give further insight into the usefulness of these markers as disease predictors.

The findings in Study **II** suggest that seropositivity to ASCA is associated with NRCD. In addition, elevated anti-I2 antibody levels persisted in NRCD patients despite dietary treatment. The results indicate that dysregulated immune responses to microbial antigens are associated with persistent mucosal inflammation and morphological damage. A larger cohort including RCD patients would help to further estimate the relation between microbial antibody responses and a complicated disease course. Given that antibody levels decrease along with the restoration of the small-bowel mucosa, ASCA might serve as an additional noninvasive marker in the follow-up of mucosal restoration and could possibly help to identify those in need of a follow-up endoscopy. However, more studies with longer follow-up periods and several antibody measurement time points would be needed to determine the accuracy of ASCA in the surveillance.

ASCA do not necessarily correlate with the presence of *Saccharomyces cerevisiae* in the intestinal mucosa, for example due to cross-reactivity of ASCA antigens with other microbial epitopes. Studies on the respective correlations of intestinal *Pseudomonas fluorescens* and *Bacteroides caccae* with anti-I2 and anti-OmpW response

would clarify the origin of these antibodies. In general, estimating the relation between microbial antibody responses and the overall composition of intestinal microbiota would give further insight into the role of these antibodies in the host-microbiota interaction.

Even though the role of microbial antibodies in the pathogenesis of coeliac disease remains unknown, increased seroreactivity in coeliac disease patients compared with controls indicates that the antibody response reflects the inflammatory processes in the intestine. Which antibody combination would be the most accurate to distinguish the patients and non-coeliac individuals is, again, a subject for further research.

In the future, moving from association to causality studies on the role of the intestinal microbiota in coeliac disease requires longitudinal prospective studies following the composition and function of the intestinal microbiota and serum microbial antibody levels before, during and after the onset of the disease.

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PUBLICATIONS

PUBLICATION I

Early Microbial Markers of Celiac Disease

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Journal of Clinical Gastroenterology, 2014, 48(7), 620–4
doi: 10.1097/MCG.0000000000000089

PUBLICATION II

Microbial Biomarkers in Patients with Nonresponsive Celiac Disease

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Digestive Diseases and Sciences, 2018, 63(12), 3434–41
doi: 10.1007/s10620-018-5285-z

Digestive Diseases and Sciences (2018) 63:3434–3441
https://doi.org/10.1007/s10620-018-5285-z

ORIGINAL ARTICLE

Microbial Biomarkers in Patients with Nonresponsive Celiac Disease

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Received: 3 April 2018 / Accepted: 11 September 2018 / Published online: 20 September 2018

Abstract

Background and Aims In nonresponsive celiac disease (NRCD), the symptoms and duodenal damage persist despite a gluten-free diet. Celiac disease patients with persistent symptoms are found to have a dysbiotic microbiota. We thus hypothesized that increased seroreactivity to the serum gluten-sensitive microbial antibodies Saccharomyces cerevisiae (ASCA), Pseudomonas fluorescens-associated sequence (I2), and Bacteroides caccae TonB-linked outer membrane protein (OmpW) is associated with NRCD.

Methods ASCA, I2 and OmpW were measured in 20 seronegative CD patients with persistent villous damage despite strict dietary treatment (NRCD group). Fifty-eight responsive patients served as CD controls (55 on gluten-free treatment) and 80 blood donors as non-CD controls.

Results At least one microbial marker was positive in 80% of NRCD patients, in 97% of untreated CD and 87% of treated CD patients, and in 44% of controls. NRCD patients had the highest frequency of ASCA positivity (65% vs 52, 20, and 0%, respectively) and also significantly higher ASCA IgA (median 14.5 U/ml) and IgG (32.5 U/ml) titers than treated CD patients (7.0 U/ml, 13.0 U/ml) and non-CD controls (4.5 U/ml, 5.8 U/ml). The frequencies of I2 and OmpW were lower in NRCD than in untreated CD (65% and 45% vs 86% and 59%, respectively), and I2 titers were higher in NRCD (median absorbance 0.76) and untreated (1.0) and treated (0.83) CD than controls (0.32). OmpW was elevated in untreated (1.1) and treated (0.94) CD patients compared with controls (0.79).

Conclusions Seropositivity and high titers of ASCA are associated with NRCD and might serve as an additional follow-up tool in CD.

Keywords Nonresponsive celiac disease · Microbiota · Saccharomyces cerevisiae · Pseudomonas fluorescence · Bacteroides caccae

Introduction

Celiac disease (CD) is an immune-mediated intestinal disease characterized by diverse symptoms, specific autoantibodies against tissue transglutaminase (tTG-ab), and small-bowel mucosal injury. The only cure is a strict gluten-free diet (GFD), initiation of which usually results in beneficial response. Some patients, however, have nonresponsive celiac disease (NRCD) characterized by persistent clinical symptoms and histological damage [1–3]. The most common reason for NRCD is ongoing gluten intake, but there may also be coexisting disorders such as irritable bowel syndrome and microscopic colitis sustaining the symptoms [1–3]. After exclusion of other etiologies, refractory celiac disease (RCD) must be ruled out. Suggested risk factors for RCD are for example older age, male gender, and seronegativity at diagnosis [4], but the fundamental reasons for the development of RCD remain unknown.

Recently, there has been growing interest in the role of abnormal gut microbiota in the development of chronic intestinal diseases [5, 6]. Alongside the well-known association with anti-Saccharomyces cerevisiae antibodies (ASCA), we and others have reported increased serological responses to Pseudomonas fluorescens-associated sequence (I2) and Bacteroides caccae TonB-linked outer membrane protein (OmpW) in IBD [7–9]. A dysbiotic microbiota has also been associated with persistent symptoms in treated CD [10] and presence of ASCA and I2 with an increased risk of a complicated course

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in IBD [11–14]. These microbial markers are also gluten-sensitive and already present in early stages of CD, even before the development of villous atrophy and serum CD-specific autoantibodies (tTG-ab) [15–18].

Based on these observations, we hypothesized that NRCD could be associated with a dysbiotic intestinal microbiota reflected by increased seroreactivity against ASCA, I2, and OmpW. In addition, since most RCD patients are negative for CD autoantibodies [19], microbial markers might serve as novel noninvasive diagnostic and follow-up tools. We tested these hypotheses by comparing microbial seroreactivity between groups of NRCD patients, responsive CD patients, and non-CD controls.

Methods

Patients and Study Design

The study was conducted in Tampere University and Tampere University Hospital. The cohort comprised 20 adult NRCD patients, 58 CD patients with dietary response to GFD, and 80 healthy blood donors (non-CD controls). CD patients underwent careful clinical, serological, and histological evaluation during the diagnostic visit, and additional blood samples were drawn for serological analyses and HLA genotyping. After the diagnosis, the patients started a GFD under the supervision of a professional dietitian, and upper gastrointestinal endoscopy and clinical assessment were carried out after 1 year. The NRCD group comprised 20 CD patients who continued to have persistent clinical symptoms and villous atrophy despite long-term (median 3.5 years) strict GFD. Besides the esogastroduodenoscopy, the patients underwent various other medical investigations such as laboratory testing, abdominal imaging, and colonoscopy to exclude other than dietary reasons for NRCD [20]. Repeat endoscopy was conducted after 1 year in 15 NRCD patients, whereas the rest five were biopsied within 2–4 years. Two of the NRCD patients developed a true refractory CD with abnormal intraepithelial lymphocyte phenotype during the follow-up. The 58 CD patients who evinced a clear clinical, serological, and histological response to GFD represented the responsive CD patients. Among these, control sera for microbial analyses after 1 year on GFD were available from 55 subjects.

The Ethical Committee of the Pirkanmaa Hospital District approved the study protocol and patient enrollment. All participants gave written informed consent.

Small-Bowel Mucosal Morphology and Immunohistochemistry

Upper gastrointestinal endoscopy with multiple duodenal biopsies was undertaken in CD and NRCD patients at diagnosis and on GFD. The biopsy samples were orientated and processed according to our standard operating procedures [21]. Mucosal inflammation and villous height–crypt depth ratio (VH/CrD) were calculated from H&E-stained paraffin sections as an average from at least three separate villous-crypt units. In addition, separate biopsies were taken for immunohistochemical analyses, embedded in optimal cutting temperature compound (Miles Labs, Elkhart, IN, USA) and stored at − 80 °C until further used. Cryostat sections were cut 5 µm thick and processed for immunohistochemistry as described elsewhere [22]. The densities of mucosal CD3 + and γδ + IELs were quantified from the frozen sections utilizing monoclonal antibodies against CD3 (Leu-4; Becton Dickinson, San Jose, CA) and TCRγ (Endogen, Woburn, MA) [22]. All evaluations were carried out without previous information on the patient's medical history or laboratory results.

Serum Antibody Tests and HLA-Typing

Serum for the celiac autoantibodies and microbial markers were drawn in connection with the endoscopy visits. Serum IgA class tissue transglutaminase antibodies (tTG-ab) were measured by a commercial enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Celikey; Phadia, Freiburg, Germany). Serum tTG-ab values ≥ 5.0 U/ml were considered positive. In case of selective IgA deficiency, the serum tests were carried out utilizing the corresponding IgG class antibodies.

Serum IgA and IgG class ASCA were measured by ELISA (Quanta Lite ASCA; INOVA Diagnostics Inc., San Diego, CA) according to manufacturer's protocol. Results ≥ 25 U/ml for IgA and IgG ASCA were regarded as positive [15]. I2 and OmpW were expressed in *E. coli* XL-1 blue and *E. coli* BL-21 (Stratagene, La Jolla, CA) strains, and antigens for the corresponding IgA ELISA test were produced using previously reported antigen purification techniques [23, 24]. The cutoff level for positivity was set at absorbance 0.5 for I2 and at 1.0 for OmpW [7].

The presence of the celiac disease-associated HLA alleles (HLA)-DQB1*02 and DQB1*0302 (DQ2 and DQ8) was analyzed in whole blood samples from all NRCD and CD patients as described elsewhere [25].

Statistical Analysis

Quantitative data were expressed as percentages or as medians with lower and upper quartiles. Kruskal–Wallis and Mann–Whitney tests were used to compare serum antibody titers and other parameters between the groups, and Dunn–Bonferroni test was used for post hoc pairwise comparisons. When comparing seropositivity to ASCA, I2, and OmpW, the groups were adjusted by age and gender by

binary logistic regression. To express overlapping of seropositivity for different microbial antibodies, the data were cross-tabulated. A p value < 0.05 was considered significant. Statistical analyses were carried out with IBM SPSS Statistics, version 23.

Results

Patients with NRCD were somewhat older and non-CD controls more often men than subjects in the other study groups (Table 1). However, adjusting by age and gender did not change the study results. Coexisting collagenous colitis was diagnosed in two and unspecific proctitis in one NRCD patients, but treatment of these diseases did not affect the CD symptoms or duodenal histology. The exact tTG-ab value at diagnosis was available from nine NRCD patients of whom in eight it was positive. Their median value was somewhat higher compared with GFD-responsive patients, but the difference was not significant (72.6 vs 31.7 U/l, respectively, $p = 0.125$). Furthermore, VH/CrD was lower in NRCD than GFD-responsive patients at diagnosis with borderline significance (median ratio 0.2 vs 0.4, respectively, $p = 0.065$).

By definition, on a GFD NRCD patients had lower VH/CrD than CD patients (Table 1). Morphology of the duodenal mucosa was fully normalized in 32 (58%) out of the 55 GFD-responsive patients after 1 year on GFD, while the rest had ongoing recovery. NRCD patients had the lowest median tTG-ab value of all groups (the value was immeasurably low in 16 out of 20 cases), but they still had median CD3 + and γδ + IEL densities almost as high as untreated CD patients (Table 1). The tTG-ab titers clearly decreased on GFD but remained marginally elevated in seven GFD-responsive patients, as well as in four NRCD patients. One of the NRCD patients had IgA deficiency and also negative IgG class tTG-ab on GFD. All of the controls had negative tTG-ab. All NRCD and CD patients had the celiac disease-associated HLA-DQ2 and/or DQ8.

Seropositivity for at least one of the three microbial markers (ASCA, I2, OmpW) was seen in 80% of NRCD patients, 97% of untreated and 87% of treated CD patients, and in 44% of non-CD controls (Fig. 1). The most conspicuous difference between the groups was observed in ASCA, seropositivity for which was even more common in NRCD (65%) than untreated CD (52%), while only 20% of the treated CD patients and none of the controls were ASCA positive (Fig. 1). Seropositivity to I2 and OmpW was significantly more common in untreated CD patients (86% and 59%, respectively) and less frequent in controls (31% and 24%) (Fig. 1), whereas no significant difference was seen between NRCD and other groups.

NRCD patients had significantly higher IgA and IgG class ASCA titers compared with responsive CD patients on a GFD and non-CD controls; the titers were also higher in untreated CD patients than in those on a GFD (Figs. 2a and 2b). Although the difference was not clinically significant, the ASCA titer medians were higher in NRCD (ASCA IgA 14.5 and IgG 32.5) than in untreated CD (10.5 and 23.5, respectively). I2 titers were significantly higher in NRCD and both CD groups compared with non-CD controls, whereas there was no significant difference between the NRCD and CD groups (Fig. 2c). OmpW titers did not differ significantly between NRCD and CD groups, but were higher in both untreated and treated CD compared with non-CD controls (Fig. 2d).

There was no correlation between age and any of the serum antibody titers within the four subgroups. In addition, the titers did not differ significantly when genders were evaluated separately (data not shown).

Discussion

The most conspicuous finding in the present study was the highest frequency of ASCA positivity in NRCD patients compared with the other study groups. In accord, NRCD

Table 1 Demographic, histological and serological data in nonresponsive and responsive celiac disease (CD) patients and in nonceliac controls

	Nonresponsive CD $n = 20$	CD at diagnosis $n = 58$	CD on a GFD $n = 55$	Controls $n = 80$
Females, %	85.0	77.6	76.4	35.0
Age, median ($Q_{1–3}$), years	50 (43–67)	45 (36–59)	46 (38–60)	41 (31–56)
VH/CrD, median ($Q_{1–3}$)	0.3 (0.1–1.0)	0.4 (0.2–0.7)	2.1 (1.5–2.6)[a]	ND
CD3 + IELs, median ($Q_{1–3}$), cells/mm	38.0 (26.0–55.0)	51.0 (35.5–69.0)	28.0 (19.5–37.5)	ND
γδ + IELs, median ($Q_{1–3}$), cells/mm	11.9 (4.7–16.8)	13.4 (9.0–17.2)	8.7 (4.7–12.7)	ND
TG2ab, median ($Q_{1–3}$), U/l	0.0 (0.0–0.0)	31.7 (8.7–71.6)	1.9 (1.1–3.6)	0.3 (0.1–0.5)

GFD gluten-free diet, *VH/CrD* villous height–crypt depth ratio, $Q_{1–3}$ lower and upper quartiles, *TG2ab* transglutaminase 2 antibodies, *IEL* intraepithelial lymphocyte, *ND* no data

[a]Biopsy was conducted in all but one GFD-responsive patient

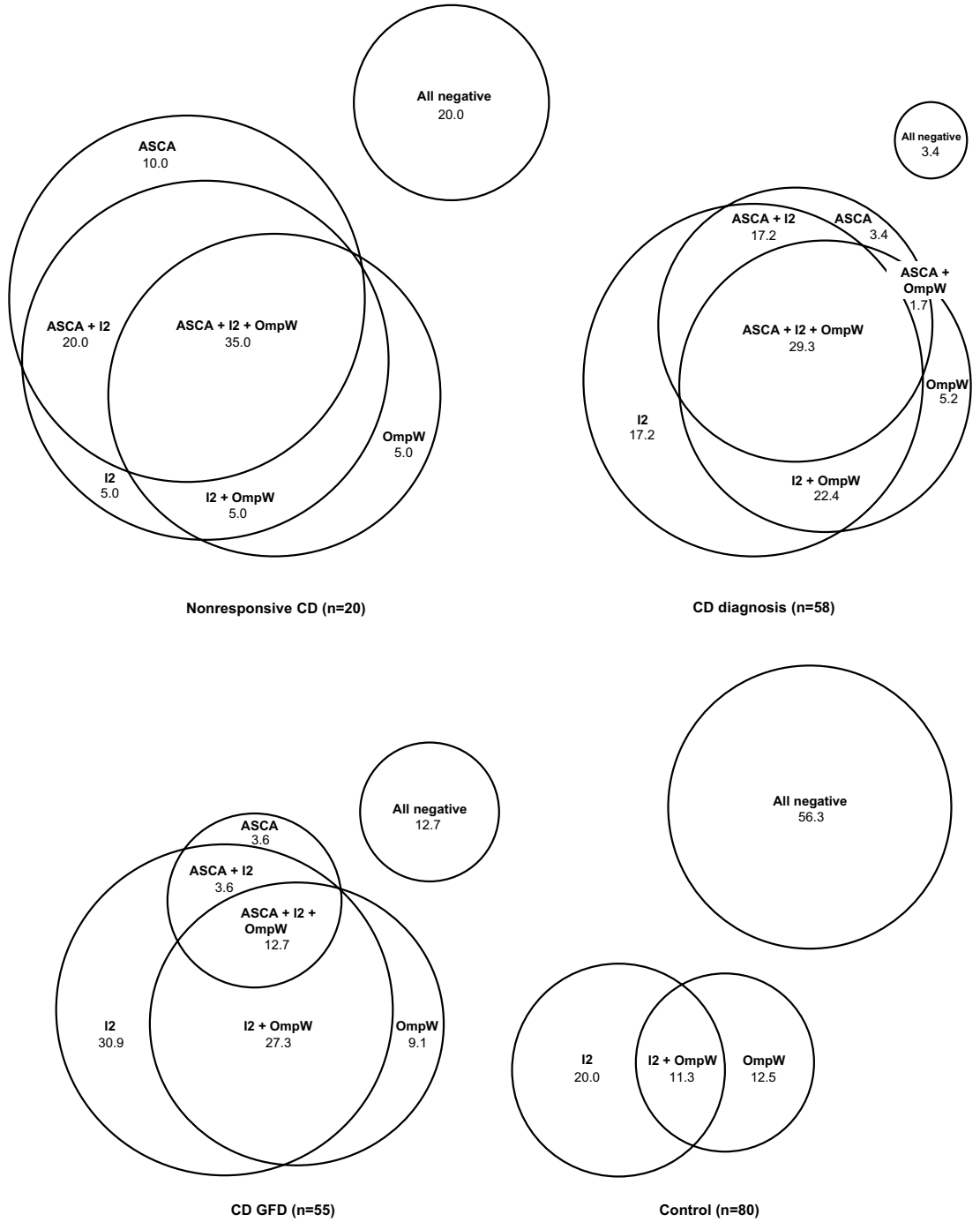

Fig. 1 Distribution of positivity to Saccharomyces cerevisiae (ASCA), Pseudomonas fluorescens-associated sequence (I2) and Bacteroides caccae TonB-linked outer membrane protein (OmpW) serum antibodies in NRCD and CD (at the time of diagnosis and on a GFD) patients and controls. The numbers are given as percentages for each antibody and their combinations (together the numbers add 100%)

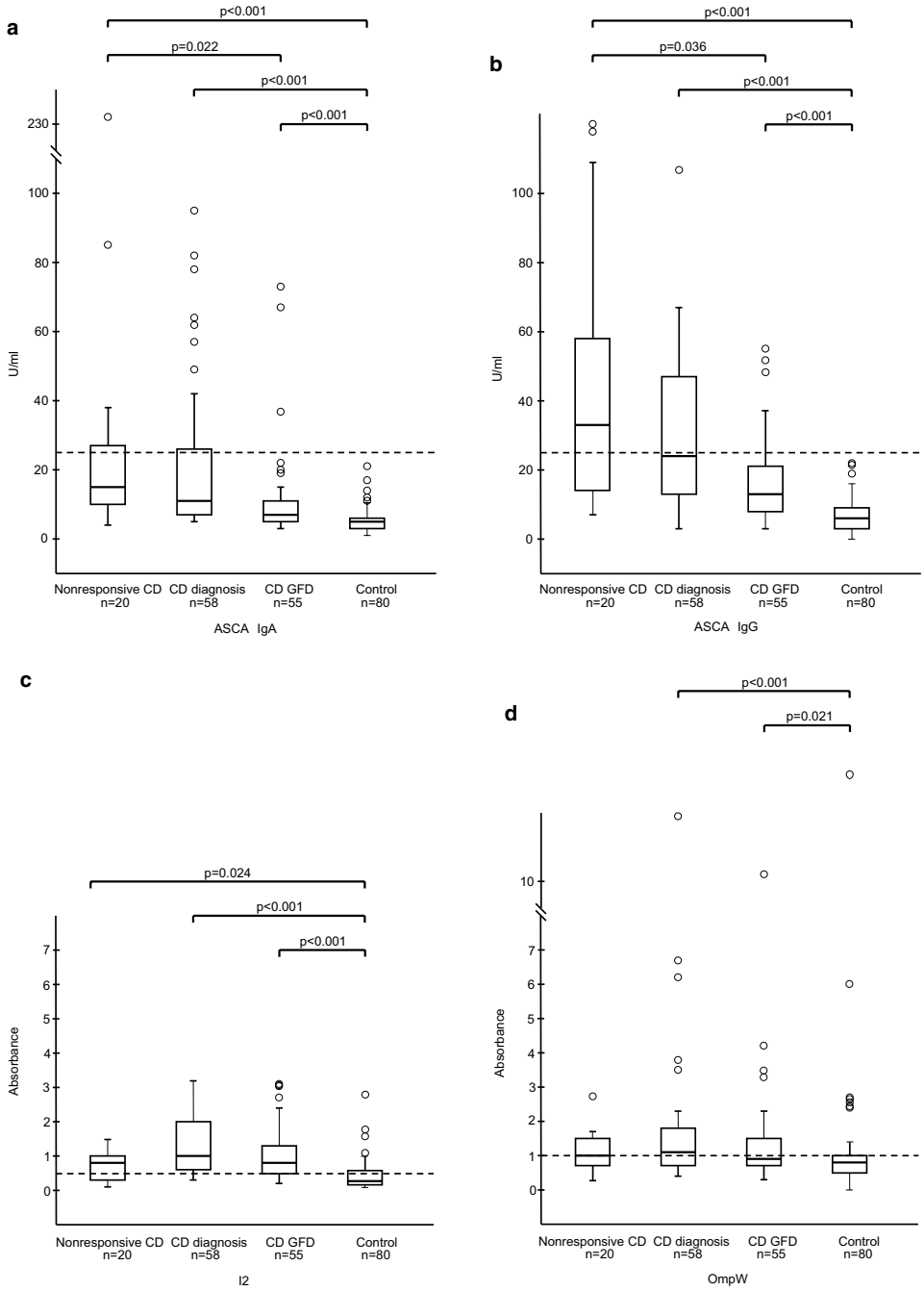

Fig. 2 Serum titers of Saccharomyces cerevisiae antibodies (ASCA) in IgA (**a**) and IgG (**b**) classes, Pseudomonas fluorescens-associated sequence (I2) (**c**) and Bacteroides caccae TonB-linked outer membrane protein (OmpW) (**d**) antibodies. Horizontal lines indicate the cutoff level for seropositivity of each antibody

patients also showed higher median ASCA levels than the GFD-responsive CD patients and non-CD controls. Besides ourselves, only Gross and colleagues have previously evaluated the role of ASCA in NRCD [26]. The authors measured IgA class ASCA titers in 35 CD patients and 27 non-CD controls and further divided the CD group into GFD-responsive ($n=26$) and nonresponsive ($n=9$). They found the median ASCA titers in the NRCD patients to be quite similar (17.3 U/ml) to our figures (14.5 U/ml). In contrast, Gross and associates observed higher ASCA titers both in the untreated (17.1 U/ml vs. 10.5 U/ml, respectively) CD patients and GFD-responsive (11.6 U/ml vs. 7.0 U/ml) treated patients, and also in non-CD controls (11.1 U/ml vs. 4.5 U/ml). One plausible explanation for these discrepancies between studies might be use of different commercial kits for the ASCA analyses. Furthermore, the selection of patients and controls and the definition of NRCD were not identical. For instance, the patients in the study by Gross and group had been on a GFD for a markedly shorter time and appeared to be only slow responders, as they had ongoing mucosal damage during the study but recovered later during follow-up [26].

In neither the present study nor in that by Gross and colleagues did the ASCA titers differ significantly between NRCD and untreated CD, very likely because NRCD patients continued to evince histological damage in the small-bowel mucosa. In contrast, both we and they found ASCA titers to decrease in CD patients responding to GFD, and the same has also been reported in a few previous studies [15–17]. Moreover, we have shown a positive correlation between the severity of small-bowel mucosal damage and ASCA titers [17]. The gluten sensitivity of ASCA and its association with the histological damage is of particular interest regarding the follow-up of celiac disease, since tTG-ab have shown limited sensitivity in the assessment of mucosal recovery [27]. Hence, new sensitive and non-invasive biomarkers for monitoring GFD are being eagerly sought.

Interestingly, seropositivity to ASCA has been shown to be less frequent in children than in adults with CD, very likely by reason of the shorter duration of gluten exposure and mucosal damage [16]. Further, even if increased, the ASCA titers in children also seem to decrease faster on GFD compared with adult patients [16]. On the other hand, RCD is practically nonexistent in children [28], indicating that the development of this severe condition requires long-term exposure to dietary gluten with ongoing small-bowel mucosal damage [4]. Gross and colleagues [26] investigated ten adults with type II RCD and found them to exhibit even higher serum ASCA titers than untreated CD patients, this finding supporting a possible relation between ASCA and disease severity. Interestingly, our data also showed a trend toward higher ASCA titers in NRCD than in CD at diagnosis. In fact in Crohn's disease, ASCA-positive patients have been reported to have a more aggressive clinical and endoscopic presentation with an increased risk of severe complications [11–13]. These findings indicate that positive ASCA signifies in general a more complicated disease course and poorer treatment response in both CD and IBD.

To our knowledge, this is the first study assessing serum antibodies to Pseudomonas fluorescens-associated sequence (I2) and Bacteroides caccae TonB-linked outer membrane protein (OmpW) in NRCD patients. In contrast to ASCA, NRCD patients did not show significantly higher I2 or OmpW titers than those in the GFD-responsive group, while seropositivity to these two markers was more frequent in untreated CD than in the NRCD and other study groups. In Crohn's disease, seroreactivity toward I2 is associated with severe fibrostenosing phenotype and a higher risk of small-bowel surgery [14]. Although we found I2 titers to be higher in NRCD than in healthy controls, there was no difference between the three CD groups, and the results do not support a major association between seroreactivity to I2 and the development of NRCD. OmpW was elevated only in the CD groups other than NRCD when compared to controls, suggesting that it might take a longer time for OmpW titers to normalize during GFD.

It has been suggested that complicated interactions between various environmental and genetic factors and gut microbiota together modulate the risk for celiac disease [29]. The role of luminal microbiota in these circumstances is supported by the finding that its composition differs between active CD and healthy individuals [30]. Interestingly, we have recently shown treated CD patients with persistent symptoms to have intestinal dysbiosis and reduced microbial diversity in comparison with GFD-responsive patients [10], and the present observation of elevated microbial antibodies in NRCD could reflect a similar ongoing imbalance in the microbiota. Nevertheless, it still remains unclear whether there is a causal connection between intestinal dysbiosis and the persistency of CD activity, or whether the altered microbiota simply reflects an ongoing pathologic process triggered by other factors.

Another intriguing question is how the microbial antigens enter into the systemic circulation and drive the production of the corresponding serum microbial antibodies in different gastrointestinal conditions. In IBD, it has been suggested that they enter the body via a disrupted intestinal mucosal barrier [31]. Similarly, even treated CD patients may have increased intestinal permeability when compared to healthy controls [32], this possibly offering a route for microbial products to enter the circulation. On the other hand, in Crohn's disease ASCA titers do not necessarily correlate with intestinal permeability [33, 34], and in the study by Gross and group no correlation was found between the degree of villous atrophy and mucosal permeability [26]. Moreover, we recently observed

seroreactivity to microbial markers already at stages of CD prior to marked histological damage [18], suggesting that there could be some other mechanism than mucosal disintegration driving the elevated serum microbial antibody levels.

The main strengths of this research were the well-defined groups of NRCD and regular CD patients. In particular, the original CD diagnoses and medical information were confirmed as well as possible, and NRCD patients had undergone extensive exclusion of coexisting conditions possibly causing nonresponsivity. In addition, more than one microbial marker was included to obtain a broader view of the microbial seroreactivity in CD. In fact, neither I2 nor OmpW have previously been studied in patients with NRCD or RCD. As a limitation, the microbial markers were not measured in NRCD patients at diagnosis, and it thus remains unclear whether the titers would have differed from the responsive patients at that point. The duration of GFD was also shorter in the responsive than nonresponsive group which could have influenced the serological outcomes. Only two NRCD patients developed abnormal lymphocyte phenotype, and we thus had no true RCD group. It must also be emphasized that, although the NRCD patients underwent extensive diagnostic workup, some conditions such as food allergies were not systemically ruled out. In addition, the causal relationship between microbial markers and CD activity could not be evaluated in the present study design and thus remains a subject for further research.

To conclude, we found seropositivity and high titers of ASCA to be associated with NRCD. This indicates that ASCA is associated with more severe disease course and poorer response to GFD and might thus serve as an additional noninvasive marker of histological recovery in CD.

Acknowledgments We would like to acknowledge Jonathan Braun, M.D., Ph.D, Professor and Chair, Pathology and Laboratory Medicine, UCLA David Geffen School of Medicine, UCLA Health System for his support.

Funding This study was supported by the Academy of Finland, the Sigrid Juselius Foundation, the Päivikki and Sakari Sohlberg Foundation, the Competitive State Research Financing of the Expert Area of Tampere University Hospital, the Foundation for Pediatric Research, the Yrjö Jahnsson Foundation and State fund, Grant number Y1023G2017.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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PUBLICATION III

First-degree Relatives of Celiac Disease Patients Have Increased Seroreactivity to Serum Microbial Markers

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Nutrients, 2020, 12(4), 1073
doi: 10.3390/nu12041073

nutrients

MDPI

Article

First-degree Relatives of Celiac Disease Patients Have Increased Seroreactivity to Serum Microbial Markers

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Received: 23 March 2020; Accepted: 8 April 2020; Published: 13 April 2020

Abstract: Risk of celiac disease (CD) is increased in relatives of CD patients due to genetic and possible environmental factors. We recently reported increased seropositivity to anti-*Saccharomyces cerevisiae* (ASCA), *Pseudomonas fluorescens*-associated sequence (anti-I2) and *Bacteroides caccae* TonB-linked outer membrane protein (anti-OmpW) antibodies in CD. We hypothesized these markers also to be overrepresented in relatives. Seropositivity and levels of ASCA, anti-I2 and anti-OmpW were compared between 463 first-degree relatives, 58 untreated and 55 treated CD patients, and 80 controls. CD-associated human leukocyte antigen (HLA)-haplotypes and transglutaminase (tTGab) and endomysium (EmA) antibodies were determined. One or more of the microbial antibodies was present in 75% of relatives, 97% of untreated and 87% of treated CD patients and 44% of the controls. The relatives had higher median ASCA IgA (9.13 vs. 4.50 U/mL, $p < 0.001$), ASCA IgG (8.91 vs. 5.75 U/mL, $p < 0.001$) and anti-I2 (absorbance 0.74 vs. 0.32, $p < 0.001$) levels than controls. There was a weak, positive correlation between tTGab and ASCA ($r = 0.31$, $p < 0.001$). Seropositivity was not significantly associated with HLA. To conclude, seropositivity to microbial markers was more common and ASCA and anti-I2 levels higher in relatives of CD patients than controls. These findings were not associated with HLA, suggesting the role of other genetic and environmental factors.

Keywords: celiac disease; relatives; microbiota; *Saccharomyces cerevisiae*; *Pseudomonas fluorescens*; *Bacteroides caccae*

1. Introduction

Celiac disease (CD) is an immune-mediated condition characterized by gluten-induced small-bowel enteropathy. Almost all patients carry human leukocyte antigen (HLA) alleles encoding DQ2 or DQ8 heterodimers [1]. These alleles are nevertheless also present in up to 35% of the general population and do not fully explain the genetic risk [2]. Recent genome-wide association studies and immunogenetic studies have identified numerous non-HLA loci and single nucleotide polymorphisms that may modify CD risk [3,4]. Partly due to shared genetic predisposition, the relatives of patients

have an increased susceptibility to CD, the average prevalence among first-degree relatives being approximately 8% [5] compared with 1%–2% in the general population [6,7].

However, only a minority of at-risk individuals develop CD, and the concordance even varies between identical twins [8,9], which implicates environmental factors. The prevalence may also vary between adjacent countries with similar genetic backgrounds and gluten consumption [10], and retrospective measurements of stored samples indicate a rise in the true incidence [6,11,12]. As one potentially associated factor, the role of intestinal microbiota in the development of CD has aroused particular interest [13–15]. Previously, we and others observed elevated levels of antibodies to microbial markers *Saccharomyces cerevisiae* (ASCA), *Pseudomonas fluorescens*-associated sequence (anti-I2) and *Bacteroides caccae* TonB-linked outer membrane protein (anti-OmpW) in inflammatory bowel disease [16–18]. We have shown increased seroreactivity to these markers also in overt CD [19] and a decrease of the antibody levels during gluten-free diet (GFD) [20]. Further, these microbial markers are detectable in early stages of the disease even before the presence of villous atrophy and serum CD-specific autoantibodies [21].

We hypothesized that close relatives of CD patients, with partially shared living environments and genetic factors, could have increased seroreactivity to microbial markers. This was investigated by comparing their frequency of seropositivity and levels of microbial antibodies with those in untreated and treated CD patients and in healthy controls.

2. Materials and Methods

2.1. Study Participants

The study was carried out at Tampere University and Tampere University Hospital. Previously diagnosed CD patients were recruited in a nationwide search through newspaper advertisements and via patient societies. Their medical records were obtained with permission, and only subjects with a biopsy-proven diagnosis were included. Relatives of these patients were invited to a screening study comprising personal interviews and measurement of CD serology. Additional blood samples were drawn for research purposes. Exclusion criteria for the relatives were previously diagnosed CD or dermatitis herpetiformis, or otherwise initiated gluten-free diet (GFD). Altogether, 3031 relatives met the inclusion criteria and entered the original screening study. Duodenal biopsy was offered for all relatives with positive CD serology. For the present study, serum samples from 463 first-degree relatives were randomly selected for the measurement of ASCA, anti-I2 and anti-OmpW. The CD control group comprised 58 biopsy-proven patients who underwent measurements of the CD serology and microbial markers at diagnosis and after one year on GFD (n = 55). In addition, 80 adult blood donors with negative CD serology served as non-CD controls.

2.2. CD Autoantibodies and Genotyping

Serum immunoglobulin A (IgA) class endomysium autoantibodies (EmA) were tested by an indirect immunofluorescence method using human umbilical cord as substrate [22]. Titers 1: ≥ 5 were deemed positive and diluted up to 1:4000 or until negative. Serum IgA class tissue transglutaminase autoantibodies (tTGab) were measured by an enzyme-linked immunosorbent assay (ELISA, INOVA diagnostics, San Diego, CA) according to the manufacturer's instructions. A cutoff ≥ 30 U/mL was applied for seropositivity. Some of the CD autoantibody-positive relatives declined the biopsy, but, due to the high specificity of EmA/tTGab [23], the vast majority of them are also likely to have CD. They were therefore analyzed as a separate group.

The CD-associated HLA DQ haplotypes (DQ2.5, DQ2.2, DQ8) were determined from the relatives and CD patients with the tagging single nucleotide polymorphism method or with the Olerup SSP DQ low-resolution kit (Olerup SSP AB, Stockholm, Sweden) as described elsewhere [24,25].

2.3. Microbial Antibodies

Serum IgA and IgG class ASCA were measured by a commercial ELISA (Quanta Lite ASCA, INOVA Diagnostics Inc., San Diego, CA) considering levels ≥ 25 U/mL positive. *E. coli* XL-1 blue and *E. coli* BL-21 (Stratagene, La Jolla, CA) strains and previously reported antigen purification techniques [26,27] were used to produce I2-GST and OmpW antigens. The serum samples were diluted 1:50, and IgA anti-I2 and anti-OmpW antibodies were measured with an in-house ELISA. For anti-I2, the cutoff level for positivity was set at absorbance 0.5. For anti-OmpW, it was set at 0.6 in children and 1.0 in adults based on our previous studies showing age differences in the normal range [16,19].

2.4. Statistical Analysis

Quantitative data are shown in tables as percentages or as medians with lower and upper quartiles. The data were cross-tabulated in order to ascertain the overlap of seropositivity for microbial antibodies in different study groups. The Kruskal–Wallis test was used to compare the differences in microbial antibody levels between the groups. Correlations between autoantibodies and microbial markers were tested with Spearman's rank correlation coefficient. Associations in the seropositivity to microbial antibodies within and between the families were also tested. The chi-square statistic for the change in the -2 log-likelihood from the constant only model to the model with "family" was used to determine whether the inclusion of "family" contributed significantly to model fit. A p value < 0.05 was considered significant. Statistical analyses were carried out with SPSS Statistics for Windows (IBM Corp., Armonk, NY, USA).

2.5. Ethical Aspects

The study protocol was approved by the Ethics Committee of the Pirkanmaa Hospital District, study identification code ETL R05183. All participants or, in the case of children, their legal guardians gave written informed consent. The paper follows the rules of the Declaration of Helsinki.

3. Results

The gender distribution was fairly equal among the relatives, whereas a majority of CD patients were women, and there were more men in the non-CD control group (Table 1). There were no major differences in the median ages between the groups (Table 1), but 49 (10.6%) of the relatives were <18 years of age, while the other groups comprised only adults.

Table 1. Demographic data on relatives of celiac disease (CD) patients, CD patients and non-celiac controls.

	Seropositive Relatives	Seronegative Relatives *	CD at Diagnosis	CD on GFD	Non-CD Controls
	$n = 49$	$n = 414$	$n = 58$	$n = 55$	$n = 80$
Females, %	42.9	57.2	77.6	76.4	35.0
Age, median (quartiles), y	41 (31–54)	42 (28–59)	45 (36–59)	46 (38-60)	41 (31–56)

* Negative serum endomysium (titer 1: < 5) and tissue transglutaminase (< 30 U/mL) antibodies. GFD, gluten-free diet.

The relatives were divided into CD autoantibody-negative ($n = 414$) and autoantibody-positive ($n = 49$) groups and were analyzed separately (Table 1). Among the autoantibody-negative relatives, seropositivity for at least one of the microbial markers was more common than in the non-CD controls but less frequent than in the CD patients (Figure 1). The most notable difference was seen in ASCA,

as 19% of the relatives without CD-autoantibodies and none of the controls were seropositive for ASCA IgA, ASCA IgG, or both. In addition, anti-I2 and anti-OmpW positivity was more common among the autoantibody-negative relatives than controls (61% and 40% vs. 31% and 24%, respectively; Figure 1).

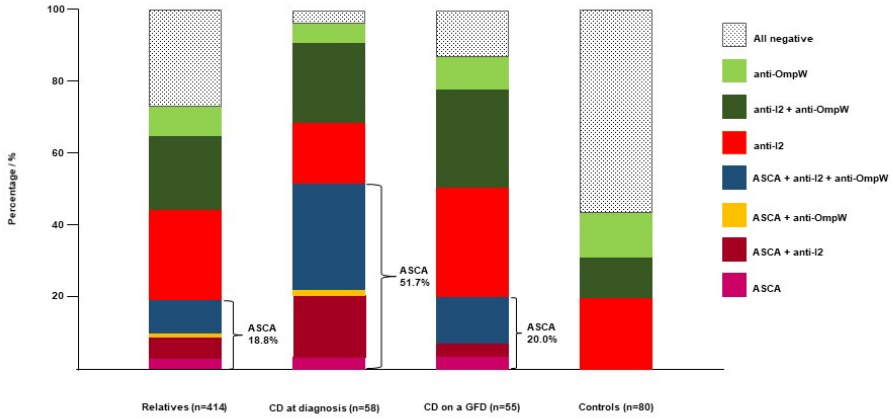

Figure 1. Distribution of seropositivity to antibodies against *Saccharomyces cerevisiae* (ASCA), *Pseudomonas fluorescens*-associated sequence (anti-I2 antibodies) and *Bacteroides caccae* TonB-linked outer membrane protein (anti-OmpW antibodies) among autoantibody-negative relatives of celiac disease (CD) patients, CD patients (at diagnosis and on a GFD) and controls.

The median levels of ASCA IgA, ASCA IgG and anti-I2 were also significantly higher in the autoantibody-negative relatives than those in the control group (Figure 2a–c), whereas anti-OmpW was higher only in untreated and treated CD patients (Figure 2d). ASCA IgG was higher in both untreated and treated CD patients and anti-I2/OmpW in untreated patients when compared with autoantibody-negative relatives (Figure 2b–d).

Altogether, 46 out of the 49 autoantibody-positive relatives had HLA-DQ2 haplotype, DQ8 haplotype, or both. As many as 86% of them showed seroreactivity to at least one microbial marker compared to 73% of the CD antibody-negative relatives, and the median levels of the microbial antibodies were also higher (ASCA IgA 11.1 vs. 8.90 U/mL, $p = 0.019$; ASCA IgG 12.8 vs. 8.37 U/mL, $p = 0.001$; absorbance for anti-I2 0.93 vs. 0.71, $p = 0.320$ and for anti-OmpW 1.00 vs. 0.81, $p = 0.022$, respectively). In contrast to the autoantibody-negative group, anti-OmpW levels were also significantly higher than in the controls (absorbance 0.79, $p = 0.043$).

Adjusting for age and gender or exclusion of children from the comparisons did not affect the results of the prevalence of seropositivity nor median levels of the microbial markers, although the medians were significantly lower in children than in adults (ASCA IgA 6.30 vs. 9.64 U/mL, $p < 0.001$; ASCA IgG 7.13 vs. 9.18 U/mL, $p = 0.070$; absorbance for anti-I2 0.34 vs. 0.79, $p < 0.001$ and for anti-OmpW 0.54 vs. 0.87, $p < 0.001$, respectively).

Seropositivity to anti-I2 and anti-OmpW was significantly more frequent between relatives in the same family than between different families ($p < 0.001$ for anti-I2 and $p = 0.001$ for anti-OmpW, respectively). In ASCA, this was observed only when autoantibody-positive relatives were also included in the analysis ($p = 0.007$).

There were no significant differences in the distribution of seropositivity across microbial markers when the relatives were categorized according to their HLA haplotypes (Table 2).

There was a weak, positive correlation between the values of tTGab and ASCA IgA (r = 0.31, $p < 0.001$), whereas correlation coefficients between the other microbial markers and tTGab or EmA were <0.3.

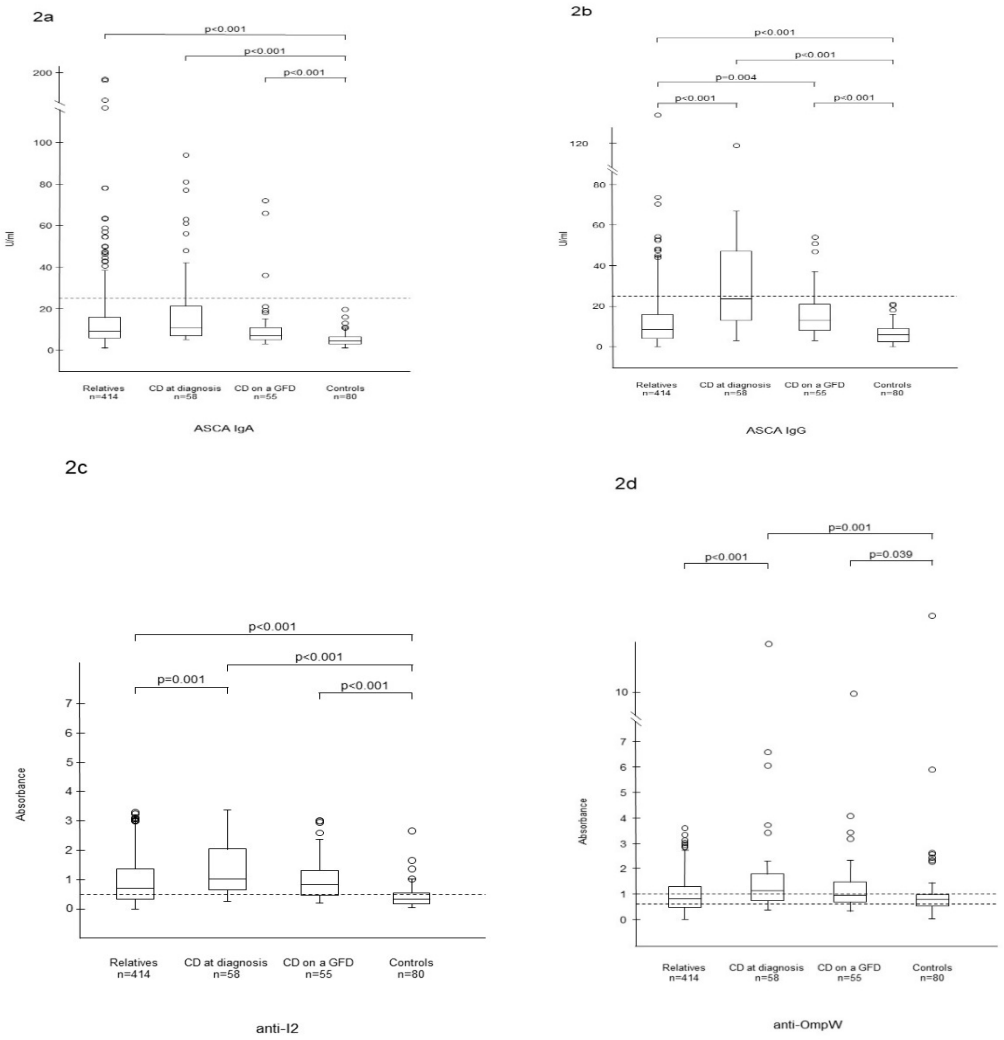

Figure 2. Serum levels of antibodies to *Saccharomyces cerevisiae* (ASCA) in IgA (**a**) and IgG (**b**) classes, *Pseudomonas fluorescens*-associated sequence (anti-I2) (**c**) and *Bacteroides caccae* TonB-linked outer membrane protein (anti-OmpW) (**d**) in autoantibody-negative relatives. Horizontal lines indicate the cutoff level for seropositivity of each antibody.

Table 2. Frequency of seropositivity to microbial markers in autoantibody-negative relatives of celiac disease patients with different human leukocyte antigen (HLA) haplotypes.

	DQ2 ***n* = 233**	**DQ8** ***n* = 67**	**DQ2 + DQ8** ***n* = 8**	**DQ2/8 Negative** ***n* = 89**
	%	%	%	%
ASCA IgA	11.2	10.4	12.5	10.1
ASCA IgG	12.9	13.4	0	14.6
Anti-I2	58.4	61.2	75.0	66.3
Anti-OmpW	39.5	35.8	25.0	43.8

ASCA, Anti-*Saccharomyces cerevisiae* antibodies; anti-I2, antibodies to *Pseudomonas fluorescens*-associated sequence; anti-OmpW, antibodies to *Bacteroides Caccae* TonB-linked outer membrane protein; DQ2, HLA-DQA1*05-DQB1*02 (DQ2.5) or HLA-DQA1*02-DQB1*02 (DQ2.2); DQ8, HLA-DQA1*03-DQB1*0302. There were no statistically significant differences between the groups in the distribution of seropositivity.

4. Discussion

The main finding of the present study was increased seroreactivity to microbial markers in the relatives of CD patients compared with controls even after the exclusion of CD autoantibody-positive individuals. This was observed particularly with ASCA and anti-I2, the median levels of which were also significantly higher than levels in the controls, although they were lower than in CD patients. To the best of our knowledge, the only study to report on this issue so far was a conducted by Da Silva et al., who investigated seropositivity to ASCA in relatives of CD patients [28]. They divided 76 relatives into EmA/tTGab negative and positive groups, while 57 individuals with negative CD autoantibodies and no family risk served as controls. Partly in contrast to us, there was a significantly higher frequency of positivity to ASCA IgA/G only in autoantibody-positive relatives compared with the controls [28]. This discrepancy may, at least in part, be explained by the smaller number of participants since there was a trend toward overrepresentation of ASCA, also among the CD autoantibody-negative relatives. There may also have been methodological differences, as the authors did not report the kits used for the ASCA measurements.

Owing to the high specificity of tTGab and EmA [23], most of the autoantibody-positive relatives were likely CD patients. Therefore, their increased seroreactivity to microbial markers is logically in line with that observed in already-diagnosed CD. By contrast, the increased frequency of seroreactivity to a part of the microbial markers in the autoantibody-negative relatives is not as easily explained. It is to be noted that Setty and colleagues [29] previously reported that tTGab-negative relatives of CD patients had signs of intestinal epithelial stress, demonstrated by ultrastructural alterations of microvilli, and increased expression of heat shock proteins and interleukin-15 along with elevated expression of activating NK receptors on intraepithelial cytotoxic T cells. Thus, even in the absence of CD autoantibodies or characteristic histological damage to the intestine, at least some of the relatives appeared to display proinflammatory responses reminiscent of CD. This raises the question of whether the observed abnormal microbial antibody production could also be implicated in this process.

Setty et al. also speculated about a possible genetic predisposition to epithelial stress [29] and suggested a possible HLA and other as yet-unidentified genetic associations. We observed no significant association between the distribution of ASCA, anti-I2 and anti-OmpW positivity and the CD-related HLA haplotypes, suggesting that at least HLA genetics does not markedly affect the serological response. In line with this, HLA DQ2/8 are not overexpressed in inflammatory bowel disease (IBD) patients [30] who also may have increased seropositivity to microbial markers [16,17]. Genetics may still play a role in microbial antibody production in intestinal diseases, as demonstrated by two studies comparing levels of microbial antibodies between monozygous and dizygous twin pairs with IBD. Amcoff et al. reported that the differences in the anti-I2 antibody levels were smaller within than between monozygous twin pairs, even if only one of them had IBD [31]. However, this was not seen in dizygous twins with one suffering from IBD and the other being healthy and having partly discordant genetics, supporting the role of genetic factors [31]. By contrast, similar ASCA levels were observed only in a subgroup of monozygous twins both having IBD [31,32]. Bearing this in mind, it is interesting that we found stronger associations of anti-I2 positivity between the relatives from the same family than between the families, whereas with ASCA this was seen only when autoantibody-positive relatives were included in the analysis. Taken together, it seems that both genetic and environmental factors have a role in the antibody production, with this varying depending on the microbial marker, but further studies are needed.

Environmental factors including gluten intake [33,34] and infections in early life [35–37] have also been associated with increased CD risk. Other suggested, although controversial [38,39], risk factors include bacterial infections and frequent use of antibiotics [40,41]. Interestingly, the incidence has been reported to vary depending on socioeconomic circumstances [10], leading to the hypothesis that slight microbial exposure increases CD risk by driving immune reactions toward autoantigens and dietary components [42]. Close relatives usually share the living milieu and may, thus, experience similar environmental modulatory effects on the microbiota and immune system that, in addition to genetics,

could give rise to parallel responses to microbial antigens. It remains unclear, however, which external factors drive these responses and whether the microbial markers have a causal role [43]. It is likely that a complex interaction between multiple factors, such as dysregulation of the immune system, changes in the epithelial barrier, and dysbiosis causes the loss of tolerance to microbial antigens [13,44–46]. In addition, a very recent study showed that *Pseudomonas fluorescens* peptides mimic gluten epitopes and activate gliadin-reactive T cells, with this cross-reactivity possibly contributing to the onset of CD [47].

We previously found most of the potential CD patients to already exhibit the microbial markers before the development of villous damage or autoantibodies [21], reflecting the situation in the relatives in the present study. Interestingly, Torres and colleagues recently showed that ASCA also predicts forthcoming Crohn's disease up to five years before the diagnosis [48]. More studies are needed to determine the role of these markers in early development of CD and whether they could be utilized to predict the disease in at-risk groups.

The main strengths of our study include the large and well-defined cohort of relatives of CD patients who underwent systematic screening for CD-associated HLA and autoantibodies and the representative control groups. As a weakness, however, large differences between the group sizes could have influenced the results. Furthermore, only the groups with relatives contained pediatric subjects, although the results remained unchanged after excluding children from the analyses. Genetic data of the non-HLA alleles were also lacking, which could be an even more significant limitation among relatives with a less marked HLA predisposition to CD. Since we did not have detailed information on the health condition of the relatives, and the histological status of their intestines remains unknown, it is possible that some of them had unreported CD or another disease affecting the results. Furthermore, dietary data of the relatives was lacking, and it is possible that cross-reactions between food antigens influenced the microbial antibody levels. ASCA is known to cross-react with other yeast strains [49], and the lack of correlation between ASCA antibodies and *Saccharomyces cerevisiae* DNA on intestinal mucosa [50] indicates the possibility of some yet-unidentified cross-reactive antigens. In accord with our previous study [51], for currently unclear reasons, ASCA levels were generally higher in the IgG class than the IgA class. By contrast, IgA class ASCA seems to be more consistently elevated in IBD [48,52]. Which of these two antibody classes is the more useful marker in CD would be an interesting subject for further research. The median duration of GFD in the CD group was only one year, which may have biased the serological results, as histological and serological recovery often take longer despite a strict diet [53]. Finally, a few adults here had surprisingly high anti-OmpW values compared with our previous studies. Although we still believe that the used cutoff was valid, we recommend that it be confirmed in other populations.

In conclusion, we found increased seroreactivity to serum microbial markers, particularly ASCA and anti-I2, in relatives of CD patients even in the absence of the disease-specific autoantibodies or other signs of active CD. This observation was not explained by the presence or absence of predisposing HLA haplotypes, thereby suggesting the role of other genetic and environmental factors.

Author Contributions: L.V.: Conceptualization, writing the original draft; S.I.: Conceptualization, supervision, writing—review and editing; H.H.: Conceptualization, methodology, writing—review and editing; P.S.: Conceptualization, methodology, writing—review and editing; K.K. (Katri Kaukinen): Conceptualization, funding acquisition, writing—review and editing; K.L.: Conceptualization, methodology, funding acquisition, writing—review and editing; K.K. (Kalle Kurppa): Conceptualization, funding acquisition, supervision, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Academy of Finland, the Finnish Medical Foundation, the Sohlberg Foundation, the Paulo Foundation, the Sigrid Juselius Foundation, the Foundation for Pediatric Research, the Competitive State Research Financing of the Expert Area of Tampere University Hospital, Helsinki University Hospital Research Fund, the Finnish Medical Foundation and the Finnish Coeliac Society.

Acknowledgments: We would like to acknowledge Jonathan Braun, Pathology and Laboratory Medicine, UCLA David Geffen School of Medicine, UCLA Health System for his support.

Conflicts of Interest: The authors declare no conflicts of interest.

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