Diagnostics and genetics in coeliac disease

Victorien Wolters

ISBN: 978-90-9024470-9

Diagnostics and genetics in coeliac disease

Thesis, University of Utrecht, with a summary in Dutch

Print: Gildeprint Drukkerijen, Enschede, The Netherlands
Lay out: Barbara Hagoort, Multimedia, UMC Utrecht
Cover: Vrije interpretatie van darmvlokken

Diagnostics and genetics in coeliac disease

Diagnostische en genetische aspecten van coeliakie (with a summary in Dutch)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 3 november 2009 des middags te 2.30 uur

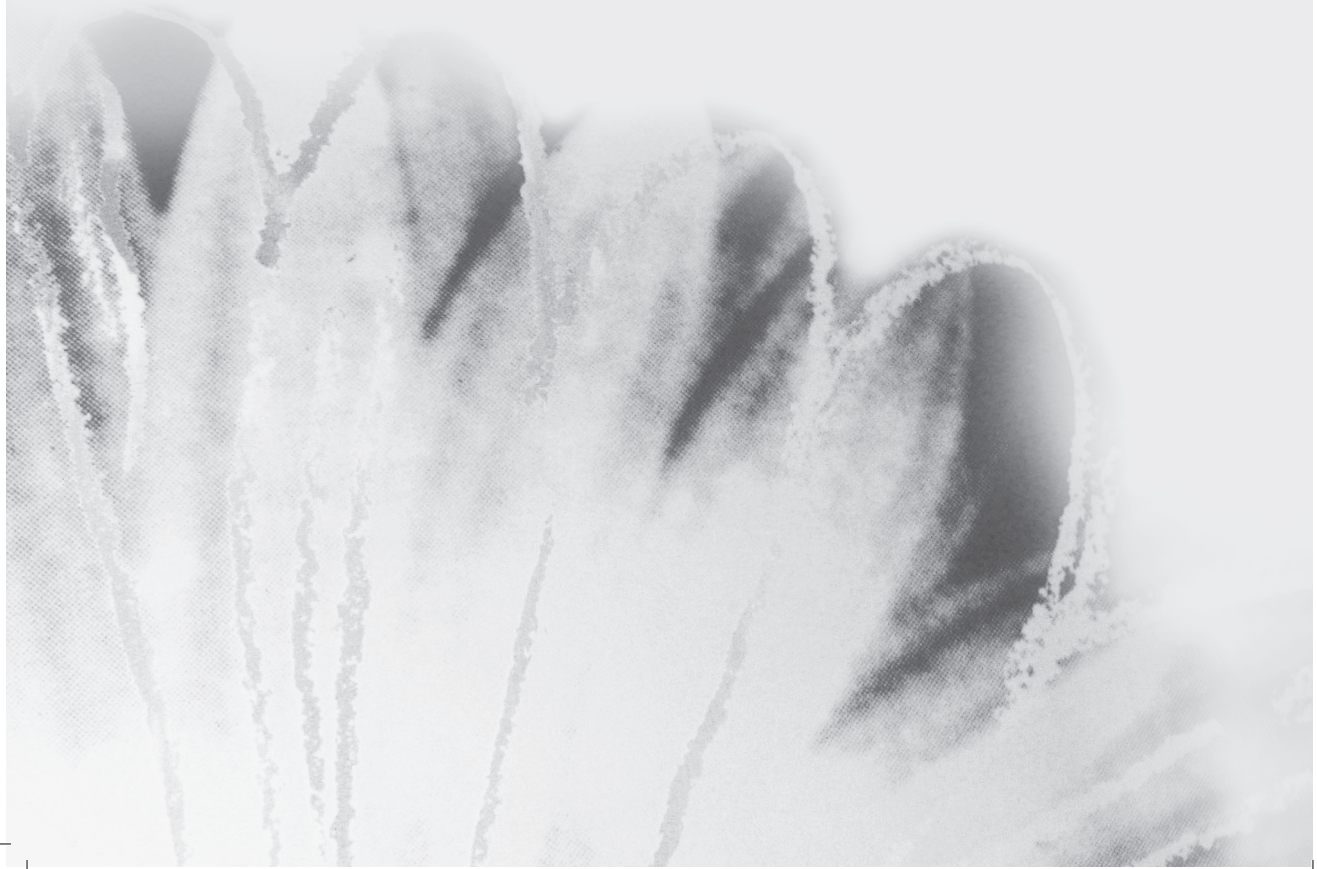
door

Victorine Maria Wolters

geboren op 19 december 1973
te Leidschendam

Promotoren:	Prof.dr. C. Wijmenga
	Prof.dr. J.L.L. Kimpen
Copromotor:	Dr. R.H.J. Houwen

Contents



Chapter 1

General introduction and outline of the thesis

General introduction

Coeliac disease (CD) is a common enteropathy of the gut with a prevalence of ~1%.[1] CD is caused by ingestion of gluten and only occurs in individuals with a genetic susceptibility. Treatment consists of a life-long gluten free diet. In recent years research interests focused on the improvement of diagnostic tests to screen patients for CD, and a better understanding of the interactions between environmental, genetic and immunologic factors.

Serologic tests and diagnosis

At present, the gold standard for the diagnosis of CD is an intestinal biopsy. Serologic tests are used to help identify children who require a small intestinal biopsy to confirm the diagnosis of CD. Initially, antifood antibodies against gliadin (IgG and IgA) were discovered in 1964 by Berger.[2] A few years later antireticuline (IgA) antibodies were identified as the first auto-antibodies.[3] A major step forward was made in 1984 when anti-endomysium antibodies (EMA) were described for the first time and subsequently became widely used because of their high specificity.[4-7] However, the technique for performing EMA is considered subjective and requires extensive training in the immunofluorescence microscopy. Therefore a new test was hoped for to overcome the need of technical skills necessary for EMA determinations. In 1997 Dieterich *et al* identified the enzyme tissue transglutaminase (tTG) as the main endomysial autoantigen.[8;9] An ELISA was newly developed which initially used guinea-pig tTG[10] and subsequently human tTG as an antigen. The IgA-tTG might better identify patients with CD than the former tests and could be useful for the monitoring of compliance with the gluten free diet once the diagnosis has been confirmed.[11-13] The first papers describing the results of IgA-tTG as a diagnostic tool for CD were promising, but not in all studies the control group was biopsied, making it difficult to calculate a reliable sensitivity and specificity.[12;13]

IgA-tTG is an immunologic (indirect) marker for small intestinal damage and correlates with mucosal changes in CD. Nevertheless the diagnosis of CD is not certain in patients with an abnormal IgA-tTG. Consequently, mucosal biopsies of the proximal small bowel are necessary to confirm the diagnosis. However, a new test, serum I-FABP, might be a direct marker of gluten induced damage to the small intestine in CD, and could therefore potentially be useful in the non-invasive diagnosis of CD.[14-16]

Confirmation of the diagnosis of CD requires at least one intestinal biopsy in all cases. After revision of the diagnostic criteria in 1990 a gluten challenge (i.e. a second and third biopsy) was deemed necessary only in children in whom the initial diagnosis was made below the age of 2 years.[17;18] Gluten challenge was not mandatory anymore in children two years or older at diagnosis nor in adults. This revision was partially based on a large study by Guandalini *et al*, who showed that in a small percentage of patients (~5%, 123/ 2523 patients) another diagnosis was made following gluten challenge.[19] The mean age of these non-confirmed coeliac patients was 8 months so the revised criteria recommended gluten challenge only for children aged two years or younger at diagnosis. However, the actual yield of a gluten challenge in this group seems to be low, as even in this age group only very

few diagnoses have to be revised.[19] One of the reasons is the improved reliability of serologic tests for CD. In a child with unequivocal histology and abnormal serology, presently many paediatricians will not use routine gluten challenge anymore to confirm the diagnosis in very young children. The question is whether there is evidence for this change in attitude.

Genetic factors

Only genetically susceptible individuals may develop CD. The strongest genetic factor that is associated with CD is HLA-DQ2 and -DQ8 and is found in virtually all CD patients.[20;21] The role of HLA in diagnosis is limited as ~30-40 % of the general population also carries HLA-DQ2 or -DQ8.[22] However, absence of HLA-DQ2 or -DQ8 virtually excludes the diagnosis of CD.[20;21] In addition twin and family studies strongly suggest an important role for non HLA genetic risk factors.[23] A number of disease susceptibility loci have been identified but many of these could not be replicated in other populations. In this thesis we focus on three genes (*PARD3, MYO9B, MAGI2*) that were found to be associated with CD.[24;25] These genes might have a role in regulating intestinal permeability, but evidence is far from complete at present.[26-28] In selected populations with supposedly an increased intestinal permeability this hypothesis might be tested further, e.g. Down syndrome (DS) patients. Up to 20% of DS patients have increased levels of antigliadin antibodies (AGA) without CD, far more than the general population.[29-33] These elevated AGA levels might be associated with an increased intestinal permeability.[34;35]

Genetic risk factors might also be important in the 2-5% of adult patients in whom, for unknown reasons the mucosa does not recover on a gluten free diet: refractory coeliac disease (RCD).[36] Two types of RCD can be recognized; RCD I and RCD II. Especially the diagnosis of RCD II has prognostic implications, as approximately half of these patients develop an enteropathy-associated T-cell lymphoma (EATL) within 5 years, whereas RCD I patients seldom develop an EATL.[37;38]

Aims of this thesis

This thesis consists of two parts. The aim of the first part is to analyze new diagnostic tests in the diagnosis of CD and to evaluate current diagnostic criteria. The aim of the second part is to analyze various genetic variants in different CD populations and to investigate whether suspected permeability genes are associated with elevated antigliadin antibodies as an indirect marker for increased intestinal permeability.

Outline of this thesis

This thesis starts with an introduction and outline of the thesis (**chapter 1**). The history of CD and the great breakthrough of paediatrician Willem Karel Dicke of the Wilhelmina Children's hospital in the treatment of this disease is described in **chapter 2**. In his thesis in 1950 he showed that the exclusion of wheat and rye lead to dramatic improve of the condition of the child and till now this is the treatment of this disease.[39;40]

Part 1. Diagnostics

In **chapter 3** the sensitivity and specificity of the tissue transglutaminase (tTG) and EMA were determined in a group of patients and controls who were all biopsied. Subsequently we investigate in **chapter 4** whether the determination of the serum level of intestinal fatty acid binding protein I-FABP can improve the results of tTG and or EMA. Furthermore, we tried to analyze if both markers are a reliable tool in the follow up of CD after introduction of the gluten free diet. In **chapter 5**, the current diagnostic criteria for the diagnosis of CD are evaluated. The ESPGHAN criteria state that in every child initially diagnosed under the age of two years, a gluten challenge is necessary. However in our experience in very few patients the diagnosis has to be revised subsequently. We therefore retrospectively investigated the results of 100 gluten challenges in children diagnosed under the age of 2 years to find scientific proof for abandoning a routine gluten challenge in this group of children.

Part 2. Genetics

Until recently the only well replicated genetic association with CD was the HLA region. In recent years non-HLA associations were identified as well. In **chapter 6** we give an overview of the recent genetic developments and the implications for clinical practice. In **chapter 7** we investigated whether the newly found *MYO9B* gene is a risk factor for the development of refractory coeliac disease (RCD) type II and enteropathy-associated T-cell lymphoma (EATL). We also investigated a possible interaction between *MYO9B* and HLA in RCD II and EATL. In **chapter 8** we analyzed three newly found non-HLA regions (*PARD3, MYO9B and MAGI2)* in a population of patients with Down syndrome (DS), who supposedly have an increased intestinal permeability. It was hypothesized that intestinal permeability, as measured by the level of antigliadin antibodies, was associated with specific variants in the three genes mentioned.

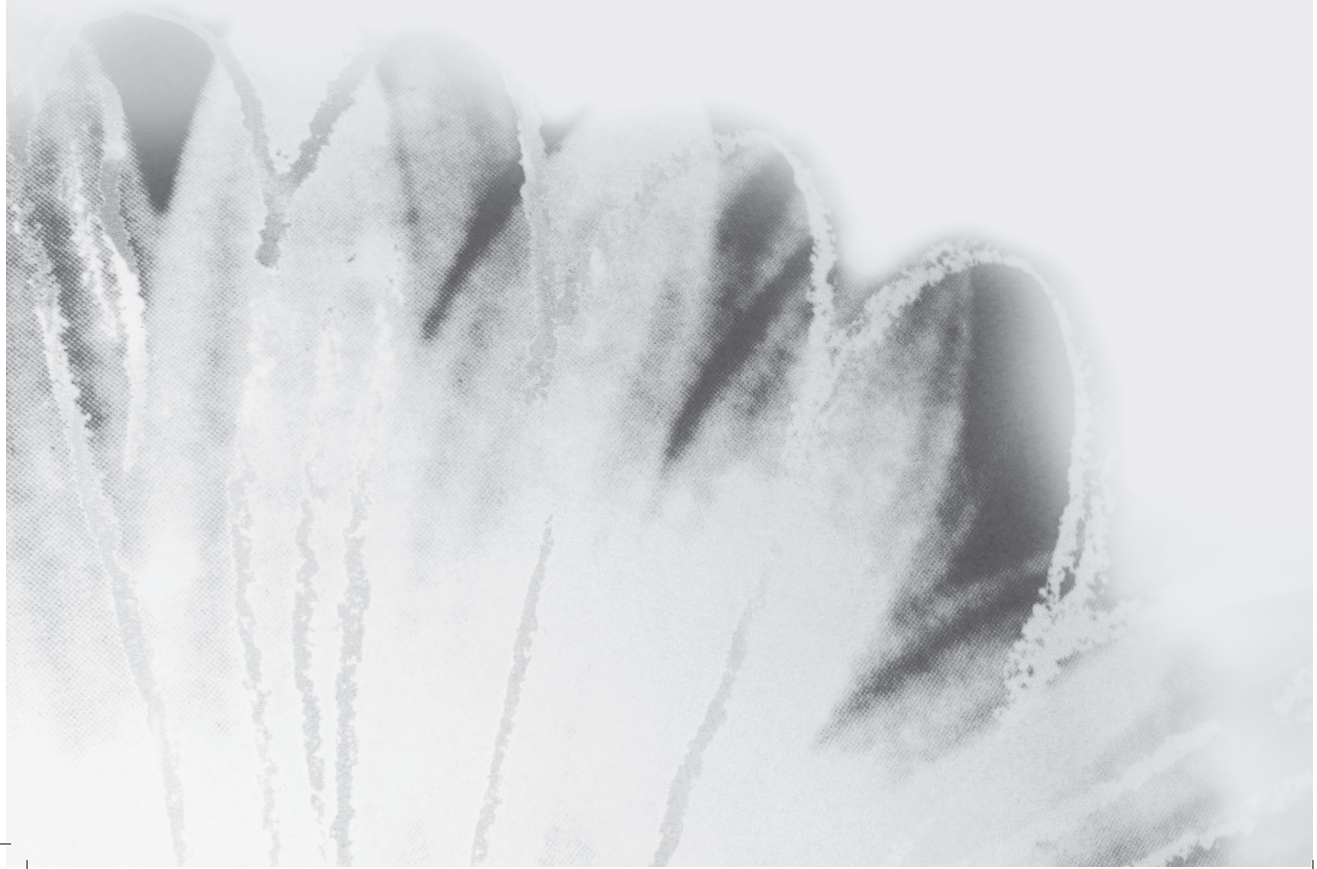
This thesis concludes with a summary and general discussion (**chapter 9**) and future directions will be given.

References

1. National Institutes of Health Consensus Development Conference Statement on Celiac Disease, June 28-30, 2004. *Gastroenterology* 2005;**128**(4 Suppl 1):S1-S9.
2. BERGER E, BUERGIN-WOLFF A, FREUDENBERG E. [DIAGNOSTIC VALUE OF THE DEMONSTRATION OF GLIADIN ANTIBODIES IN CELIAC DISEASE.]. *Klin Wochenschr* 1964;**42**:788-90.
3. Seah PP, Fry L, Rossiter MA *et al*. Anti-reticulin antibodies in childhood coeliac disease. *Lancet* 1971;**2**(7726):681-2.
4. Burgin-Wolff A, Gaze H, Hadziselimovic F *et al*. Antigliadin and antiendomysium antibody determination for coeliac disease. *Arch Dis Child* 1991;**66**(8):941-7.
5. Rossi TM, Tjota A. Serologic indicators of celiac disease. *J Pediatr Gastroenterol Nutr* 1998;**26**(2):205-10.
6. Russo PA, Chartrand LJ, Seidman E. Comparative analysis of serologic screening tests for the initial diagnosis of celiac disease. *Pediatrics* 1999;**104**:75-8.
7. Chorzelski TP, Beutner EH, Sulej J *et al*. IgA anti-endomysium antibody. A new immunological marker of dermatitis herpetiformis and coeliac disease. *Br J Dermatol* 1984;**111**(4):395-402.
8. Dieterich W, Ehnis T, Bauer M *et al*. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med* 1997;**3**(7):797-801.
9. Dieterich W, Laag E, Schopper H *et al*. Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* 1998;**115**(6):1317-21.
10. Sulkanen S, Halttunen T, Laurila K *et al*. Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterology* 1998;**115**(6):1322-8.
11. Burgin-Wolff A, Dahlbom I, Hadziselimovic F *et al*. Antibodies against human tissue transglutaminase and endomysium in diagnosing and monitoring coeliac disease. *Scand J Gastroenterol* 2002;**37**(6):685-91.
12. Sardy M, Odenthal U, Karpati S *et al*. Recombinant human tissue transglutaminase ELISA for the diagnosis of gluten-sensitive enteropathy. *Clin Chem* 1999;**45**(12):2142-9.
13. Sblattero D, Berti I, Trevisiol C *et al*. Human recombinant tissue transglutaminase ELISA: an innovative diagnostic assay for celiac disease. *Am J Gastroenterol* 2000;**95**(5):1253-7.
14. Derikx JP, Evennett NJ, Degraeuwe PL *et al*. Urine based detection of intestinal mucosal cell damage in neonates with suspected necrotising enterocolitis. *Gut* 2007;**56**(10):1473-5.
15. Derikx JP, Vreugdenhil AC, Van den Neucker AM *et al*. A Pilot Study on the Noninvasive Evaluation of Intestinal Damage in Celiac Disease Using I-FABP and L-FABP. *J Clin Gastroenterol* 2009.
16. Kanda T, Fujii H, Tani T *et al*. Intestinal fatty acid-binding protein is a useful diagnostic marker for mesenteric infarction in humans. *Gastroenterology* 1996;**110**(2):339-43.
17. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. *Arch Dis Child* 1990;**65**(8):909-11.
18. Meeuwisse G.W. Diagnostic criteria in coeliac disease. *Acta Paediatr Scand* 1970;**59**:461.

19. Guandalini S, Ventura A, Ansaldi N *et al*. Diagnosis of coeliac disease: time for a change? *Arch Dis Child* 1989;**64**(9):1320-4.
20. Karell K, Louka AS, Moodie SJ *et al*. HLA types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2.heterodimer: results from the European Genetics Cluster on Celiac Disease. *Hum Immunol* 2003;**64**(4):469-77.
21. Margaritte-Jeannin P, Babron MC, Bourgey M *et al*. HLA-DQ relative risks for coeliac disease in European populations: a study of the European Genetics Cluster on Coeliac Disease. *Tissue Antigens* 2004;**63**(6):562-7.
22. Sollid LM. Coeliac disease: dissecting a complex inflammatory disorder. *Nat Rev Immunol* 2002;**2**(9):647-55.
23. Greco L, Romino R, Coto I *et al*. The first large population based twin study of coeliac disease. *Gut* 2002;**50**(5):624-8.
24. Monsuur AJ, de Bakker PI, Alizadeh BZ *et al*. Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat Genet* 2005;**37**(12):1341-4.
25. Wapenaar MC, Monsuur AJ, van Bodegraven AA *et al*. Associations with tight junction genes PARD3 and MAGI2 in Dutch patients point to a common barrier defect for coeliac disease and ulcerative colitis. *Gut* 2008;**57**(4):463-7.
26. Izumi Y, Hirose T, Tamai Y *et al*. An atypical PKC directly associates and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of Caenorhabditis elegans polarity protein PAR-3. *J Cell Biol* 1998;**143**(1):95-106.
27. Post PL, Tyska MJ, O'Connell CB *et al*. Myosin-IXb is a single-headed and processive motor. *J Biol Chem* 2002;**277**(14):11679-83.
28. Wu X, Hepner K, Castelino-Prabhu S *et al*. Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2. *Proc Natl Acad Sci U S A* 2000;**97**(8):4233-8.
29. Ruch W, Schurmann K, Gordon P *et al*. Coexistent coeliac disease, Graves' disease and diabetes mellitus type 1 in a patient with Down syndrome. *Eur J Pediatr* 1985;**144**(1):89-90.
30. Failla P, Ruberto C, Pagano MC *et al*. Celiac disease in Down's syndrome with HLA serological and molecular studies. *J Pediatr Gastroenterol Nutr* 1996;**23**(3):303-6.
31. Bonamico M, Rasore-Quartino A, Mariani P *et al*. Down syndrome and coeliac disease: usefulness of antigliadin and antiendomysium antibodies. *Acta Paediatr* 1996;**85**(12):1503-5.
32. Gale L, Wimalaratna H, Brotodiharjo A *et al*. Down's syndrome is strongly associated with coeliac disease. *Gut* 1997;**40**(4):492-6.
33. Rumbo M, Chirdo FG, Ben R *et al*. Evaluation of coeliac disease serological markers in Down syndrome patients. *Dig Liver Dis* 2002;**34**(2):116-21.
34. Lerner A, Lebenthal E. The controversy of the use of anti-gluten antibody (AGA) as a diagnostic tool in celiac disease. *J Pediatr Gastroenterol Nutr* 1991;**12**(4):407-9.
35. Savilahti E, Viander M, Perkkio M *et al*. IgA antigliadin antibodies: a marker of mucosal damage in childhood coeliac disease. *Lancet* 1983;**1**(8320):320-2.
36. Wahab PJ, Meijer JW, Mulder CJ. Histologic follow-up of people with celiac disease on a gluten-free diet: slow and incomplete recovery. *Am J Clin Pathol* 2002;**118**(3):459-63.
37. Al Toma A, Verbeek WHM, Mulder CJ. Survival in patients with Refractory Coeliac Disease and Enteropathy associated T cell Lymphoma. *Gut* 2007;**56**(10):1373-8.

38. Meijer JW, Mulder CJ, Goerres MG *et al*. Coeliac disease and (extra)intestinal T-cell lymphomas: definition, diagnosis and treatment. *Scand J Gastroenterol Suppl* 2004; **241**:78-84.
39. Dicke WK. Simple dietary treatment for the syndrome of Gee-Herter. *Ned Tijdsch Geneesk* 1941:1715-1716.
40. Dicke WK. Coeliac disease. Investigation of the harmful effects of certain types of of cereal on patients with coeliac disease (Thesis). University of Utrecht, The Netherlands, 1950 (in Dutch).



Chapter 2

History of coeliac disease, from Dicke till present (in Dutch)

Geschiedenis van coeliakie; van Dicke tot nu

Wolters VM
Stoop JW
Mulder CJJ
Houwen RHJ

Samenvatting

- Coeliakie is een aandoening waarbij individuen, op basis van een genetische predispositie, een vlokatrofie van het dunne darmslijmvlies ontwikkelen bij gebruik van gluten, een eiwit dat aanwezig is in tarwe, rogge en gerst. De wereldwijde prevalentie van coeliakie is hoog: 0,3-1%.
- Hoewel het klassieke symptomentrias, diarree, malaise en groeiachterstand/gewichtsverlies, nog steeds wordt gezien, hebben veel patiënten aspecifieke symptomen als anemie, osteoporose of klachten passend bij het prikkelbare darmsyndroom.
- Serologisch onderzoek met behulp van IgA-anti-endomysium (IgA-EMA) en IgA-anti tissue transglutaminase antistoffen (IgA-tTG) geeft een goede indicatie voor het al dan niet aanwezig zijn van coeliakie. Een dunne darmbiopt blijft echter nodig voor de definitieve diagnose.
- Met een glutenvrij dieet, een behandeling oorspronkelijk geïntroduceerd door de Nederlandse kinderarts Dicke, verdwijnen zowel de bij coeliakie behorende klachten als de onderliggende vlokatrofie.
- De etiologie van deze immuungemedieerde aandoening is deels opgehelderd. Naast HLA-DQ2 of -DQ8 zijn ook andere niet HLA gerelateerde genetische factoren geassocieerd. De verwachting is dat de genetische basis van coeliakie de komende jaren verder opgehelderd zal worden.

Summary

- Coeliac disease (CD) is an immune-mediated enteropathy following the ingestion of gluten in the genetically predisposed. It is characterized by a permanent intolerance for gluten proteins present in dietary wheat, rye and barley. Several large population based studies estimate the prevalence at 0,3-1%.
- Although the classical gastrointestinal malabsorption syndrome, characterized by diarrhoea, steatorrhoea and weight loss still occurs, nowadays more patients are diagnosed with non- specific symptoms such as anemia, osteoporosis or abdominal discomfort.
- Current serologic tests, IgA-anti-endomysium (IgA-EMA) and IgA-anti tissue transglutaminase (IgA-tTG) antibodies, are the first diagnostic modality to employ when coeliac disease is suspected. However a small bowel biopsy remains the gold standard when diagnosing coeliac disease.
- With a strict gluten-free diet, originally introduced by the Dutch pediatrician Willem Karel Dicke, both the symptoms of coeliac disease and the histological changes in the intestinal mucosa disappear.
- Our understanding of the key steps underlying the intestinal inflammatory response in coeliac disease has increased dramatically in recent years. The disease is strongly linked to HLA-DQ2 and HLA-DQ8, though other non HLA genetic factors seem to be involved as well. It is to be expected that the genetic basis of coeliac disease will be further elucidated during the coming years.

Inleiding

Coeliakie is een immuun-gemedieerde aandoening die ontstaat na de ingestie van gluten bij personen met een genetische predispositie. De prevalentie wordt geschat op 0,3-1%.[1-4] Coeliakie wordt gekenmerkt door een levenslange intolerantie voor gluten, die voorkomen in tarwe, rogge en gerst. Bij het ontstaan van coeliakie spelen vele factoren een rol, waarbij in ieder geval de omgevingsfactor gluten essentieel is. Het betrokken glutenpeptide ('gliadine') is relatief resistent tegen enzymatische afbraak in het maag-darmkanaal en wordt in het darmepitheel gemodificeerd door tissue transglutaminase. Het kan vervolgens door het HLA-DQ2 of -DQ8 aan T cellen gepresenteerd worden, waarna schade aan het darmepitheel ontstaat wat kan leiden tot vlokatrofie.[5] Hoewel hiermee een aantal stappen in de pathogenese van coeliakie zijn opgehelderd blijft onduidelijk hoe de vlokatrofie precies tot stand komt. Ook de initiële trigger waardoor de orale tolerantie voor gluten wordt gebroken is nog onbekend.

Begripsontwikkeling van het ziektebeeld

De eerste aanwijzingen voor een ziektebeeld wat zou kunnen passen bij coeliakie werd al tussen de 1e en 2e eeuw beschreven door de arts Aretaeus. Hij beschreef een ziekte waarbij onverteerd voedsel en dunne ontlasting werd gezien en noemde dit 'coeliac disease' (buik ziekte) met een chronisch karakter.[6] Eind 19e eeuw beschreef Dr. Samuel Gee, arts en docent geneeskunde, voor het eerst een ziekte met de nu klassieke symptomen van diarree, malaise en groeiachterstand.[7] Aangezien dit beeld met name werd gezien bij Engelsen die uit de tropen terugkeerden, is in die gevallen waarschijnlijk sprake geweest van tropische spruw. Dit ziektebeeld kwam echter ook voor bij jonge kinderen en volwassenen die nooit buiten Engeland waren geweest. Dit werd de 'coeliac affection' ofwel 'buikgevoeligheid' genoemd. Gee beschreef naast de klassieke symptomen ook de spierzwakte, abdominale distensie en het chronische karakter van de ziekte.[7] Daarnaast merkte hij op dat de patiënt meestal een kind was tussen de 1 en 5 jaar oud. Hij opperde toen al dat de ziekte door een dieet zou moeten worden genezen. Gee kon geen oorzaak vinden. Ook in Nederland werden kort hierna de eerste volwassen patiënten met dit ziektebeeld beschreven. Herter liet vervolgens in 1908 zien dat er bij deze patiënten sprake was van een indrukwekkende vetmalabsorptie, waarna gesproken werd van het syndroom van Gee-Herter. Herter vond verder dat vetten beter werden getolereerd dan koolhydraat bevattende voedingsmiddelen. In 1918 werd door Still opgemerkt dat brood niet goed werd verdragen en Howland bevestigde in 1921 een intolerantie voor koolhydraat bevattende voedingsmiddelen.[8;9]

De echte doorbraak vond eind dertiger jaren van de vorige eeuw plaats. Toen ontstond bij de Nederlandse kinderarts Willem Karel Dicke het vermoeden dat er een verband bestond tussen de ingestie van tarwe en het ontstaan van de ziekteverschijnselen bij coeliakie (figuur 1).[10] Dit idee werd in de loop van de Tweede Wereldoorlog versterkt, toen bleek dat de klachten van coeliakie patiënten ten tijde van broodschaarste afnamen. Hij beschreef dit in zijn proefschrift in 1950 aan de Universiteit van Utrecht.[11] Dicke toonde aan dat introductie van tarwe-eiwit en rogge ('gluten'), maar niet van tarwezetmeel, bij coeliakie patiënten

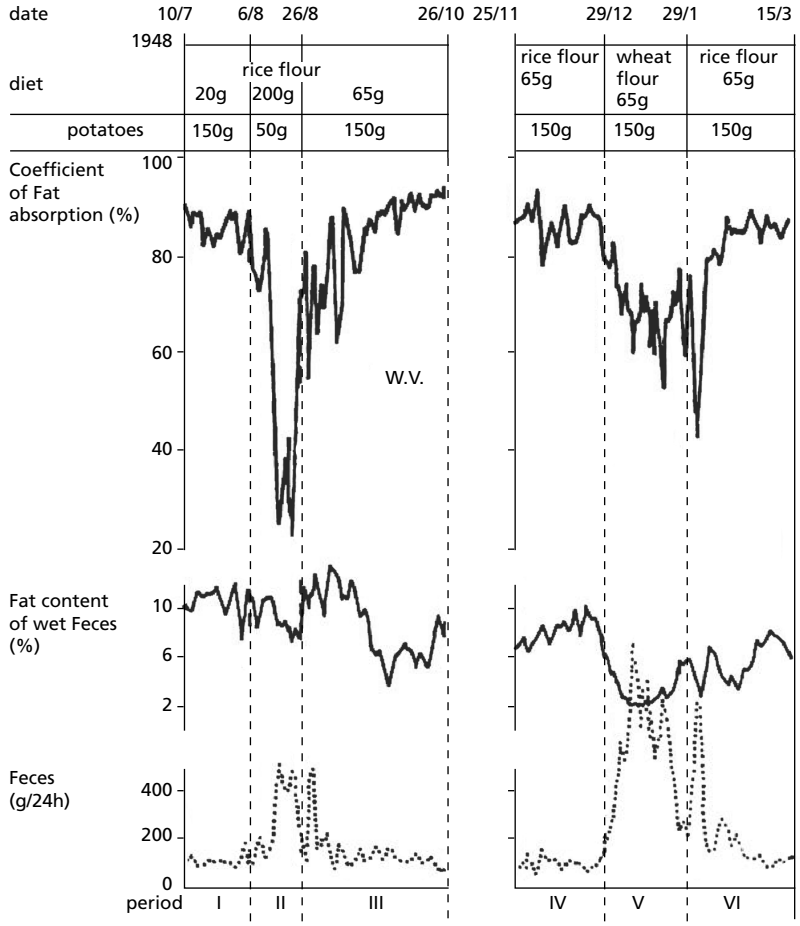
gepaard gaat met vetmalabsorptie. Een glutenvrij dieet leidde tot een zeer uitgesproken klinische verbetering en ook een sterke reductie van de vetmalabsorptie. In figuur 2 is een van de onderzochte patiënten te zien die op een rijstebloem houdend dieet staat en daarop een goede vetabsorptie heeft (87.34%) een normale faecesproductie van 66 gram faeces/ dag en in goede klinische conditie is. Op 6 augustus 1948 werd gestart met brood en aardappelen waarna de vetabsorptie daalde (51.62%) en de faecesproductie vervier-voudigde naar 272.81 gram faeces/ dag. De patiënt werd bleek en lusteloos, kreeg buikpijn en zijn eetlust en conditie verslechterde. Dicke toonde vervolgens samen met zijn collega's Weijers en van de Kamer aan dat een glutenvrij dieet als behandeling voor coeliakie zeer effectief is.[11;12] Dit is sindsdien dan ook de geëigende behandeling.

Figuur 1

Prof. Dr. W.K. Dicke
1905-1962

Vlokatrofie als gevolg van coeliakie werd ontdekt door Paulley in 1954 die dunne darm biopten tijdens operatie onderzocht.[13] Enkele jaren later werd het mogelijk om peroraal een dunne darm biopsie te verrichten, waardoor het aantal biopsieën toenam en de histologische afwijkingen verder werden beschreven.[14] De diagnostische criteria voor coeliakie werden vervolgens gebaseerd op de aanwezigheid van vlokatrofie in een dunne darmbiopsie, met herstel van de laesies op een glutenvrij dieet, en het opnieuw ontstaan van een vlokatrofie na glutenbelasting.[15] De richtlijn is in de loop der jaren vervolgens geleidelijk aangepast omdat nu duidelijk is dat de afwijkingen die kunnen worden gevonden bij coeliakie een spectrum vormen.[16]

Figuur 2



De histologische veranderingen bij coeliakie werden voor het eerst systematisch ingedeeld door Dr. M. Marsh. Bij deze naar hem genoemde classificatie laat een biopt met Marsh 0 geen afwijkingen zien, Marsh I alleen een toegenomen infiltratie van lymfocyten in het villusepitheel, Marsh II de eerder genoemde infiltratie van lymfocyten in combinatie met crypthyperplasie, terwijl bij Marsh III al deze afwijkingen worden gezien in combinatie met vlokatrofie. Marsh III is verder onderverdeeld in partiële, subtotale en totale vlokatrofie (respectievelijk Marsh IIIA, B en C).[17] Voor de diagnose van coeliakie is de aanwezigheid van vlokatrofie nog steeds de gouden standaard. De aanwezigheid van Marsh II kan echter ook compatibel zijn met coeliakie, met name wanneer er ook positieve transglutaminase-antistoffen en/ of endomysium antistoffen (tTG en/ of EMA) wordt gevonden. De aanwezigheid van alleen Marsh I in het duodenum biopt is niet specifiek maar zou ook kunnen passen bij coeliakie in combinatie met positieve serologie (tTG en/ of EMA).[16] Inmiddels is duidelijk dat een glutenbelasting slechts noodzakelijk is bij een minderheid van de patiënten bij wie er twijfel is over de diagnose.[18;19]

Serologische testen

In dunne darmbiopten van onbehandelde patiënten met coeliakie worden naast vlokatrofie ook sterk toegenomen aantallen lymfocyten gezien. Dit suggereert dat er sprake is van immuun gemedieerde darmschade. Bij serologisch onderzoek naar dit proces werd aanvankelijk getest op antistoffen tegen reticuline en gliadine. De sensitiviteit en specificiteit van deze testen waren echter nog niet optimaal.[20] IgA-anti-endomysium antistoffen (IgA-EMA) en IgA-anti tissue transglutaminaseantistoffen (IgA-tTG) bleken vervolgens een aanzienlijk betere associatie te vertonen met de aanwezigheid van coeliakie.[21] Bij totale of vrijwel totale vlokatrofie zijn de sensitiviteit en specificiteit van deze testen hoger dan 95%, indien er althans geen sprake is van een IgA deficiëntie, een aandoening die geassocieerd is met het voorkomen van coeliakie.[20] Bij deze laatste patiëntengroep zal men daarom relatief snel overgaan tot het verrichten van een dunne darmbiopsie, indien er althans klachten aanwezig zijn die kunnen passen bij coeliakie. Bij coeliakie patiënten kunnen IgA-EMA en IgA-tTG ontbreken, vooral wanneer de vlokatrofie niet totaal is.[22;23] De afwezigheid van deze antistoffen sluit coeliakie dus niet uit en bij een blijvende klinische verdenking op deze ziekte zal zeker een dunne darmbiopsie moeten plaatsvinden. Verder komen IgA-tTG of IgA-EMA soms voor bij personen zonder coeliakie.[20;24;25] Ook dit is een reden waarom histologisch onderzoek van een dunne darmbiopsie nodig blijft als sluitstuk van het diagnostisch proces. Voor de follow up van coeliakie patiënten wordt in het algemeen tTG gebruikt.[26;27] Echter studies bij volwassenen laten zien dat tTG niet goed correleert met dieetfouten; in 52% van de gevallen van gluten inname was de tTG negatief.[28] Recent is er een nieuwe marker ontdekt, 'intestinal fatty acid binding protein' (I-FABP), die direct lijkt te correleren met darm schade. Dit zou in de toekomst een veelbelovende marker kunnen worden voor de toekomst in de follow up van coeliakie patiënten.[29]

Diagnose

De diagnose coeliakie wordt in de meeste gevallen gesteld op de kinderleeftijd, tussen 9 en 24 maanden, of veel later, tussen het 30e en 50e levensjaar. Het klassieke trias van diarree, malaise en en gewichtsverlies is nog steeds de meest voorkomende presentatie maar wordt - relatief- steeds minder gezien.[30,31] Vele patiënten bij wie momenteel de ziekte wordt vastgesteld hebben mildere symptomen zoals atypische buikklachten, passend bij het prikkelbare darmsyndroom, dyspepsie, of zelfs niet primair intestinale klachten zoals anemie, osteoporose of onbegrepen moeheid (tabel 1). Dit brede scala van klachten waarmee patiënten zich kunnen presenteren maakt dat de drempel om serologisch onderzoek te verrichten naar het voorkomen van deze aandoening laag moet liggen.

Tabel 1 Atypische symptomen van coeliakie

- Buikpijn
- Opgeblazen gevoel
- Dyspepsie
- Anemie
- Onbegrepen moeheid
- Osteoporose
- Verminderde vruchtbaarheid
- Geïsoleerd gewicht <2 SD
- Geïsoleerde lengtegroeiachterstand bij kinderen
- Hypotonie bij kinderen

De genetische achtergrond van coeliakie

Coeliakie is een multifactoriële aandoening waarbij naast omgevingsfactoren (gluten) vooral genetische factoren een rol spelen, zoals ook blijkt uit een concordantie van meer dan 70% voor deze ziekte bij monozygote tweelingen.[32] Een belangrijke component van deze erfelijke predispositie wordt gevormd door de HLA klasse II moleculen DQ2 en DQ8, welke bij een deel van de bevolking aanwezig zijn op antigeen presenterende cellen. Deze HLA moleculen binden glutenpeptides en presenteren deze vervolgens aan CD4+ T cellen. Vrijwel alle coeliakie patienten zijn in het bezit van ofwel HLA-DQ2 (90-95%) ofwel HLA-DQ8 (5-10%).[33;34] Afwezigheid sluit coeliakie vrijwel uit. Echter bij 35% van de bevolking is HLA-DQ2 of HLA-DQ8 aanwezig terwijl slechts bij één tot enkele procenten van deze groep coeliakie ontstaat.[35;36] HLA-DQ2 of HLA-DQ8 is dus noodzakelijk voor de ontwikkeling van coeliakie maar niet voldoende. Geschat wordt dat 40% van het genetisch risico hierdoor verklaard wordt.[37;38] Het is inmiddels mogelijk om ook andere genen betrokken bij multifactoriële ziekten zoals coeliakie op te sporen en te identificeren door bestudering van grote groepen (verwante) patiënten. Met behulp van deze methode werden gebieden gevonden, respectievelijk op chromosoom 2, 5, 6, 7, 9, 10, 15 en 19 welke zijn geassocieerd met het ontstaan van coeliakie.[39-50]

Binnen het locus op chromosoom 19 werd recent één gen geïdentificeerd, Myosine IXB, dat

verantwoordelijk lijkt te zijn voor deze associatie.[50] Myosine IXB codeert voor een myosine molecuul dat een rol speelt in de actine opbouw van epitheliale enterocyten en tight junctions en dus mogelijk van belang is voor de epitheliale permeabiliteit. Het bezit van deze genetische variant zou secundair kunnen leiden tot een toegenomen darmpermeabiliteit, waardoor passage van immunogene peptides naar het darmepitheel kan plaatsvinden. Dit zou een belangrijke rol kunnen spelen in de fase voorafgaand aan de ontstekingsreactie in het darmslijmvlies die aanleiding geeft tot de symptomen bij coeliakie. Helaas werd deze associatie niet gerepliceerd in andere coeliakie patiënten populaties. Op chromosoom 2 werd ook een gebied gevonden dat geassocieerd lijkt met coeliakie. Hierin ligt onder andere het CTLA-4 gen dat een rol speelt in het beperken van de immuunrespons.[51-54] Een bepaalde variant van dit gen komt vaker voor bij coeliakie patiënten en leidt tot een lagere mRNA concentratie van dit molecuul.[52] Deze bevinding werd wel gerepliceerd. Recent werd een nieuwe techniek, een genoom brede associatie scan (GWAS), verricht waarbij 8 nieuwe gebieden werden geïdentificeerd waarvan 7 gebieden genen bevatten die een veronderstelde immunologische functie hebben.[56;57] Naast HLA werd de duidelijkste associatie gevonden op chromosoom 4q27. Deze regio codeert voor verscheidene genen waaronder de cytokines IL2 en IL21. Ook bij diabetes type 1 en reuma werd precies in deze regio een associatie gevonden. Mogelijk dat deze regio een rol speelt bij het ontstaan van verschillende auto-immunziekten wat verder onderzoek zal moeten uitwijzen.

Prevalentie coeliakie is hoog en veel patiënten hebben atypische klachten

De prevalentie van coeliakie is hoog. Verschillende serologische studies in Europa, Zuid Amerika en de VS maken waarschijnlijk dat 0,3-1% van de populatie coeliakie heeft, veelal zonder dat dit bekend is bij de betrokken persoon.[1] Globaal is van iedere 8 coeliakie patiënten slechts bij één de diagnose bekend en bij 7 niet, zodat de prevalentie van klinisch gediagnostiseerde coeliakie aanzienlijk lager ligt.[58;59] De implicaties hiervan voor de volksgezondheid zijn onbekend, aangezien het natuurlijk beloop van niet herkende (en dus niet behandelde) coeliakie nog onvoldoende is onderzocht. Er zijn studies die een lagere botdichtheid, anemie en een suboptimale voedingsstatus tonen bij patiënten met niet herkende coeliakie.[60-63] Daarintegen werd een verminderd risico gevonden op cardiovasculair lijden, het meest waarschijnlijk door de lagere body mass index in de groep van niet herkende coeliakie patiënten.[63] De groepen waren echter te klein om definitieve conclusies te kunnen trekken en verder onderzoek is noodzakelijk. Pas wanneer meer bekend is over de gezondheidsrisico's van niet herkende coeliakie kan antwoord worden gegeven op de vraag of populatiescreening geïndiceerd is om niet herkende coeliakie patiënten op te sporen.

Nieuwe therapeutische en preventieve opties?

Het verbeterde inzicht in de pathogenese van coeliakie heeft geleid tot het formuleren van nieuwe therapeutische strategieën. Zo is inmiddels bekend dat gliadine rijk is aan prolineresiduen, waardoor de digestie in het darmlumen moeizaam verloopt, en er relatief veel immuunreactieve peptidebrokstukken in het darmlumen aanwezig blijven. Wanneer het mogelijk zou zijn deze brokstukken verder af te breken zou er wellicht geen immuunres-

pons meer plaatsvinden. De prolylpeptidases afkomstig van Aspergillus niger zijn in dit opzicht veelbelovend, omdat ze resistent zijn tegen een lage pH en in staat zijn om in vitro de betrokken peptide brokstukken zover af te breken dat er geen stimulatie van T-cellen meer ontstaat.[64] Het is echter nog niet bekend of het ook mogelijk is de concentratie van de immunogene gliadine brokstukken in de darm van patiënten zover te reduceren dat er geen immuunrespons meer optreedt.
Een andere veelbelovende optie is om tarwe te ontdoen van immunogene gliadine peptiden door selectief telen en/of genetische modificatie. Enkele varianten van tarwe hebben namelijk al minder immunogene eigenschappen.[65;66] Dit gemodificeerde tarwe zal wellicht niet dezelfde eigenschappen hebben en op dezelfde manier gebruikt kunnen worden bij de bereiding van brood of pasta's, doch de vraag is in hoeverre dit een bezwaar vormt. Ook zou geprobeerd kunnen worden om de T-cel gemedieerde schade aan de darmvlokken te blokkeren door middel van immuuntherapie. Zo kon bij diabetes mellitus type 1 met behulp van CD3 antistoffen het te gronde gaan van betacellen onderdrukt worden.[67;68] Ook inductie van immuuntolerantie zal wellicht mogelijk worden nu de immunogene epitopen van het gliadine eiwit zijn geïdentificeerd. Op dit moment wordt er vanuit Leiden een Europese studie gecoördineerd die onderzoekt of het vroeg geven van kleine hoeveelheden gluten tolerantie kan induceren en daardoor het ontstaan van coeliakie kan voorkomen (www.preventcd.com).[69]

De komende jaren zal duidelijk moeten worden welke van deze nieuwe therapeutische opties een aanvulling op, of vervanging van het glutenvrije dieet zullen kunnen gaan vormen.

Conclusie

Dankzij de observaties van Willem Karel Dicke is het nu al meer dan vijftig jaar mogelijk om deze vroeger vaak fataal verlopende ziekte adequaat te behandelen. Waar men destijds nog dacht dat het hier om een relatief zeldzame ziekte ging, die eigenlijk alleen bij kinderen voorkwam, is inmiddels duidelijk dat coeliakie een hoge prevalentie kent. Omdat de meeste patiënten geen of weinig symptomen hebben zal de drempel voor serologisch onderzoek laag moeten liggen bij elke patiënt met klachten welke zouden kunnen passen bij coeliakie.

De pathogenese van coeliakie is inmiddels voor een belangrijk deel opgehelderd. Het gaat om een genetisch bepaalde, immuungemedieerde aandoening. HLA-DQ2 en -DQ8 zijn essentieel voor de ontwikkeling van dit ziektebeeld, doch niet voldoende. Andere, deels nog onbekende genen zijn eveneens noodzakelijk. Te verwachten valt dat, nu de genoom brede associatie scans mogelijk zijn, de komende jaren ook andere genen betrokken bij deze aandoening geïdentificeerd zullen worden.

References

1. Dube C, Rostom A, Sy R *et al*. The prevalence of celiac disease in average-risk and at-risk Western European populations: a systematic review. *Gastroenterology* 2005;**128**:S57-S67.
2. Bingley PJ, Williams AJ, Norcross AJ *et al*. Undiagnosed coeliac disease at age seven: population based prospective birth cohort study. *BMJ* 2004;**328**:322-323.
3. Fasano A, Berti I, Gerarduzzi T *et al*. Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. *Arch Intern Med* 2003;**163**:286-292.
4. Gomez JC, Selvaggio GS, Viola M *et al*. Prevalence of celiac disease in Argentina: screening of an adult population in the La Plata area. *Am J Gastroenterol* 2001;**96**:2700-2704.
5. van de Wal Y, Kooy YM, Drijfhout JW *et al*. Peptide binding characteristics of the coeliac disease-associated DQ(alpha1*0501, beta1*0201) molecule. *Immunogenetics* 1996;**44**:246-253.
6. Adams F. The extant works of Aretaeus the Cappadocian.: London Sydenham Society; 1856.
7. Gee, S. On the coeliac affection. *St Bartholomew´s Hosp Rep* 1888;**24**:17-20.
8. Sauer LW. Celiac disease (chronic intestinal indigestion). *Am J Dis Child* 1925;**34**:934-939.
9. Still GF. The Lumleian lectures on coeliac disease. *Lancet* 1918;**2**:163-166.
10. Dicke WK. Simple dietary treatment for the syndrome of Gee-Herter. *Ned Tijdsch Geneesk* 1941;**85**:1715-1716.
11. Dicke WK. Coeliac disease. Investigation of the harmful effects of certain types of of cereal on patients with coeliac disease (Thesis). University of Utrecht, The Netherlands, 1950 (in Dutch).
12. DICKE WK, WEIJERS HA, VAN DE KAMER JH. Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. *Acta Paediatr* 1953;**42**:34-42.
13. Paulley JW. Observations on the aetiology of idiopathic steatorrhoea. *Br Med J* 1954;**2**:1318-1321.
14. RUBIN CE, BRANDBORG LL, PHELPS PC, *et al.* Studies of celiac disease. I. The apparent identical and specific nature of the duodenal and proximal jejunal lesion in celiac disease and idiopathic sprue. *Gastroenterology* 1960;**38**:28-49.:28-49.
15. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. *Arch Dis Child* 1990;**65**:909-911.
16. Hill ID, Dirks MH, Liptak GS *et al*. Guideline for the diagnosis and treatment of celiac disease in children: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. *J Pediatr Gastroenterol Nutr* 2005;**40**:1-19.
17. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992;**102**:330-354.
18. Mearin ML, Kneepkens CM, Houwen RH. [Diagnosis of celiac disease in children; guidelines for pediatric gastroenterologists. Section of Pediatric Gastroenterology, Dutch Society of Pediatrics]. *Ned Tijdschr Geneeskd* 1999;**143**:451-455.
19. Wolters VM, van de NC, Gerritsen SA *et al*. Is gluten challenge really necessary for the diagnosis of coeliac disease in children younger than age 2 years? *J Pediatr Gastroenterol Nutr* 2009;**48**:566-570.

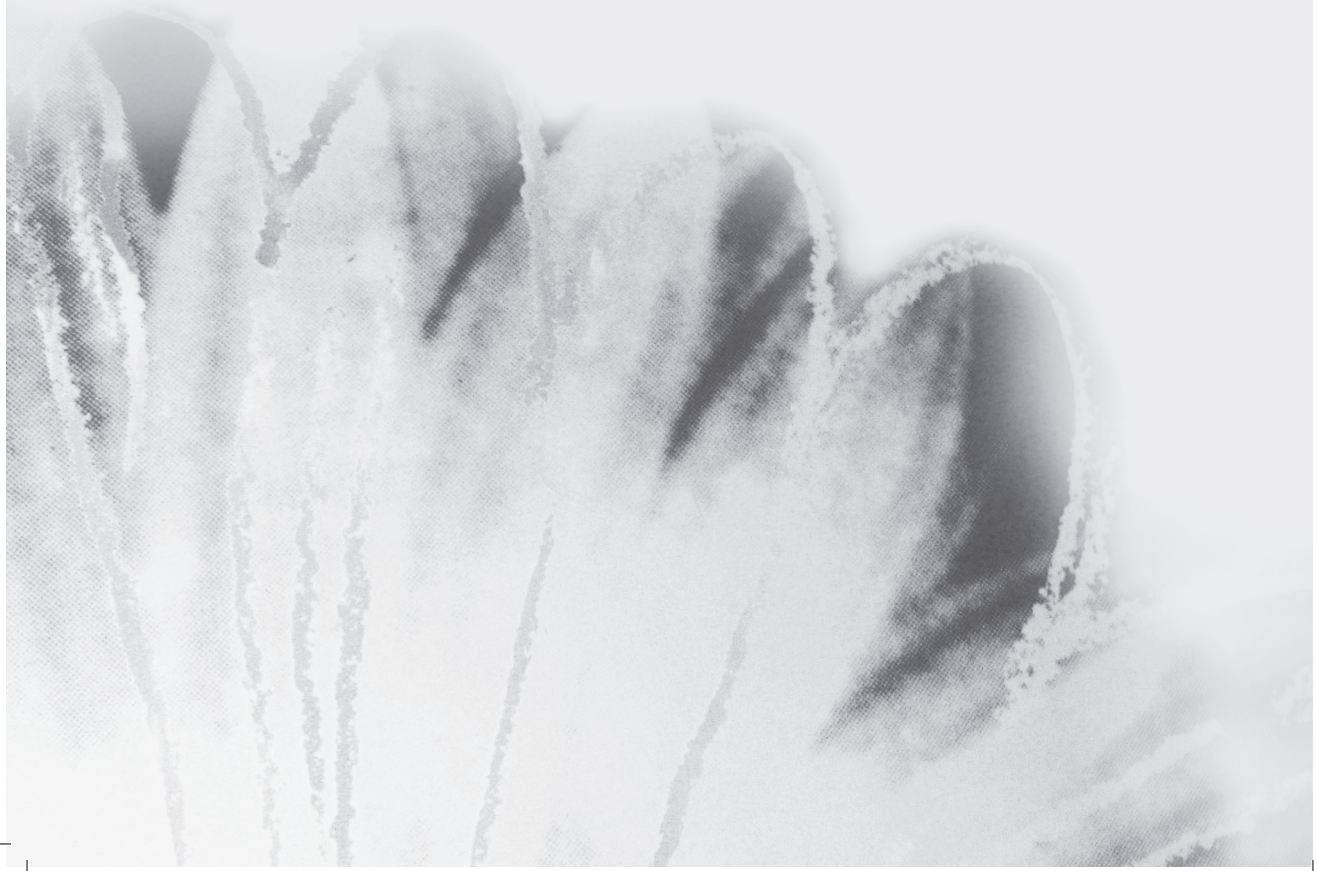
20. Rostom A, Dube C, Cranney A *et al*. The diagnostic accuracy of serologic tests for celiac disease: a systematic review. *Gastroenterology* 2005;**128**:S38-S46.
21. Wolters V, Vooijs-Moulaert AF, Burger H *et al*. Human tissue transglutaminase enzyme linked immunosorbent assay outperforms both the guinea pig based tissue transglutaminase assay and anti-endomysium antibodies when screening for coeliac disease. *Eur J Pediatr* 2002;**161**:284-287.
22. Rostami K, Kerckhaert JP, Tiemessen R *et al*. The relationship between anti-endomysium antibodies and villous atrophy in coeliac disease using both monkey and human substrate. *Eur J Gastroenterol Hepatol* 1999;**11**:439-442.
23. Tursi A, Brandimarte G, Giorgetti GM. Prevalence of antitissue transglutaminase antibodies in different degrees of intestinal damage in celiac disease. *J Clin Gastroenterol* 2003;**36**:219-221.
24. Carroccio A, Iannitto E, Di PL *et al*. Screening for celiac disease in non-Hodgkin's lymphoma patients: a serum anti-transglutaminase-based approach. *Dig Dis Sci* 2003;**48**:1530-1536.
25. Quaglia S, Chinello M, Ferrara F *et al*. Non celiac patients anti-tissue Transglutaminase antibodies: immunological and functional characterization. ICDS Amsterdam 2009: Abstract p116.
26. Burgin-Wolff A, Dahlbom I, Hadziselimovic F. Antibodies against human tissue transglutaminase and endomysium in diagnosing and monitoring coeliac disease. *Scand J Gastroenterol* 2002;**37**:685-691.
27. Pietzak MM. Follow-up of patients with celiac disease: achieving compliance with treatment. *Gastroenterology* 2005;**128**:S135-S141.
28. Vahedi K, Mascart F, Mary JY *et al*. Reliability of antitransglutaminase antibodies as predictors of gluten-free diet compliance in adult celiac disease. *Am J Gastroenterol* 2003;**98**:1079-1087.
29. Derikx JP, Vreugdenhil AC, Van den Neucker AM *et al*. A Pilot Study on the Noninvasive Evaluation of Intestinal Damage in Celiac Disease Using I-FABP and L-FABP. *J Clin Gastroenterol* 2009
30. Steens RF, Csizmadia CG, George EK et al. A national prospective study on childhood celiac disease in the Netherlands 1993-2000: an increasing recognition and a changing clinical picture. *J Pediatr* 2005;**147**:239-240.
31. Rampertab SD, Pooran N, Brar P et al. Trends in the presentation of celiac disease. *Am J Med* 2006;**119**:355.e9-14,
32. Greco L, Romino R, Coto I *et al*. The first large population based twin study of coeliac disease. *Gut* 2002;**50**:624-628.
33. Karell K, Louka AS, Moodie SJ *et al*. HLA types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the European Genetics Cluster on Celiac Disease. *Hum Immunol* 2003;**64**:469-477.
34. Margaritte-Jeannin P, Babron MC, Bourgey M *et al*. HLA-DQ relative risks for coeliac disease in European populations: a study of the European Genetics Cluster on Coeliac Disease. *Tissue Antigens* 2004;**63**:562-567.
35. Sollid LM, Markussen G, Ek J *et al*. Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. *J Exp Med* 1989;**169**:345-350.

36. Sollid LM. Coeliac disease: dissecting a complex inflammatory disorder. *Nat Rev Immunol* 2002;**2**:647-655.
37. Petronzelli F, Bonamico M, Ferrante P *et al*. Genetic contribution of the HLA region to the familial clustering of coeliac disease. *Ann Hum Genet* 1997;**61**:307-317.
38. Risch N. Assessing the role of HLA-linked and unlinked determinants of disease. *Am J Hum Genet* 1987;**40**:1-14.
39. Greco L, Corazza G, Babron MC *et al*. Genome search in celiac disease. *Am.J Hum.Genet.* 1998;**62**:669-675.
40. Greco L, Babron MC, Corazza GR *et al*. Existence of a genetic risk factor on chromosome 5q in Italian coeliac disease families. *Ann Hum Genet* 2001;**65**:35-41.
41. Holopainen P, Naluai AT, Moodie S *et al*. Candidate gene region 2q33 in European families with coeliac disease. *Tissue Antigens* 2004;**63**:212-222.
42. Liu J, Juo SH, Holopainen P *et al*. Genomewide linkage analysis of celiac disease in Finnish families. *Am J Hum Genet* 2002;**70**:51-59.
43. van Belzen MJ, Meijer JW, Sandkuijl LA *et al*. A major non-HLA locus in celiac disease maps to chromosome 19. *Gastroenterology* 2003;**125**:1032-1041.
44. van Belzen MJ, Vrolijk MM, Meijer JW *et al*. A genomewide screen in a four-generation Dutch family with celiac disease: evidence for linkage to chromosomes 6 and 9. *Am J Gastroenterol* 2004;**99**:466-471.
45. Woolley N, Holopainen P, Ollikainen V *et al*. A new locus for coeliac disease mapped to chromosome 15 in a population isolate. *Hum Genet* 2002;**111**:40-45.
46. King AL, Moodie SJ, Fraser JS *et al*. CTLA-4/CD28 gene region is associated with genetic susceptibility to coeliac disease in UK families. *J Med Genet* 2002;**39**:51-54.
47. Babron MC, Nilsson S, Adamovic S *et al*. Meta and pooled analysis of European coeliac disease data. *Eur J Hum Genet* 2003;**11**:828-834.
48. Percopo S, Babron MC, Whalen M *et al*. Saturation of the 5q31-q33 candidate region for coeliac disease. *Ann Hum Genet* 2003;**67**:265-268.
49. Wapenaar MC, Monsuur AJ, van Bodegraven AA *et al*. Associations with tight junction genes PARD3 and MAGI2 in Dutch patients point to a common barrier defect for coeliac disease and ulcerative colitis. *Gut* 2008;**57**:463-467.
50. Monsuur AJ, de Bakker PI, Alizadeh BZ *et al*. Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat Genet* 2005;**37**:1341-1344.
51. Holopainen P, Arvas M, Sistonen P *et al*. CD28/CTLA4 gene region on chromosome 2q33 confers genetic susceptibility to celiac disease. A linkage and family-based association study. *Tissue Antigens* 1999;**53**:470-475.
52. Naluai AT, Nilsson S, Samuelsson L *et al*. The CTLA4/CD28 gene region on chromosome 2q33 confers susceptibility to celiac disease in a way possibly distinct from that of type 1 diabetes and other chronic inflammatory disorders. *Tissue Antigens* 2000;**56**:350-355.
53. Popat S, Hearle N, Hogberg L *et al*. Variation in the CTLA4/CD28 gene region confers an increased risk of coeliac disease. *Ann Hum Genet* 2002;**66**:125-137.
54. Rioux JD, Karinen H, Kocher K *et al*. Genomewide search and association studies in a Finnish celiac disease population: Identification of a novel locus and replication of the HLA and CTLA4 loci. *Am J Med Genet* 2004;**130**:345-350.

55. van Belzen MJ, Mulder CJ, Zhernakova A *et al*. CTLA4 +49 A/G and CT60 polymorphisms in Dutch coeliac disease patients. *Eur J Hum Genet* 2004;**12**:782-785.
56. Hunt KA, Zhernakova A, Turner G *et al*. Newly identified genetic risk variants for celiac disease related to the immune response. *Nat Genet* 2008;**40**:395-402.
57. van Heel DA, Franke L, Hunt KA *et al*. A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. *Nat Genet* 2007;**39**:827-829.
58. Catassi C, Fabiani E, Ratsch IM *et al*. The coeliac iceberg in Italy. A multicentre antigliadin antibodies screening for coeliac disease in school-age subjects. *Acta Paediatr Suppl* 1996;**412**:29-35.
59. Catassi C, Ratsch IM, Fabiani E *et al*. Coeliac disease in the year 2000: exploring the iceberg. *Lancet* 1994;**343**:200-203.
60. Corazza GR, Di SA, Cecchetti L *et al*. Influence of pattern of clinical presentation and of gluten-free diet on bone mass and metabolism in adult coeliac disease. *Bone* 1996;**18**:525-530.
61. Corazza GR, Di SA, Sacco G *et al*. Subclinical coeliac disease: an anthropometric assessment. *J Intern Med* 1994;**236**:183-187.
62. Mustalahti K, Collin P, Sievanen H *et al*. Osteopenia in patients with clinically silent coeliac disease warrants screening. *Lancet* 1999;**354**:744-745.
63. West J, Logan RF, Hill PG *et al*. Seroprevalence, correlates, and characteristics of undetected coeliac disease in England. *Gut* 2003;**52**:960-965.
64. Stepniak D, Spaenij-Dekking L, Mitea C *et al*. Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. *Am J Physiol Gastrointest Liver Physiol* 2006;**291**:621-9.
65. Molberg O, Uhlen AK, Jensen T *et al*. Mapping of gluten T-cell epitopes in the bread wheat ancestors: implications for celiac disease. *Gastroenterology* 2005;**128**:393-401.
66. Spaenij-Dekking L, Kooy-Winkelaar Y, van Veelen P *et al*. Natural variation in toxicity of wheat: potential for selection of nontoxic varieties for celiac disease patients. *Gastroenterology* 2005;**129**:797-806.
67. Belghith M, Bluestone JA, Barriot S *et al*. TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat Med* 2003;**9**:1202-1208.
68. Keymeulen B, Vandemeulebroucke E, Ziegler AG *et al*. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *N Engl J Med* 2005;**352**:2598-2608.
69. Troncone R, Ivarsson A, Szajewska H, Mearin ML. Review article: future research on coeliac disease - a position report from the European multistakeholder platform on coeliac disease (CDEUSSA). *Aliment Pharmacol Ther* 2008;**27**:1030-1043.

Part 1

Diagnostics



Chapter 3

Human tissue transglutaminase enzyme linked immunosorbent assay outperforms both the guinea pig based tissue transglutaminase assay and anti-endomysium antibodies when screening for coeliac disease

Wolters VM
Vooijs-Moulaert AFSR
Burger H
Brooimans R
De Schryver JEAR
Rijkers GT
Houwen RHJ

Eur J Pediatr 2002;161(5):284-287

Abstract

Anti-endomysium antibodies (EMA) and antigliadin antibodies (AGA) are widely used when-screening for coeliac disease (CD), although their specificity and sensitivity are suboptimal. The guinea pig tissue transglutaminase (tTG) assay also did not prove to be superior. A newly developed enzyme linked immunosorbent assay (Celikey), based on human tTG, might however have a better performance. We therefore investigated the sensitivity and specificity of this human IgA-tTG assay in 101 patients with aspecific gastrointestinal complaints and compared this to guinea pig IgA-tTG, AGA and EMA. A total of 52 patients with CD were investigated and 49 patients without CD. All had a small bowel biopsy. Our results showed that human IgA-tTG had a sensitivity of 96% and a specificity of 100%. Guinea pig IgA-tTG had a sensitivity of 96% and a specificity of 92%. EMA had a sensitivity of 92% and a specificity of 90%. Both IgA-AGA and IgG-AGA had a sensitivity of 83% whilst having a specificity of 86% and 80% respectively. Conclusion: both the human IgA tissue transglutaminase enzyme linked immunosorbent assay and the guinea pig IgA tissue transglutaminase assay could better identify patients with CD than IgA anti-endomysium antibodies. Although in a larger series of control patients the specificity for the human IgA tissue transglutaminase enzyme linked immunosorbent assay might fall below 100%, in our opinion this is currently the serological method of choice in identifying patients with CD in the absence of IgA deficiency.

Abbreviations

AGA antigliadin antibodies, CD coeliac disease, ELISA enzyme-linked immunosorbent assay, EMA anti-endomysium antibodies, tTG tissue transglutaminase.

Introduction

Coeliac disease (CD) is an auto-immune enteropathy triggered by the ingestion of gluten in genetically-susceptible individuals.[1] On intestinal biopsy a flattened jejunal mucosa is seen. This typical intestinal damage resolves completely upon elimination of gluten-containing grains from the diet. The prevalence of CD in the general population might be as high as 0.5%.[2-4] However, not all patients present with the classical triad of growth failure, chronic diarrhoea and abdominal distention. The remainder will either manifest less specific symptoms such as constipation, abdominal pain or anaemia or will have no symptoms at all.[4;5] Serological markers for CD were developed primarily to detect CD in the group of patients presenting with these atypical symptoms. Initially IgG and IgA antibodies against gliadin were used. However, IgG antibodies to gliadin are not very specific because elevated levels can be found in other diseases and in healthy individuals.[6;7] IgA antibodies against gliadin are more specific, but in most studies more than 10% of the patients with CD are missed.[6;8] Nevertheless, those tests are still widely used because of low costs. Until now the 'test' indicating the presence of anti-endomysium antibodies EMA was the most reliable in predicting CD. Although this test is more sensitive and specific than the antigliadin antibody (AGA) assays, nevertheless 5%–10% of patients with CD are missed, while some children without CD are falsely labelled as patients.[6-8] Therefore, a more reliable serological test for CD is needed. Recently tissue transglutaminase (tTG) was identified as the self-antigen for endomysial antibodies.[9] As guinea pig tTG, but not human tTG, was directly available, the first enzyme linked immunosorbent assays (ELISAs) developed used this substrate. However, the sensitivity of this test did not exceed that of the EMA.[10-14] These results might have been caused by the only partial homology between guinea pig tTG and human tTG. The aim of the present study was to evaluate the diagnostic potential of a newly developed ELISA based on human tTG, as compared to the guinea pig IgA-tTG assay, EMA, IgA-AGA and IgG-AGA in a biopsy-controlled study.

Patients and methods

Patients

In this retrospective study we examined 101 serum samples from patients with suspected CD. All patients were seen during the period 1996–2000 in the Department of Paediatric Gastroenterology of the Wilhelmina Children's Hospital in Utrecht, The Netherlands. Patients had various clinical symptoms such as chronic diarrhoea, growth failure, abdominal distension, constipation, abdominal pain or anaemia. In all patients several duodenal samples were taken during endoscopy from the second to third part of the duodenum. In 52 patients, histology was considered to be diagnostic for CD as the small intestinal mucosa revealed (sub) total villous atrophy in combination with crypt hyperplasia, an inflammatory infiltrate in the lamina propria and an increased intraepithelial lymphocyte count. In 49 patients, histological examination revealed no abnormalities or only non specific changes.

Methods

Anti-endomysial antibodies

EMA were determined by indirect immunofluorescence on frozen sections of slides of distal monkey oesophagus (ImmunoGlo, Immco Diagnostics, Buffalo, N.Y.) according to the manufacturers instructions. Sera were diluted 1:5 and incubated for 30 min at room temperature with the tissue sections. Slides were then washed in PBS and incubated for 30 min with FITC conjugated goat antihuman IgA/IgG. Slides were washed again, embedded in mounting medium and examined by fluorescence microscopy. A positive result was defined as a reticular pattern of immunofluorescence in the muscularis mucosae at a serum dilution equal to or greater than 1:5.[15]

IgA and IgG antibodies to gliadin

A two-step indirect ELISA technique was used to determine AGA. Wells of flat-bottomed 96-well microtitre plates (Greiner, Frickenhausen, Germany) were coated with, via alcohol extraction, purified gliadin (10 lg/ml) in sodium-carbonate buffer (pH 9.6) overnight at room temperature, as described by Volta *et al*.[16] The plates were then washed three times and incubated for 1 h at room temperature under constant shaking (Milenia Micromix 4, Diagnostic Products Cooperation, Los Angeles, Calif.) with serial 3-fold dilutions of patient sera, starting with a 1:10 dilution. The plates were washed again and incubated for a further 1 h at room temperature with a 1:1000 dilution of alkaline phosphatase conjugated goat anti-human IgA or anti-human IgG antibodies (Organon Teknika, Boxtel, The Netherlands). The plates were then washed and incubated with p-nitrophenolphosphate. Colour development was stopped with 2.4 N NaOH and the optical density measured at 405 nm using a Milenia ELISA reader (Diagnostic Products, Los Angeles). AGA titres were expressed relative to a standard preparation to which 1000 U/ml IgA-AGA and 1000 U/ ml IgG-AGA were assigned. Antibody titres were considered positive if IgA-AGA exceeded 4 U/ml and IgG-AGA exceeded 150 U/ml.[15]

Guinea pig IgA tissue transglutaminase enzyme linked immunosorbent assay

For the guinea pig tTG ELISA, 96-well microtitre plates were coated overnight at room temperature with 10 lg/ml guinea pig tTG (No.90195, Fluka) in 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl 2 (pH 7.5). The plates were washed and incubated for 1 h with 10 mg/ml bovine serum albumin in PBS. The remainder of the procedure was identical to the AGA assay. Anti-tTG antibody titres were expressed relative to a reference pool of control sera to which 100 U/ml IgA anti-tTG was assigned. Values of IgA-tTG above 1 U/ml were considered positive.

Human IgA tissue transglutaminase enzyme linked immunosorbent assay

For determination of IgA antibodies to recombinant human tTG, the Celikey tTG ELISA kit (Pharmacia and Upjohn Diagnostics, Freiburg, Germany) was used. The assay was performed according to the manufacturer's instructions. Values of IgA-tTG higher than 8 U/ml in patient sera (relative to the standard preparation provided with the kit) were considered positive.

Data analysis

The sensitivity and specificity of the screening tests were calculated using the biopsy results as the gold standard. The sensitivity denotes the proportion of CD-patients with a positive screening test, and the specificity the proportion of non CD-patients with a negative test. All results are presented as percentages with exact binomial 95% confidence intervals.

Results

A total of 52 samples from children with biopsy-verified CD were collected (14 males, 38 females, mean age 4.0 years, range 1.1–14.4 years). Clinical and serological improvement was shown in all patients after termination of gluten ingestion. Human IgA-tTG was elevated in all but two patients, giving a sensitivity of 96%. Fifty patients had elevated guinea pig IgA-tTG (sensitivity 96%) and in 48, IgA-EMA was positive (sensitivity 92%). IgA-AGA was elevated in 43 patients (sensitivity 83%) and IgG-AGA was elevated in 43 patients (sensitivity 83%) (Table 1). A total of 49 samples from children with biopsy verified non CD were collected as a disease control group (29 males, 20 females, mean age 5.1 years, range 0.8–19.2 years). These control patients had symptoms suggestive of CD, but had a normal intestinal biopsy. None of the patients had elevated human IgA-tTG, thus giving a specificity of 100%. Forty-five patients had no elevation of guinea pig IgA-tTG (specificity 92%) and 44 were IgA-EMA negative (specificity 90%). IgA-AGA was normal in 42 patients (specificity 86%) and IgG-AGA was not elevated in 39 patients (specificity 80%) (Table 1).
The human IgA-tTG ELISA had the highest sensitivity (96%) and specificity (100%). Nevertheless, in two CD patients, both with a normal total serum IgA, a normal human IgA-tTG was found. As the guinea pig IgA-tTG failed to identify two other patients in this series, a combination of both would have identified all patients with CD if we would have considered a positive result on either one of these tests diagnostic for CD (sensitivity 100%). However, a misdiagnosis of CD would have occurred in 4 out of 49 control patients (specificity 92%).

Table 1 Sensitivity and specificity of different screening tests for CD. The 95% confidence intervals are given in parentheses

	IgA-AGA (%)	IgG-AGA (%)	IgA-EMA (%)	Guinea pig IgA-tTG (%)	Human IgA-tTG (%)
Sensitivity	83 (70–92)	83 (70–92)	92 (81–98)	96 (87–100)	96 (87–100)
Specificity	86 (73–94)	80 (66–90)	90 (78–97)	92 (80–98)	100 (93–100)

Discussion

Although at least one intestinal biopsy is still needed[17], serological assays are helpful to screen persons at risk for CD. For this purpose IgA- and IgG-AGA are widely used, mainly due to their low costs. Nevertheless, IgA-EMA is a more specific marker for CD, but disadvantages of this test are the high costs, the limited availability of monkey oesophagus, the time-consuming technique and the interobserver variation. Therefore, after the recent identification of tTG as the auto-antigen of CD[9], a guinea pig based tTG ELISA was developed by several groups, hoping that this would provide a more reliable serological test for CD. However, sensitivity and specificity were not superior to IgA-EMA [10-14], probably due to only partial homology between guinea pig tTG and human tTG. Thus, the next step was the development of an ELISA based on human tTG. In our hands this new test, human IgA-tTG, correlated very well with CD with a sensitivity of 96% and a specificity of 100%. Although in a larger series, a false-positive result might be obtained with this test, as is also indicated by the confidence intervals for the specificity, the human IgA-tTG ELISA seems to be the best test for identifying patients with CD and its implementation might result in less unnecessary intestinal biopsies.

Patients included in this study had complaints suggestive of CD. It can be expected that a lower reliability will be obtained when using the human IgA-tTG ELISA for population screening, as was the case for the well validated EMA test in a mass screening project in the Netherlands.[3] In that study almost 50% of the subjects positive for EMA had a normal small intestinal biopsy.

In the follow-up of patients with CD, IgA-AGA is widely used as an indicator of dietary compliance [6;18], although the EMA seems to be more sensitive to detect long standing periods of gluten intake.[6] As human IgA-tTG ELISA gives quantitative values, like IgA-AGA, and has proven to be a more sensitive- and specific test toidentify CD, this test might be used in the future in the follow-up of patients with established CD. However, further investigations are required to evaluate the role of human IgA-tTG in monitoring the compliance with a gluten-free diet.

Selective IgA deficiency is frequently associated with CD with an incidence 10–15-fold higher in CD patients than in the general population.[19;20] Obviously for subjects with selective IgA deficiency, neither the human IgA-tTG nor the IgA-EMA tests are reliable screening tools. Human IgG-tTG might be an option in those cases. However, in one study sensitivity was as low as 47%.[21] Guinea pig based IgG-tTG also revealed a disappointingly low sensitivity of 23% and 44% respectively.[13;18] However, in a small group of untreated CD patients with selective IgA deficiency, Cataldo *et al*.[22] described a 100% sensitivity for both guinea pig IgG-tTG and IgG1 EMA. Because of these conflicting results, for the moment it seems sensible to test for IgA deficiency when screening for CD. In the presence of IgA deficiency and complaints that may be due to CD, an intestinal biopsy is needed.[19]

So far, only four studies have described the predictive value of human IgA-tTG in the diagnosis of CD. The results from these studies, two describing an ELISA [21;23] and two a radioligand assay [24;25], are comparable to our results, with a sensitivity of 93%–100%. The high specificity given in those studies (98%–99%) might be less reliable because intestinal biopsies were not performed in the majority of the control groups.

Four biopsy-controlled studies have been published describing the results for the guinea pig IgA-tTG test as a tool in the diagnostic work-up of CD. The results from those four studies [10;13;18;26], giving a sensitivity of 85%–95% and a specificity of 90%–98%, are comparable with our results for guinea pig based tTG (sensitivity of 96% and a specificity of 92%). Furthermore, several non-biopsy controlled studies describing the use of a guinea pig IgA-tTG ELISA have been published recently, giving comparable sensitivities and specificities. [11;12;23;27-29]

We conclude that this new serological screening test for CD, human IgA-tTG ELISA, is an excellent tool to screen for CD in patients with gastrointestinal complaints in the absence of IgA deficiency. This assay may also prove useful for the screening of populations at low or medium risk for CD.

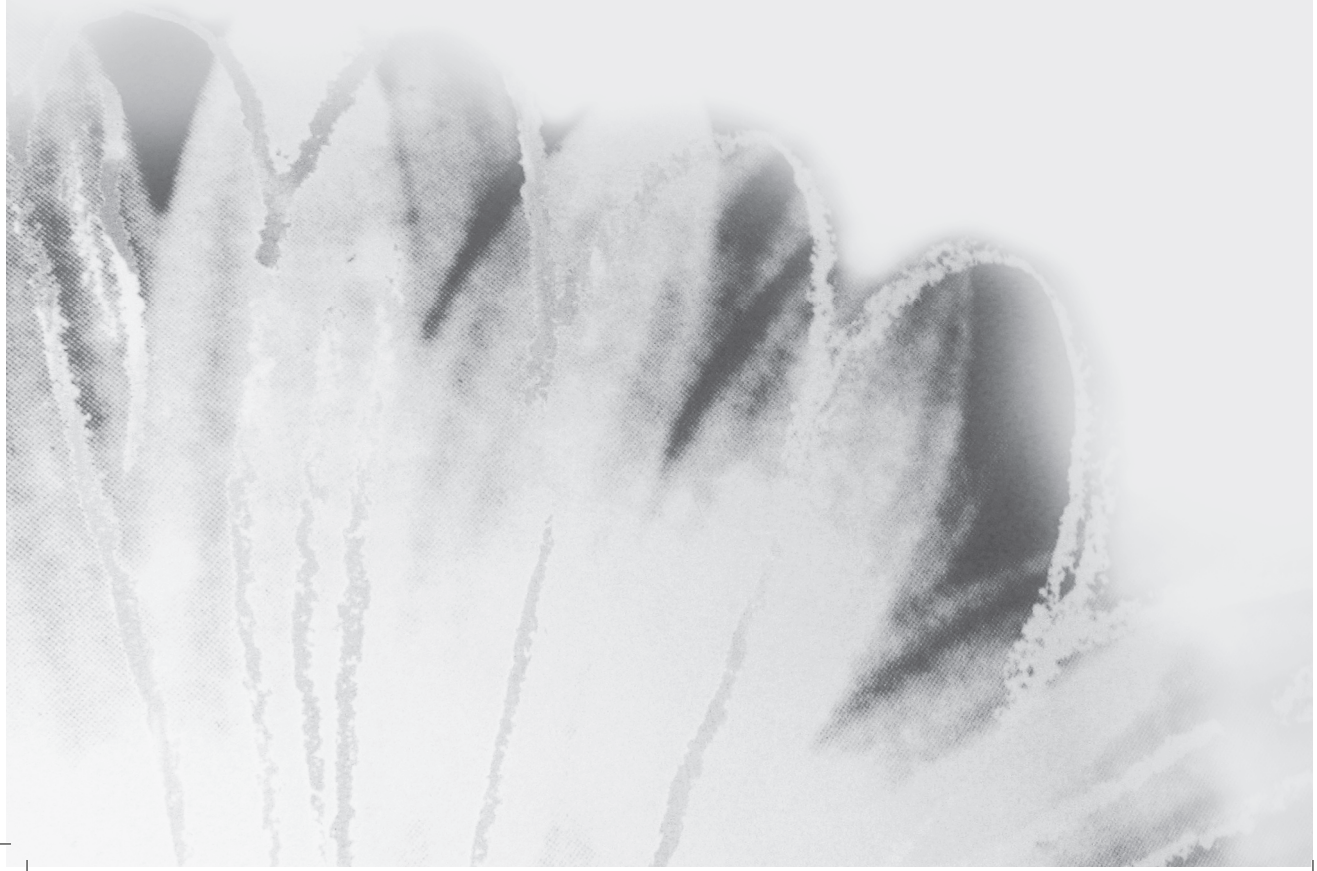
Acknowledgements

We gratefully acknowledge the expert technical assistance of Mrs. E. Venema, M. Maas, and C. Engels. The authors also wish to thank Mrs. W. van der Hoeven for her contribution to the patient database. The Celikey kits used in this study were kindly donated by Pharmacia Upjohn. However, no other financial support was received, nor have the authors any financial interest in this company.

References

1. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992;**102**(1):330-54.
2. Catassi C, Fabiani E. The spectrum of coeliac disease in children. *Baillieres Clin Gastroenterol* 1997;**11**(3):485-507.
3. Csizmadia CG, Mearin ML, von Blomberg BM *et al*. An iceberg of childhood coeliac disease in the Netherlands. *Lancet* 1999;**353**(9155):813-4.
4. Maki M, Collin P. Coeliac disease. *Lancet* 1997;**349**(9067):1755-9.
5. George EK, Mearin ML, Franken HC *et al*. Twenty years of childhood coeliac disease in The Netherlands: a rapidly increasing incidence? *Gut* 1997;**40**(1):61-6.
6. Burgin-Wolff A, Gaze H, Hadziselimovic F *et al*. Antigliadin and antiendomysium antibody determination for coeliac disease. *Arch Dis Child* 1991;**66**(8):941-7.
7. Rossi TM, Tjota A. Serologic indicators of celiac disease. *J Pediatr Gastroenterol Nutr* 1998;**26**(2):205-10.
8. Russo PA, Chartrand LJ, Seidman E. Comparative analysis of serologic screening tests for the initial diagnosis of celiac disease. *Pediatrics* 1999;**104**(1 Pt 1):75-8.
9. Dieterich W, Ehnis T, Bauer M *et al*. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med* 1997;**3**(7):797-801.
10. Biagi F, Ellis HJ, Yiannakou JY *et al*. Tissue transglutaminase antibodies in celiac disease. *Am J Gastroenterol* 1999;**94**(8):2187-92.
11. Dahele AV, Aldhous MC, Humphreys K *et al*. Serum IgA tissue transglutaminase antibodies in coeliac disease and other gastrointestinal diseases. *QJM* 2001;**94**(4):195-205.
12. Gillett HR, Freeman HJ. Comparison of IgA endomysium antibody and IgA tissue transglutaminase antibody in celiac disease. *Can J Gastroenterol* 2000;**14**(8):668-71.
13. Lock RJ, Pitcher MC, Unsworth DJ. IgA anti-tissue transglutaminase as a diagnostic marker of gluten sensitive enteropathy. *J Clin Pathol* 1999;**52**(4):274-7.
14. Troncone R, Maurano F, Rossi M *et al*. IgA antibodies to tissue transglutaminase: An effective diagnostic test for celiac disease. *J Pediatr* 1999;**134**(2):166-71.
15. Hillhorst MI, Rijkers G, Blomberg BME. *et al*. Sensitiviteit en specificiteit van de anti-gliadine en anti-endomysium antilichaam bepaling. *Tijdschr Kindergeneeskd* 1996;**64**:104-7.
16. Volta U, Lenzi M, Lazzari R *et al*. Antibodies to gliadin detected by immunofluorescence and a micro-ELISA method: markers of active childhood and adult coeliac disease. *Gut* 1985;**26**(7):667-71.
17. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. *Arch Dis Child* 1990;**65**(8):909-11.
18. Valletta EA, Mastella G. Adherence to gluten-free diet and serum antigliadin antibodies in celiac disease. *Digestion* 1990;**47**(1):20-3.
19. Cataldo F, Marino V, Ventura A *et al*. Prevalence and clinical features of selective immunoglobulin A deficiency in coeliac disease: an Italian multicentre study. Italian Society of Paediatric Gastroenterology and Hepatology (SIGEP) and «Club del Tenue» Working Groups on Coeliac Disease. *Gut* 1998;**42**(3):362-5.

20. Collin P, Maki M, Keyrilainen O *et al*. Selective IgA deficiency and coeliac disease. *Scand J Gastroenterol* 1992;**27**(5):367-71.
21. Baldas V, Tommasini A, Trevisiol C *et al*. Development of a novel rapid non-invasive screening test for coeliac disease. *Gut* 2000;**47**(5):628-31.
22. Cataldo F, Lio D, Marino V *et al*. IgG(1) antiendomysium and IgG antitissue transglutaminase (anti-tTG) antibodies in coeliac patients with selective IgA deficiency. Working Groups on Celiac Disease of SIGEP and Club del Tenue. *Gut* 2000;**47**(3):366-9.
23. Hansson T, Dahlbom I, Hall J *et al*. Antibody reactivity against human and guinea pig tissue transglutaminase in children with celiac disease. *J Pediatr Gastroenterol Nutr* 2000;**30**(4):379-84.
24. Bazzigaluppi E, Lampasona V, Barera G *et al*. Comparison of tissue transglutaminase-specific antibody assays with established antibody measurements for coeliac disease. *J Autoimmun* 1999;**12**(1):51-6.
25. Seissler J, Boms S, Wohlrab U *et al*. Antibodies to human recombinant tissue transglutaminase measured by radioligand assay: evidence for high diagnostic sensitivity for celiac disease. *Horm Metab Res* 1999;**31**(6):375-9.
26. Sulkanen S, Halttunen T, Laurila K *et al*. Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterology* 1998;**115**(6):1322-8.
27. Dieterich W, Laag E, Schopper H *et al*. Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* 1998;**115**(6):1317-21.
28. Sugai E, Selvaggio G, Vazquez H *et al*. Tissue transglutaminase antibodies in celiac disease: assessment of a commercial kit. *Am J Gastroenterol* 2000;**95**(9):2318-22.
29. Vitoria JC, Arrieta A, Arranz C *et al*. Antibodies to gliadin, endomysium, and tissue transglutaminase for the diagnosis of celiac disease. *J Pediatr Gastroenterol Nutr* 1999;**29**(5):571-4.



Chapter 4

Plasma I-FABP levels are elevated in children with celiac disease and rapidly recover after gluten withdrawal

Vreugdenhil AC
Wolters VM
Van den Neucker AM
van Bijnen AA
Houwen RH
Buurman WA

Abstract

Background

Non-invasive tools for evaluation of intestinal damage are needed to further improve the accuracy of serologic tests in the diagnosis of celiac disease (CD) and to enable monitoring of mucosal healing after gluten elimination. Intestinal fatty acid binding protein (I-FABP) is potentially useful since this small cytosolic enterocyte protein is a sensitive marker for intestinal mucosal damage. Our aim was to investigate whether serum I-FABP levels can reliably identify CD in children with a positive serological screening test for CD. We also studied the recovery of I-FABP levels after initiation of a gluten free diet (GFD).

Methods

I-FABP levels were analyzed retrospectively in 49 children with biopsy proven CD. Blood was collected before biopsy and repeatedly after the GFD was started. The control group consisted of 19 patients with a positive screening for CD (elevated IgA-tTG and IgA-EMA antibodies) but without histological characteristics matching CD.

Results

Initial I-FABP concentrations in CD (median 458 pg/ml) were significantly ($p<0.001$) elevated compared to controls (median 0 pg/ml). Forty out of 49 CD patients had an elevated serum I-FABP level, whereby I-FABP concentrations correlated with Marsh stage. In the control group only 2 of 19 children were found to have elevated I-FABP levels, in whom one subsequently was diagnosed with CD after a gluten challenge. In all CD patients I-FABP levels decreased quickly after GFD and normalized in 80% of patients within 12 weeks.

Conclusion

I-FABP improve the reliability of the current serologic parameters (tTG and EMA) in the diagnosis of CD and in monitoring the response to GFD. Future studies are needed to investigate whether serology in combination with I-FABP can replace the intestinal biopsy.

Introduction

A major problem in the clinical management of celiac disease (CD) is the absence of non-invasive methods to confirm the diagnosis in patients with positive screening tests. Although the measurement of IgA antibodies against tissue transglutaminase (IgA-tTG) and endomysium (IgA-EMA) is a sensitive tool to identify patients who need an intestinal biopsy, encountering elevated serum IgA-tTG and/or IgA-EMA levels is not specific enough to justify a life-long diet.[1] In addition, in children under the age of 24 months these serologic tests are not always reliable diagnostic markers for CD.[2-4] Consequently, before a final diagnosis can be made, mucosal biopsies of the proximal small bowel are necessary,[5;6] which is an invasive and expensive procedure with a risk of complications.

For evaluation of mucosal healing after elimination of gluten from the diet serum IgA-EMA and IgA-tTG levels are now widely used. Although these antibodies accurately represent intestinal damage in the diagnostic phase and are very useful for selecting children who need a biopsy [3;7], various studies demonstrated a discrepancy between the levels of these antibodies and disease activity in the follow-up of patients.[1;8-11] This might be due to a long half-life and consequently a slow disappearance of antibodies from the circulation after elimination of gluten.[8;12] These concerns emphasize the need for a new methodology that accurately enables evaluation of therapy, non-compliance and unintended gluten ingestion.

In this study the utility of Intestinal Fatty Acid Binding Protein (I-FABP), a marker for small intestinal damage, in diagnosing CD in children with positive serological screening tests and follow-up of disease activity was studied. I-FABP is a very small (15 kDa) unbound cytosolic protein present in enterocytes which is released rapidly into the blood after cellular damage.[13] Previous studies have identified I-FABP as a circulating and urinary marker for intestinal epithelial cell damage in mesenterial thrombosis, necrotising enterocolitis and in the early phase of sepsis.[14-16]

In our earlier studies, the expression of I-FABP in different segments of the human intestine was evaluated. We found that I-FABP is predominantly expressed in the mucosa of the small intestine. Moreover, I-FABP is particular highly expressed in cells present at the tops of the villi.[17] Since villous atrophy in the small intestine is the hallmark of CD, we considered I-FABP to be a potential marker of damage to the small intestine in CD. A pilot study in a small group of children and adults with biopsy proven CD indeed demonstrated elevated I-FABP levels in patients as compared to healthy volunteers.[17] Therefore, I-FABP might be helpful to determine the presence of small intestinal mucosal damage in children with enhanced titers of IgA-EMA and/or IgA-tTG.

The present study evaluates whether measurement of serum I-FABP levels might improve the reliability of the current serologic screening tests for CD and can discriminate between mucosal damage and a normal mucosa in a large group of children with elevated IgA-tTG and/or IgA-EMA. In addition, the normalization of serum I-FABP levels after gluten elimination and therefore the potential of I-FABP levels for monitoring mucosal healing, were analyzed.

Materials and methods

Study population

Sixty-eight children (0-17 years old) with a clinical suspicion of CD and elevated titers of IgA-tTG or IgA-EMA and who had visited the Maastricht University Medical Centre or Wilhelmina Children's Hospital/ UMC Utrecht between 2000 and 2008 were retrospectively studied. Of these children serum left over from the original IgA-tTG or IgA-EMA analysis had been stored at -80°C. These samples had been collected at a maximum of three months before CD was diagnosed or excluded by small intestinal biopsy.

In 49 children the diagnosis was confirmed by establishing duodenal villous atrophy at histological examination. These biopsies were further staged according to Marsh's classification; stage IIIA partial villous atrophy, stage IIIB subtotal villous atrophy and IIIC total villous atrophy.(18) All children with biopsy proven CD started a gluten free diet (GFD). Follow-up serum samples were collected at different time points after withdrawal of gluten. For 41 children at least one follow-up serum sample was obtained within the first half year after starting the GFD. In 19 children the histological evaluation of the duodenal biopsy excluded the diagnosis of CD, i.e. no villous atrophy was seen. These children are further mentioned as controls. From the hospital charts, the following data were recorded: age, gender, screening tests of IgA-EMA (Scimedx, Denville, NJ, USA and ImmunoGlo, Immco Diagnostics, Buffalo, N.Y) and IgA-tTG (Phadia GmbH, Freiburg, Germany and Phadia, Nieuwegein, The Netherlands) and histological assessment of the small intestinal biopsy.

Serum I-FABP concentrations were determined using a highly specific commercially available ELISA that selectively detects human I-FABP (standard: 20-5000 pg/ml), kindly provided by HBT (Uden, the Netherlands). The laboratory technician performing the assays was not aware of the histological findings in the patients. This study was performed according to the guidelines of the local ethics committees.

Statistical analyses

Statistical analysis was performed with SPSS 15.0 for Windows (SPSS Inc, Chicago, IL). Serum I-FABP concentrations are presented as median and range. Concentrations of serum I-FABP were compared between patients with CD and controls using Mann-Whitney U test. Patients with biopsy proven CD were stratified for Marsh stage, and I-FABP concentrations in the different Marsh stage groups were compared using Mann Whitney U test. A p-value below 0.05 was considered statistically significant.

In order to find the cut-off point of I-FABP serum levels that most accurately discriminates patients with CD from healthy individuals, a receiver operating characteristic (ROC) curve was drawn by plotting sensitivity against 1-specificity for all possible serum I-FABP thresholds. The overall accuracy of serum I-FABP in detecting CD was summarized using the area under the curve (AUC), with an AUC of 0.5 indicating no discrimination ability. Ninety-five percent confidence intervals (CI) for the sensitivity and specificity were calculated for the best cut-off values, defined as the maximal sum of sensitivity and specificity.

Results

Baseline characteristics of patients with celiac disease and controls

Sixty-eight patients with elevated titers of IgA-tTG or IgA-EMA were included in this study (Table 1). In 49/68 patients the diagnosis of CD was confirmed by duodenal biopsy. A total of 16 biopsies were classified as Marsh stage IIIA, 18 as Marsh stage IIIB and 15 as Marsh stage IIIC. All 49 children with biopsy proven CD had elevated titers of IgA-tTG and IgA-EMA. In 19 children the biopsy did not fulfill the criteria for CD (further mentioned as *controls*). In this control group, 9 children showed elevated titers of IgA-tTG and IgA-EMA, while 10 children only had elevated EMA-IgA titers. In this control group the biopsies were classified as Marsh 0 in 17 children and Marsh I in 2 children.

Table 1 Characteristics of study groups

	Celiac disease (n=49)	Controls (n=19)
Female/male	26/23	12/7
Age (years)	6.7 ± 4.5	8.5 ± 4.0
Positive IgA-tTG (%)	100	47
Positive IgA-EMA (%)	100	100

IgA-tTG: >10 U/ml=positive. IgA-EMA: titer 1:10 (starting dilution) = positive. IgA-tTG: anti-tissue transglutaminase antibodies; IgA-EMA: anti-endomysium antibodies.

I-FABP levels in plasma are significantly elevated in patients with celiac disease compared to controls and correlate with Marsh stage

I-FABP levels were measured in serum of 68 children with a clinical suspicion of CD and elevated titres of either IgA-tTG and/or IgA-EMA. The serum I-FABP concentration in patients with biopsy proven CD (median value 458 pg/ml; range 0-2990 pg/ml) was significantly elevated compared to controls (median value 0 pg/ml; range 0-485 pg/ml; $p<0.001$, Figure 1). The ability of serum I-FABP levels to discriminate patients with CD from children without CD in children with a positive antibody screening was analyzed by a ROC curve (Figure 2). The ROC curve for serum I-FABP yielded a high AUC (0.92 (95% CI: 0.84-0.99) $p<0.001$) with an optimal cut-off value of 224 pg/ml. In 40/49 patients with biopsy proven CD I-FABP levels were above the cut-off value and therefore truly positive. In 9 of the patients I-FABP levels were false-negative. Biopsies of 6 of these patients were classified as Marsh stage IIIA and 3 patients as Marsh stage IIIB. In all patients classified as Marsh stage IIIC I-FABP levels were above the cut-off value. Moreover, I-FABP concentrations in patients with Marsh stage IIIA (median value 278 pg/ml, range 0-707 pg/ml), IIIB (median value 428 pg/ml, range 21-1944 pg/ml) and IIIC (median value 724 pg/ml, range 392-2990 pg/ml) were significantly elevated compared to controls (Figure 3). The difference in I-FABP levels between Marsh stage IIIA and IIIB was not significant. Patients with Marsh stage IIIC were found to have significantly higher I-FABP levels than patients classified as Marsh stage IIIA, IIIB and controls (Figure 3).

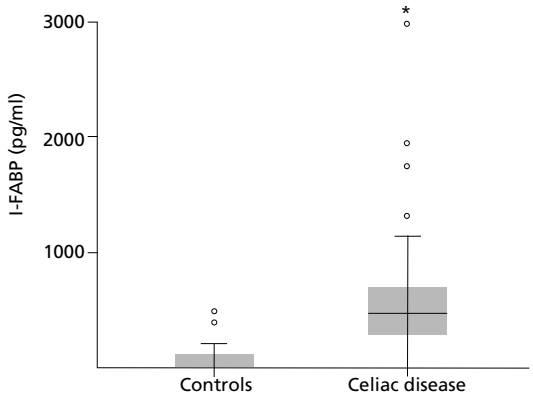


Figure 1 Concentrations of serum I-FABP in patients with celiac disease compared to controls

Boxplot of serum I-FABP levels in the study groups showing the median (central horizontal lines), the 25th percentile (lower box border), the 75th percentile (upper box border), and range of values.
O Extreme values. * Significant difference p<0.001

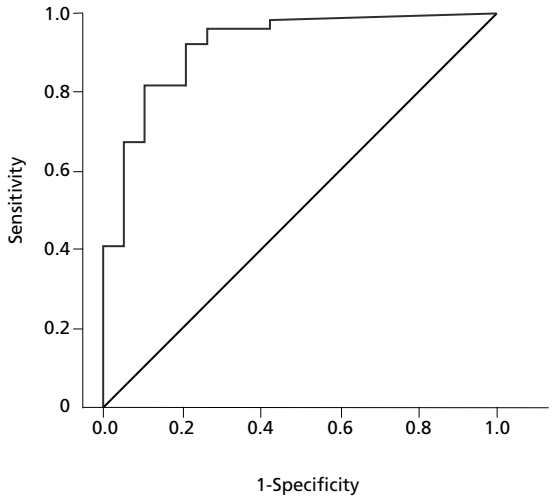


Figure 2 A receiver operating characteristic (ROC) curve of sensitivity against 1-specificity for all possible serum I-FABP thresholds

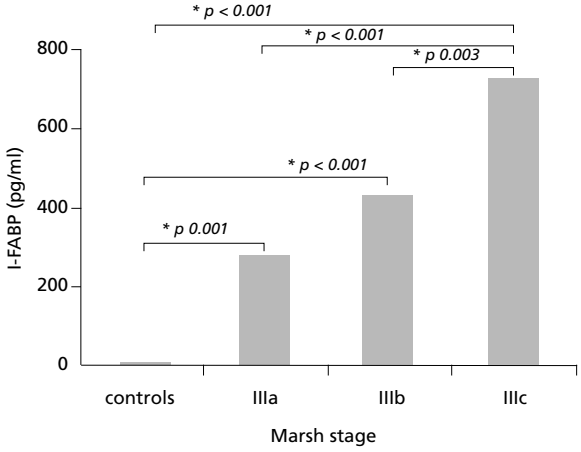


Figure 3 Serum I-FABP levels stratified for Marsh stage, expressed as medians

In 17/19 controls with abnormal serology but without histologic confirmation of the diagnosis, I-FABP levels were below the cut-off value and therefore in agreement with the histology. Two patients in this control group showed elevated I-FABP levels. IgA-tTG and IgA-EMA titers were also elevated in these two patients. Histological evaluation of the biopsies in one of these patients showed an increased mitotic activity in the crypts and an increased number of lymfocytes in the lamina propria, but without villous atrophy. Hence the diagnosis of CD could not be made initially. Therefore, the child's normal gluten containing diet was supplemented with 10 grams of purified gluten per day during 3,5 months. A subsequent biopsy now showed villous atrophy. The parents of the other child, a boy with Down syndrome, did not allow a gluten challenge. Follow-up of this boy demonstrated a persistent elevated titer of IgA-tTG above 100 U/ml.

Rapid recovery of I-FABP levels after starting a gluten free diet

Most interestingly, in 100% of the children with biopsy proven CD and an initially elevated serum I-FABP concentration, the I-FABP level decreased rapidly after gluten withdrawal. I-FABP levels were measured within 7 weeks after starting a GFD in 11 patients (Figure 4). Within 7 weeks I-FABP levels decreased 61% and the I-FABP concentration was below the cut-off value in 5/11 (45%) of patients. Within 12 weeks after elimination of gluten from the diet 16/20 (80%) of children showed normalization of I-FABP levels. In the other 4 patients, I-FABP levels were reduced by 57% compared to the initial value. In 23/25 (92%) of patients I-FABP levels dropped below the cut off value within 26 weeks of gluten elimination. The response of serum IgA-tTG was much slower: after 7, 12 and 26 weeks of the start of a GFD these levels had been normalized in respectively 0% (0/11), 30% (6/20) and 52% (13/25) of patients.

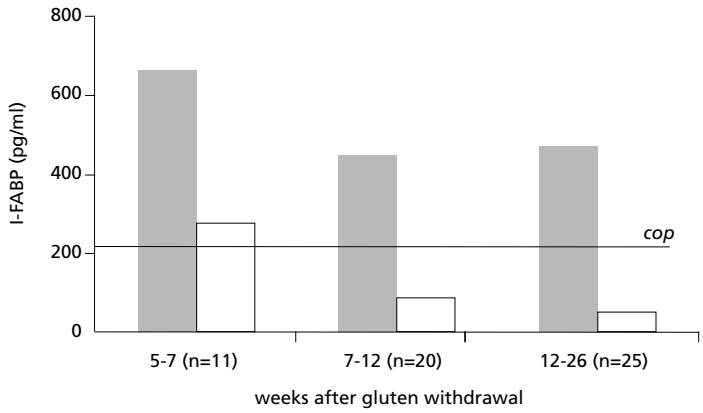


Figure 4 Serum I-FABP levels after gluten withdrawal

I-FABP levels were measured between 5-7 weeks (n=11), between 7-12 weeks (n=20) and between 12-26 weeks (n=25) after initiation of the gluten free diet. Data are expressed as medians. Grey bars represent the initial I-FABP levels at the time of the biopsy of the subgroups, open bars represent I-FABP levels at the time of follow-up. Cop is cut off point.

Discussion

Destruction of enterocytes induced by a disturbed immune response to gluten, resulting in villous atrophy is the hallmark of CD. Apoptosis of enterocytes in the crypts and on the tip of villi is increased in untreated CD.[19] These latter enterocytes contain abundant amounts of I-FABP,[17] a small cytosolic protein and marker of intestinal damage. In a pilot study we demonstrated that the ongoing apoptosis of enterocytes in patients with active CD resulted in elevated serum levels of I-FABP.[17] We now show that this enables non-invasive evaluation of villous atrophy.

In children with positive serological screening tests for CD (elevated IgA-tTG or IgA-EMA), elevated serum I-FABP levels were associated with villous atrophy in 40 out of 42 patients on the first biopsy. In one of the remaining children discrete histological abnormalities prompted us to do a gluten challenge, confirming the diagnosis of CD subsequently, as villous atrophy was now found. The other child classified as a control but with raised I-FABP levels had Down syndrome, a group in whom false positive IgA-tTG and IgA-EMA can be found.[20] The high specificity of I-FABP combined with serological screening, might help to abandon the small intestinal biopsy in the diagnosis of CD, especially when our findings are confirmed in a larger group of patients. Furthermore, the specificity of raised I-FABP levels in the diagnosis of CD in children with a positive celiac screening might be further optimized by repeated measurement of I-FABP levels after gluten withdrawal, since decreasing I-FABP levels in response to a gluten free diet will further support the diagnosis of CD.

In 9/49 children with biopsy proven CD I-FABP levels were below the calculated cut-off value. I-FABP levels represent destruction of differentiated enterocytes, so it is likely that I-FABP levels depend on both the severity of villous atrophy and the extent of disease activity in the small intestine. In CD villous atrophy can be patchy or restricted to the most proximal part of the duodenum.[21] We speculate that the low I-FABP levels in patients with histological confirmed villous atrophy might be explained by the involvement of only a small part of the intestine and/or mild mucosal damage. Consistent with this hypothesis we observed that most patients with false negative I-FABP levels showed mild villous atrophy (mostly Marsh stage IIIA) whereas all patients with severe villous atrophy (Marsh stage IIIC) had elevated levels of I-FABP. We conclude that low I-FABP levels do not exclude CD. Therefore, in children with a positive screening test and normal I-FABP levels a duodenal biopsy is still necessary to exclude or confirm CD. However, even in mild villous atrophy (Marsh stage IIIA) I-FABP levels were significantly elevated as compared to controls, implying that despite a lower sensitivity also mild villous atrophy can be detected by measuring I-FABP levels.

It is of utmost interest that I-FABP levels were demonstrated to recover quickly after starting a GFD. Within 7 weeks of GFD I-FABP levels normalized in 5/11 (45%) of patients. After 12 and 26 weeks of GFD, respectively 80% and 92% of the children were found to have normal I-FABP levels. This contrasted favorably with the recovery of serum IgA-tTG levels, which were normal in 0%, 30% and 52% at 7, 12 and 26 weeks respectively. This relatively slow

response of serum IgA-tTG was also found in a recent study that evaluated the recovery of IgA-tTG in children after starting a GFD.[22] In contrast to these antibodies, I-FABP has a very short half life, which most likely contributes to the difference in recovery time between I-FABP and IgA-tTG. Moreover I-FABP directly represents mucosal damage and healing, whereas measurement of antibodies represent activation of the immune response related to CD and not intestinal damage. In addition, the reliability of the serological tests for monitoring histological response to treatment is still under discussion. Consequently serum I-FABP levels might be more useful than serum IgA-tTG levels in monitoring the effect of a GFD.

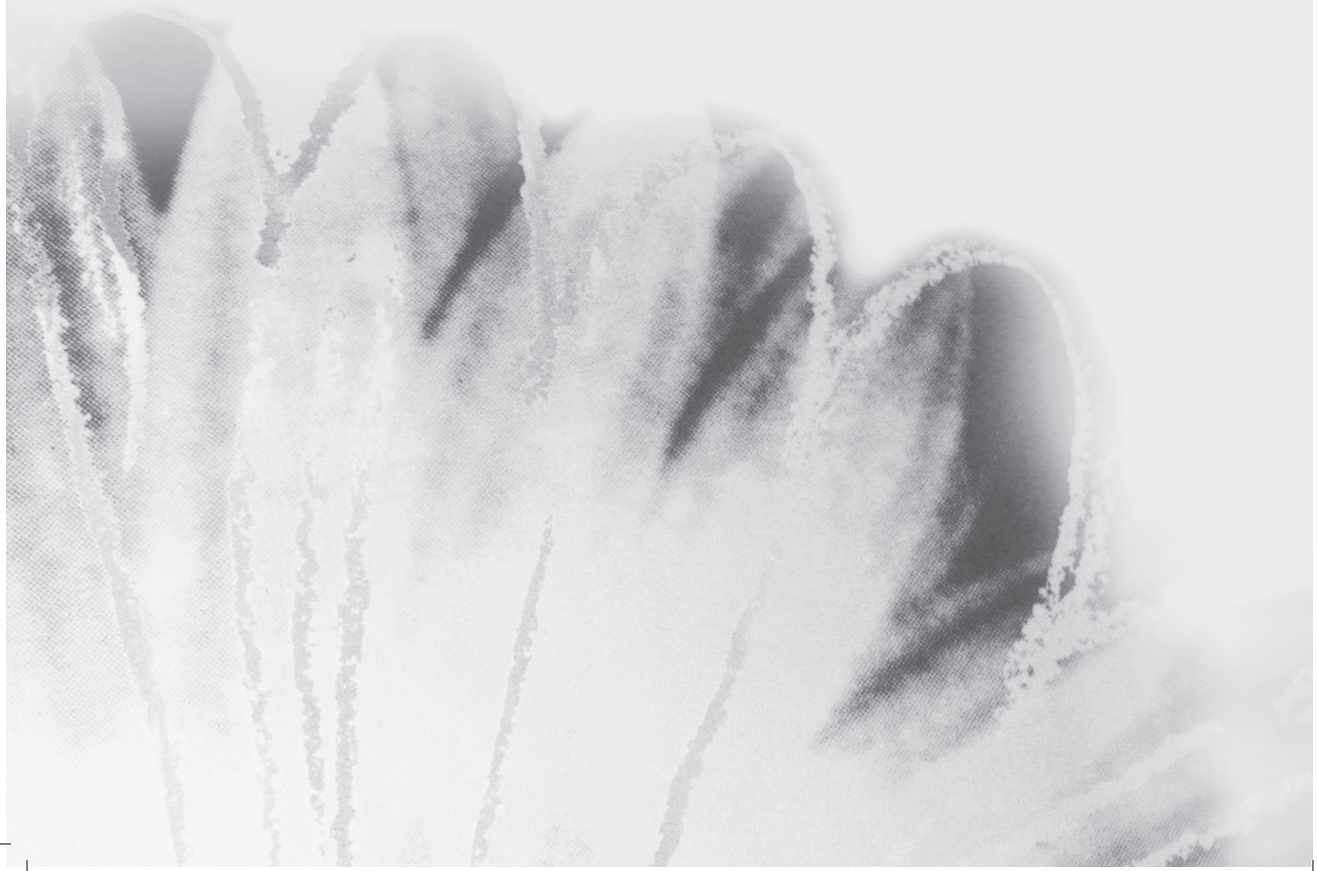
In conclusion we showed in this study that elevated serum I-FABP levels non-invasively confirm the diagnosis of CD in almost all children with abnormal IgA-tTG and IgA-EMA. The high reliability of the combination of tests might help to abandon the small intestinal biopsy in the diagnosis of CD in a substantial number of patients. Nevertheless in children with normal I-FABP levels and an abnormal serology it remains necessary to obtain duodenal biopsies. We also found that I-FABP levels recover very rapidly after elimination of gluten, which implies that I-FABP might serve as a new tool for close monitoring of diet effects on mucosal healing.

References

1. Rostom A, Dube C, Cranney A *et al*. The diagnostic accuracy of serologic tests for celiac disease: a systematic review. *Gastroenterology* 2005;**128**(4 Suppl 1):S38-S46.
2. Burgin-Wolff A, Gaze H, Hadziselimovic F *et al*. Antigliadin and antiendomysium antibody determination for coeliac disease. *Arch Dis Child* 1991;**66**(8):941-7.
3. Fabiani E, Catassi C. The serum IgA class anti-tissue transglutaminase antibodies in the diagnosis and follow up of coeliac disease. Results of an international multi-centre study. International Working Group on Eu-tTG. *Eur J Gastroenterol Hepatol* 2001;**13**(6):659-65.
4. Lagerqvist C, Dahlbom I, Hansson T *et al*. Antigliadin immunoglobulin A best in finding celiac disease in children younger than 18 months of age. *J Pediatr Gastroenterol Nutr* 2008;**47**(4):428-35.
5. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. *Arch Dis Child* 1990;**65**(8):909-11.
6. Hill ID, Dirks MH, Liptak GS *et al*. Guideline for the diagnosis and treatment of celiac disease in children: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. *J Pediatr Gastroenterol Nutr* 2005;**40**(1):1-19.
7. Llorente MJ, Sebastian M, Fernandez-Acenero MJ *et al*. IgA antibodies against tissue transglutaminase in the diagnosis of celiac disease: concordance with intestinal biopsy in children and adults. *Clin Chem* 2004;**50**(2):451-3.
8. Pietzak MM. Follow-up of patients with celiac disease: achieving compliance with treatment. *Gastroenterology* 2005;**128**(4 Suppl 1):S135-S141.
9. Simell S, Kupila A, Hoppu S *et al*. Natural history of transglutaminase autoantibodies and mucosal changes in children carrying HLA-conferred celiac disease susceptibility. *Scand J Gastroenterol* 2005;**40**(10):1182-91.
10. Tursi A, Brandimarte G, Giorgetti GM. Lack of usefulness of anti-transglutaminase antibodies in assessing histologic recovery after gluten-free diet in celiac disease. *J Clin Gastroenterol* 2003;**37**(5):387-91.
11. Vahedi K, Mascart F, Mary JY *et al*. Reliability of antitransglutaminase antibodies as predictors of gluten-free diet compliance in adult celiac disease. *Am J Gastroenterol* 2003;**98**(5):1079-87.
12. Burgin-Wolff A, Dahlbom I, Hadziselimovic F *et al*. Antibodies against human tissue transglutaminase and endomysium in diagnosing and monitoring coeliac disease. *Scand J Gastroenterol* 2002;**37**(6):685-91.
13. Derikx JP, van Waardenburg DA, Thuijls G *et al*. New Insight in Loss of Gut Barrier during Major Non-Abdominal Surgery. *PLoS ONE* 2008;**3**(12):e3954.
14. Derikx JP, Evennett NJ, Degraeuwe PL *et al*. Urine based detection of intestinal mucosal cell damage in neonates with suspected necrotising enterocolitis. *Gut* 2007;**56**(10):1473-5.
15. Derikx JP, Poeze M, van Bijnen AA *et al*. Evidence for intestinal and liver epithelial cell injury in the early phase of sepsis. *Shock* 2007;**28**(5):544-8.
16. Kanda T, Fujii H, Tani T *et al*. Intestinal fatty acid-binding protein is a useful diagnostic marker for mesenteric infarction in humans. *Gastroenterology* 1996;**110**(2):339-43.

17. Derikx JP, Vreugdenhil AC, Van den Neucker AM *et al*. A Pilot Study on the Noninvasive Evaluation of Intestinal Damage in Celiac Disease Using I-FABP and L-FABP. *J Clin Gastroenterol* 2009.
18. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992;**102**(1):330-54.
19. Ciccocioppo R, Di SA, Parroni R *et al*. Increased enterocyte apoptosis and Fas-Fas ligand system in celiac disease. *Am J Clin Pathol* 2001;**115**(4):494-503.
20. Shamaly H, Hartman C, Pollack S *et al*. Tissue transglutaminase antibodies are a useful serological marker for the diagnosis of celiac disease in patients with Down syndrome. *J Pediatr Gastroenterol Nutr* 2007;**44**(5):583-6.
21. Bonamico M, Mariani P, Thanasi E *et al*. Patchy villous atrophy of the duodenum in childhood celiac disease. *J Pediatr Gastroenterol Nutr* 2004;**38**(2):204-7.
22. Hansson T, Dahlbom I, Rogberg S *et al*. Recombinant human tissue transglutaminase for diagnosis and follow-up of childhood coeliac disease. *Pediatr Res* 2002;**51**(6):700-5.

4



Chapter 5

Is gluten challenge really necessary for the diagnosis of coeliac disease in children under the age of two years?

Wolters VM
Nadort C van de
Kneepkens CMF
Gerritsen SAM
Ten Kate FJW
Gijsbers CFM
Schweizer JJ
Nikkels PGJ
Benninga MA
Houwen RHJ

J Pediatr Gastroenterol Nutr 2009;48(5):566-70

Abstract

Introduction

In the diagnosis of coeliac disease (CD) gluten challenge is recommended for children under the age of two years at initial biopsy.

Objective

To investigate the diagnostic yield of gluten challenge in this group of children.

Methods

We included children aged 2 years or younger analysed for possible CD and having villous atrophy at initial small bowel biopsy in the period 1993-2004. We subsequently identified all patients who underwent a complete gluten challenge.

Results

We identified 333 children with possible CD. In 100 children (30%) a gluten challenge was performed, with the diagnosis being confirmed in 97. Retrospectively, in two of the three children without mucosal relapse, data available before gluten challenge did not justify the initial diagnosis of CD. In the third patient transient gluten intolerance could not be excluded. At first biopsy, the two children without mucosal relapse had negative serologic parameters, while the third patient had IgA anti-gliadin antibodies, but no IgA anti-endomysium antibodies (EMA). Indeed all patients with EMA at diagnosis had a relapse at gluten challenge.

Conclusion

Routine gluten challenge in children less than 2 years at initial diagnosis of CD has an extremely low diagnostic yield. We suggest that routine gluten challenge in this group of patients is not necessary when patients have villous atrophy in combination with EMA. Therefore, a revision of the current diagnostic criteria has to be considered.

Abbreviations

CD: Coeliac disease; CI: 95% confidence interval; IgA-AGA: IgA antigliadin antibodies; IgG-AGA: IgG anti-gliadin antibodies; IgA-ARA: IgA anti-reticuline antibodies; EMA: IgA anti-endomysium antibodies; tTG: IgA anti-tissue transglutaminase antibodies.

Introduction

Diagnostic criteria for coeliac disease (CD) were established for the first time in 1969 by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) at the Interlaken symposium.[1;2] At that time CD was defined as a permanent disorder which required three criteria: an abnormal jejunal mucosa on a gluten containing diet, clear improvement of villous structure when taking a gluten free diet, and deterioration of the mucosa during gluten challenge. In 1990 these criteria were revised as the need for routine gluten challenge had been questioned.[3] The revision was partially based on a large study by Guandalini *et al,* who studied 2523 patients diagnosed by strict adherence to the ESPGHAN protocol.[4] In a small percentage of patients (~5%, 123/ 2523 patients) in this study, following gluten challenge another diagnosis was made such as cow's milk sensitive enteropathy and transient gluten intolerance. The mean age of these non confirmed coeliac patients was 8 months. Obviously other causes of enteropathy can occur in this young age group, so the revised criteria recommended gluten challenge only for children aged two years or less at diagnosis. Gluten challenge was not mandatory anymore in children two years or older at diagnosis nor in adults.

Re-exposing a child to gluten for a considerable period has its disadvantages. It can cause symptoms like diarrhoea, abdominal pain and irritability. It can also be potentially harmful to the child's growing potential.[5] Furthermore, small bowel biopsy causes considerable distress, while general anaesthesia or sedation during biopsy has a potential risk of side effects and complications. In addition, the actual yield of gluten challenge in patients below 2 years of age at initial biopsy seems to be low, as even in this age group only very few diagnoses have to be revised.[4]

Finally, since the publication of the ESPGHAN recommendations in 1990, the reliability of serologic tests for CD has improved considerably. In a child with unequivocal histology and abnormal serology, presently many paediatricians will not use routine gluten challenge anymore to confirm the diagnosis in young children. As no evidence is available to support this change in attitude, we investigated the results of gluten challenge in children aged 2 years or younger at the initial diagnosis of CD to determine whether gluten challenge could also be abandoned in this group of children.

Materials and methods

We retrospectively investigated data of children aged 2 years or younger at initial biopsy who were analyzed for possible CD between 1993 and 2004 in four Dutch children's hospitals (departments of Paediatric Gastroenterology of the University Medical Centre, Utrecht; Academic Medical Centre, Amsterdam; VU Medical Centre, Amsterdam; and Juliana Children's Hospital/ Haga Teaching Hospital, The Hague, The Netherlands), and who had villous atrophy at initial small bowel biopsy. We identified all patients who completed a gluten challenge before October 1, 2006. The methods and results of these gluten challenges were evaluated.

A second small intestinal biopsy was taken before gluten challenge. Regarding the actual challenge, three centres (University Medical Centre, VU Medical Centre, Juliana Children's Hospital) adhered to the same gluten challenge protocol, using initially an intake of at least 175 mg/kg/day of gluten powder, in addition to an otherwise gluten free diet, with at least 375 mg/kg/day of gluten powder after 2 weeks, with a maximum of 20 grams of gluten daily.[6;7] Dietary instructions were given by a specialised nurse or dietician. In one centre (Academic Medical Centre) no gluten powder was given and patients started with a regular gluten containing diet. After at least 3 months of gluten challenge a third small intestinal biopsy was taken, as in most patients mucosal relapse is to be expected within 3 months.[8] In children with clear clinical symptoms, this final biopsy was done earlier. CD was considered to be proven if the mucosa showed villous atrophy (Marsh 3a or higher).[3]

In the three patients without mucosal relapse, all small bowel biopsies taken were revised by a pathologist experienced in the diagnosis of CD (FJTK, PGJN). In all 100 patients who underwent a gluten challenge and in the 233 who did not, the results of serologic tests at initial diagnosis were retrieved from the medical file, if possible. Furthermore, we also investigated the number of pathologists involved in the assessment of the biopsies obtained from the gluten challenged patients.

Results

We studied the data of 333 children who were investigated for CD before the age of 2 years and had villous atrophy at initial small bowel biopsy (174 patients in the University Medical Centre, Utrecht; 89 in the Academic Medical Centre, Amsterdam; 38 in the Juliana Children's Hospital/ Haga Teaching Hospital, The Hague; and 32 in the VU Medical Centre, Amsterdam, The Netherlands).

Out of 333 children, a full gluten challenge according to the ESPGHAN recommendations was performed in 100 children (38 males, 62 females).[3] Clinical presentation, serologic- and histological results were similar in both the challenged and non challenged groups (table 1). In both groups the vast majority of patients presented with classical symptoms of malabsorption such as failure to thrive since gluten introduction and diarrhoea (challenged vs non challenged: 81% and 87% respectively; NS). Only a minority had atypical symptoms such as failure to thrive since birth or severe anemia (challenged vs non challenged: 5% and 4% respectively), while presentation could not be retrieved in 14% of challenged vs 9% of non challenged patients. Serologic results could also be retrieved for the majority of patients (challenged vs non challenged: 89% and 78%) and were compatible with CD in almost all patients both in the challenged and non challenged group. Results for histologic evaluation were also similar in both groups. Microscopy was typical for CD in 98% and 97% respectively. Histology was inconclusive in the remaining 2-3%. In these 7 patients villous atrophy was found, but combined with a substantial increased amount of neutrophiles in the lamina propria or no increase in the number of intra-epithelial lymphocytes.
A typical clinical presentation, combined with abnormal serology and typical histology was found in 69 (69%) of challenged patients and 154 (66%) of non challenged patients. Either an atypical presentation, normal serology or inconclusive histology was present in 12% of challenged and 11% of non challenged patients. For 19 (19%) patients in the challenged group either the clinical presentation or the serology could not be retrieved. Similarly for 53 patients (23%) in the non challenged group one of these parameters was not available.

During the study period only 100 out of the 233 patients had had a gluten challenge, with most challenges being performed in the University Medical Centre in Utrecht (68/174; 40%) and the Academic Medical Centre in Amsterdam (23/89; 26%), these centres indicating that they tried to comply with the ESPGHAN recommendations. The other two centres (Juliana Children's Hospital in the Hague and the VU Medical Centre in Amsterdam) expressed a more liberal view, which was reflected in the lower number of challenges at these centres, respectively 6/38, (16%) and 3/32 (10%). The difference in challenge rate between these two groups of centres is significant ($P<0.001$).

Table 1 Clinical presentation, serologic- and histological results in two groups (gluten challenge vs no gluten challenge)

	Gluten challenge (N=100)	No gluten challenge (N=233)
Clinical presentation		
- typical	81 (81%)	202 (87%)
- atypical	5 (5%)	10 (4%)
- screening	-	1 (0.4%)
- unknown	14 (14%)	20 (9%)
Serology results		
- all tests performed abnormal	77 (77%)	163 (70%)
- mixed; some positive and some negative	7 (7%)	17 (7%)
- negative	3 (3%)	-
- IgA deficiency	1 (1%)	1 (0.4%)
- suspected IgA deficiency	1 (1%)	-
- unknown	11 (11%)	52 (22%)
Histology results		
- typical (Marsh III)	98 (98%)	228 (97%)
- inconclusive	2 (2%))	5 (3%)

Table 2 Characteristics of the three patients with villous atrophy at initial biopsy but who did not relapse after gluten challenge

Patient	Clinical features	Laboratory results	Pathology	Conclusion
1	- Failure to thrive - 2 years follow-up; no complaints, normal growth on diet containing gluten	- Negative serology (IgA-AGA, IgA-ARA and EMA) - Absence of HLA-DQ2 and -DQ8 genes	- Giardia lamblia infestation in biopsy (initially overlooked)	- No coeliac disease
2	- Failure to thrive - Severe social problems - 9 years follow-up; no complaints, normal growth on diet containing gluten	- Negative serology (IgA-AGA and IgA-ARA)		- Transient gluten intolerance? - Emotional failure to thrive?
3	- Acute vomiting and diarrhoea, failure to thrive and anemia - 4 years follow-up; no complaints, normal growth on diet containing gluten	- Partial negative serology (IgA-AGA positive, IgG-AGA and EMA negative)	- At re-evaluation of initial biopsy, diagnosis was judged incorrect (no increased intra-epithelial lymfocytes)	- No coeliac disease

The reason to refrain from performing gluten challenge was not stated in the files of 169 out of 233 patients (73%). In 34 (14%) patients either the parents or the paediatrician did not want to challenge with gluten or considered this unnecessary. Furthermore, in 26 patients (11%) a gluten challenge was started but not completed mostly because symptoms recurred, while in 4 patients the gluten challenge was started but patients were not included in this study as the challenge was completed after October 1st 2006 (n=2) or the biopsies were taken in other hospitals (n=2).

The mean duration of gluten challenge was 4.2 months (range 0.7-26.7). The mean age at gluten challenge was 3.7 years (range 2.0-8.7) and the mean age at initial biopsy was 1.4 years (range 0.8-2.0). At the second small intestinal biopsy, performed before gluten challenge, a completely normal intestinal mucosa (Marsh 0) was found in 85 patients. In 15 patients mucosal histology was more or less abnormal with increased intra-epithelial lymphocytes (Marsh I) in 8 patients, crypt hyperplasia (Marsh II) in 3 patients and villous atrophy (Marsh III) in 4 patients. After gluten challenge villous atrophy (Marsh III) was found in 97 patients (97%) with the histology deteriorating in the 4 patients with villous atrophy before the challenge had started. In only three children (3%) the small bowel biopsy showed no abnormalities before and after respectively 3, 9 and 15 months of gluten challenge (*table 2).* These three children did not have any symptoms during gluten challenge. In two of the three children, serology was negative at the time of diagnosis and before the introduction of a gluten free diet. In one patient IgA antigliadin antibodies (IgA-AGA) were positive (24 U/ml, normal value < 4 U/ml), while IgG antigliadin (IgG-AGA) and IgA endomysium antibodies (EMA) were negative.

In table 3 all serology results of 97 challenged patients are shown. The 3 patients who did not relapse were not included in this table. In 86 out of the 97 children who relapsed after gluten challenge, serology results were available for analysis; in two patients no serology was performed and in 9 patients the results could not be retrieved. Abnormal serologic results for all tests performed at initial diagnosis were seen in 77 of the 86 children. In 51 out of 57 patients who relapsed and were tested for EMA this test was positive (sensitivity 89%, 95% confidence interval (CI) 79-95%), while in 75 out of 78 patients IgA-AGA was positive (sensitivity 96%, (CI 89-99%)). In seven children who relapsed (mean age at first diagnosis 17,4 months, range 14-21 months) IgA-AGA and/or IgG-AGA could be detected at the time of initial diagnosis, while EMA and/or IgA anti-reticuline antibodies (IgA-ARA) were negative. In only one child, 11 months old at initial diagnosis, all serologic tests done (IgA-AGA and EMA) were negative, while total serum IgA was normal.

During the total study period in none of the 4 participating hospitals a single pathologist was dedicated to see the biopsies of all patients who had a gluten challenge. In fact it turned out that over 20 pathologists were involved in the assessment of these biopsies.

Table 3 Serology results at first biopsy in the 97 patients who did relapse after gluten challenge

Serology results	No of patients
All serologic tests done abnormal	77
- IgA-AGA pos	11
- IgA-AGA and IgG-AGA pos	8
- IgA-AGA and EMA pos	17
- IgA-AGA, IgG-AGA and EMA pos	16
- IgA-AGA, EMA and tTG pos	2
- IgA-AGA, IgG-AGA, EMA and tTG pos	5
- EMA pos	7
- IgA-ARA and IgA-AGA pos	6
- IgA-ARA, IgA-AGA and EMA pos	4
- Known IgA deficiency with IgG-AGA pos	1
Serologic tests partly normal	8
- IgA-AGA neg, IgG-AGA pos, EMA inconclusive	1
- IgA-AGA pos, IgG-AGA pos, EMA neg	2
- IgA-AGA pos, EMA neg	1
- IgA-ARA neg, IgA-AGA pos	1
- IgG-AGA pos, IgA-AGA neg, IgA-ARA neg, EMA neg, tTG neg. (Suspected IgA deficiency; total IgA unknown)	1
- IgA-AGA pos, IgG-AGA pos, IgA-ARA neg	1
- IgA-AGA pos, IgG-AGA neg	1
All serologic tests done negative	1
- IgA-AGA neg, EMA neg	1
Patients in whom results could not retrieved	9
Serology not performed	2
Total	97 patients

Discussion

We retrospectively evaluated the data of 333 children who were diagnosed with CD before the age of 2 years. This study shows that only 30% of these children underwent a formal gluten challenge as proposed by the 1990 ESPGHAN guidelines. More importantly, the initial diagnosis of CD was rejected in only 3 out of the 100 children in whom gluten challenge was performed. In these three non-relapsers highly sensitive and specific serology (EMA, IgA-ARA) was negative at diagnosis, although one patient had positive IgA-AGA.

Only few studies, in which a biopsy at initial diagnosis was performed in at least 90% of the patients, have investigated the percentage of children who did not relapse at gluten challenge. In these studies the percentage of non-relapsers varied between 3-7% and these children were usually diagnosed with cow's milk sensitive enteropathy or transient gluten intolerance.[4;9-11] The largest study, by Guandalini *et al*, investigated 2523 children and found 5% of non-relapsers with a mean age of 8 months (age range not published).[4] This study initiated the 1990 ESPGHAN recommendation to only perform routine gluten challenge in children < 2 years at diagnosis. Subsequently, Danielsson *et al* performed a study similar to ours in 62 children under the age of two. In this study 3/62 (4.5%) patients did not relapse.[10] In contrast, O'Halloran *et al* reported that 25% of 35 children diagnosed under the age of 2 years showed no mucosal relapse after at least 6 months of gluten containing diet.[12] However, the initial diagnosis of CD was unreliable in this study, as not all patients had an initial biopsy, and in one-third (13/ 40) of non-relapsers of all ages the histological abnormalities at initial diagnosis were only mild. The authors even suggested that retrospectively in these 13 patients a gluten free diet might not have been necessary anyway. It should be noted that in none of these studies serological investigations were done.
In contrast, in our study serology results were available for the vast majority of patients. All patients with a positive EMA and/or tTG at initial diagnosis relapsed at gluten challenge. A further six patients with CD proven at gluten challenge had a normal or inconclusive EMA at the time of the initial biopsy. This results in a sensitivity of 89% which is somewhat lower than published values that generally exceed 95%, but is in agreement with studies that indicate that EMA is less sensitive in children less than two years of age.[13;14] In this group IgA-AGA and/or IgG-AGA are almost always abnormal, as was also found in our study. However generally sensitivity of these two markers is considerably lower as compared to EMA and tTG.[15] They may also be false positive, as was indeed the case in our patient 3. In the other two non-relapsers all serology done was negative, consistent with the normal mucosa found after gluten challenge.

There are some limitations to the present study. First, our study had a retrospective design. Also mucosal relapse may take a long time and a second control biopsy after several years of gluten intake was not performed in the three patients who did not relapse.[10;11;16] However, in two of these three non-relapsers the initial diagnosis of CD was undoubtedly incorrect and the third patient had no clinical symptoms during 9 years on a gluten containing diet.
Furthermore we tried to retrieve the reason why a large group of patients was not

challenged, but the cause could only be identified in 27% of cases. Although we had expected that in the challenged group the diagnosis might be less solid, in fact our data (table 1) show that no differences existed in clinical, serological and histological characteristics between the challenged group and the non challenged group. Therefore it seems that the patients who were challenged are not a selected group, and that the results probably can be extrapolated to the entire group. In fact at the onset of the study physicians in two centres (University Medical Centre, Utrecht and Academic Medical Centre, Amsterdam) indicated that they tried to comply with the ESPGHAN recommendations, and persuade parents to have their children challenged. In these centres the challenge rate was 40 and 26% respectively. In the two other centres (Juliana Children's Hospital and VU Medical Centre) some doubt existed whether a challenge under two years of age was really necessary. Consequently, the challenge rate in these centres at 16% and 10% was significantly lower ($P<0.001$).

A further limitation is the considerable number of pathologists that investigated the biopsies, which might have resulted in interobserver differences and reduced the personal experience for each pathologist. However, this phenomenon is probably common in daily practice. In fact the mean number of cases handled by each pathologist in this study (<1 challenged patient/ pathologist/ year) is probably not that different from the situation in smaller centres. Therefore our results might be more generally applicable.

As all our patients with a typical presentation, abnormal serology and villous atrophy at presentation relapsed when a challenge was done, we suggest that routine gluten challenge should not be mandatory anymore in children younger than 2 years at diagnosis, if clinical symptoms are indeed compatible with CD and the characteristic histological changes (Marsh III(17)) in the intestinal biopsy sample are found in combination with EMA and/or tTG. Symptoms should disappear and serology should normalise after initiation of the gluten free diet. However, gluten challenge might still be useful in children with a) aspecific clinical symptoms; b) equivocal histological changes in the small bowel biopsy sample; c) conflicting serological test results; and d) incomplete disappearance of symptoms or no improvement of serology during gluten-free diet. Individual circumstances should determine the precise approach to a particular patient. A revision of the current diagnostic criteria established by ESPGHAN should be considered.

Acknowledgements

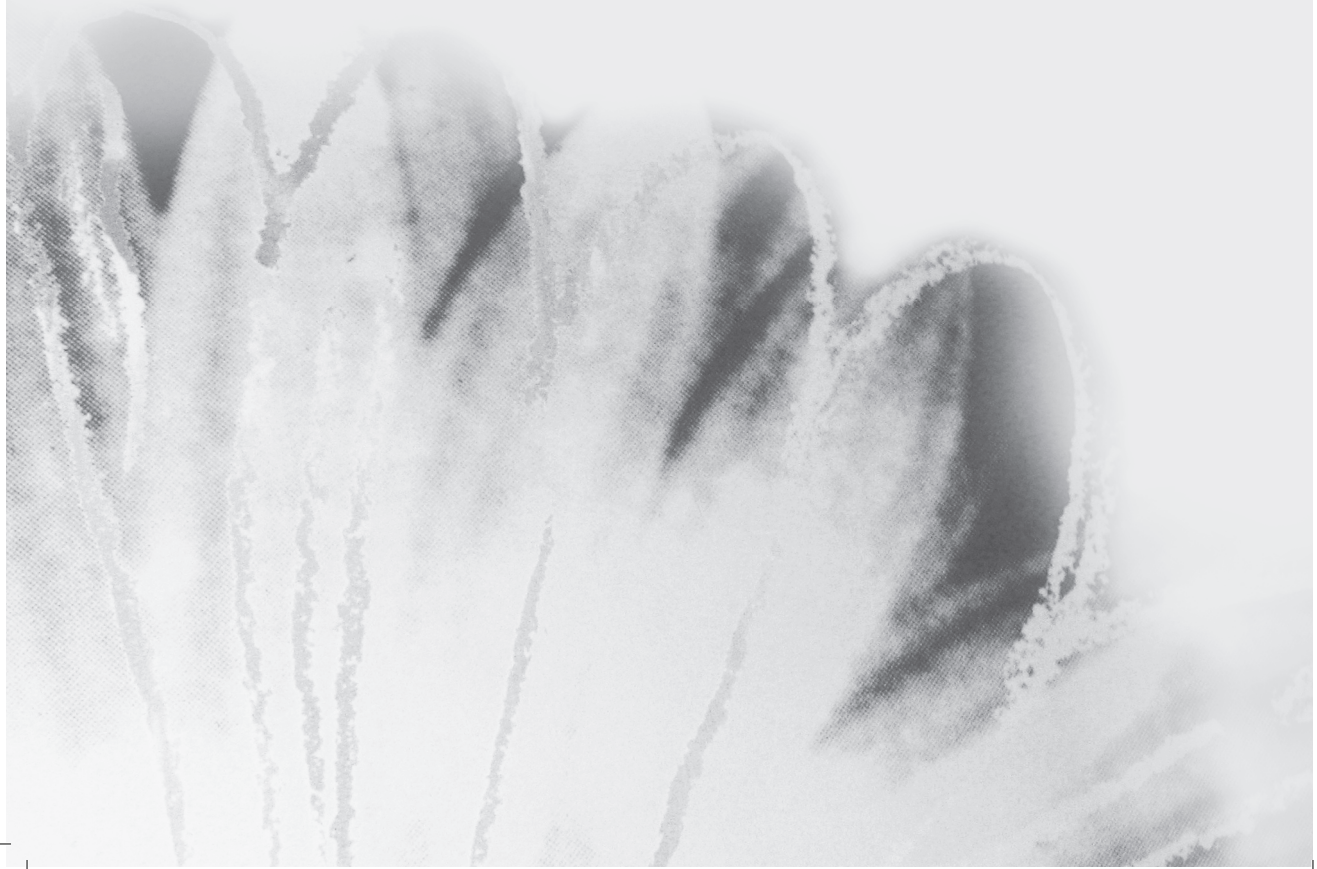
We thank Winny van der Hoeven, Janet Feenstra, Gideon Hajer and Irene Wilson, WKZ/UMC Utrecht, and Joyce Goris-de Geus, IJsselmeer Hospital, Lelystad, and Wieke Verbeek, VUMC, Amsterdam, The Netherlands for their help. We are grateful to the paediatricians who referred their patients to our hospital.

References

1. McNeish AS, Harms HK, Rey J *et al*. The diagnosis of coeliac disease. A commentary on the current practices of members of the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN). *Arch Dis Child* 1979;**54**(10):783-6.
2. Meeuwisse G.W. Diagnostic criteria in coeliac disease. *Acta Paediatr Scand* 70 A.D.;**59**:461.
3. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. *Arch Dis Child* 1990;**65**(8):909-11.
4. Guandalini S, Ventura A, Ansaldi N *et al*. Diagnosis of coeliac disease: time for a change? *Arch Dis Child* 1989;**64**(9):1320-4.
5. McNeish AS. Coeliac disease: duration of gluten-free diet. *Arch Dis Child* 1980;**55**(2):110-1.
6. Littlewood JM. Coeliac disease in childhood. *Baillieres Clin Gastroenterol* 1995;**9**(2):295-327.
7. Packer SM, Charlton V, Keeling JW *et al*. Gluten challenge in treated coeliac disease. *Arch Dis Child* 1978;**53**(6):449-55.
8. Hamilton JR, McNeill LK. Childhood celiac disease: response of treated patients to a small uniform daily dose of wheat gluten. *J Pediatr* 1972;**81**(5):885-93.
9. Mayer M, Greco L, Troncone R *et al*. Early prediction of relapse during gluten challenge in childhood celiac disease. *J Pediatr Gastroenterol Nutr* 1989;**8**(4):474-9.
10. Danielsson L, Stenhammar L, Astrom E. Is gluten challenge necessary for the diagnosis of coeliac disease in young children? *Scand J Gastroenterol* 1990;**25**(9):957-60.
11. Shmerling DH, Franckx J. Childhood celiac disease: a long-term analysis of relapses in 91 patients. *J Pediatr Gastroenterol Nutr* 1986;**5**(4):565-9.
12. O'Halloran ET, Read M, Barry RG *et al*. The management of coeliac disease. *Ir Med J* 1998;**91**(6):199-202.
13. Agardh D, Carlsson A, Lynch K *et al*. Using radioligand-binding assays to measure tissue transglutaminase autoantibodies in young children. *Acta Paediatr* 2004;**93**(8):1046-51.
14. Burgin-Wolff A, Gaze H, Hadziselimovic F *et al*. Antigliadin and antiendomysium antibody determination for coeliac disease. *Arch Dis Child* 1991;**66**(8):941-7.
15. Rostom A, Dube C, Cranney A *et al*. The diagnostic accuracy of serologic tests for celiac disease: a systematic review. *Gastroenterology* 2005;**128**(4 Suppl 1):S38-S46.
16. Kuitunen P, Savilahti E, Verkasalo M. Late mucosal relapse in a boy with coeliac disease and cow's milk allergy. *Acta Paediatr Scand* 1986;**75**(2):340-2.
17. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992;**102**(1):330-54.

Part 2

Genetics



Chapter 6

The genetic background of celiac disease and its clinical implications

Wolters VM
Wijmenga C

Am J Gastroenterol 2008;103(1):190-195

Abstract

Celiac disease (CD) is a complex genetic disorder with multiple contributing genes. Linkage studies have identified several genomic regions which probably contain CD susceptibility genes. The most important genetic factors identified are HLA-DQ2 and HLA-DQ8, which are necessary but not sufficient to predispose to CD. The associations found in non-HLA genome-wide linkage and association studies are much weaker. This might be because a large number of non-HLA genes contribute to the pathogenesis of CD. Hence, the contribution of a single predisposing non-HLA gene might be quite modest. Practically all CD patients carry HLA-DQ2 or HLA-DQ8, while the absence of these molecules has a negative predictive value for CD close to 100%. Genetic risk profiles for CD would be helpful in clinical practice for predicting disease susceptibility and progression.

Introduction

Celiac disease (CD) is a common enteropathy with a strong genetic risk. It is characterized by a permanent intolerance for gluten proteins present in dietary wheat, rye and barley. It affects approximately 1:100-300 individuals,[1-3] although only 1 person in ~8 is aware of being affected because the symptoms may be mild or non-specific.[4] Environmental, genetic and immunologic factors are important in the pathogenesis of CD.

Genes play a key role in CD and considerable progress has been made in identifying some of those responsible for CD. The roles of HLA-DQ2 and HLA-DQ8 are well known as almost all patients carry the genes encoding these heterodimers.[5;6] Non-HLA genes also contribute to the development of CD, but these associations are less evident. Genetic linkage analyses have identified susceptibility loci on various chromosomes, such as 2, 5, 6, 9, 15 and 19, revealing the complexity of CD.[7-16]

Gluten is the most important environmental factor. Gluten proteins provoke the disease as the high proline content of gluten is relatively resistant to proteolytic digestion in the intestinal tract.[17;18] The undigested gluten peptides are deamidated by tissue transglutaminase, which results in a better binding capacity to the pocket of HLA-DQ2 or HLA-DQ8 molecules on antigen-presenting cells (see figure 1). This complex is presented to CD4+ T cells and the ensuing immune response causes inflammation and intestinal tissue damage. A direct response of the epithelium via the innate immune system also plays a role.

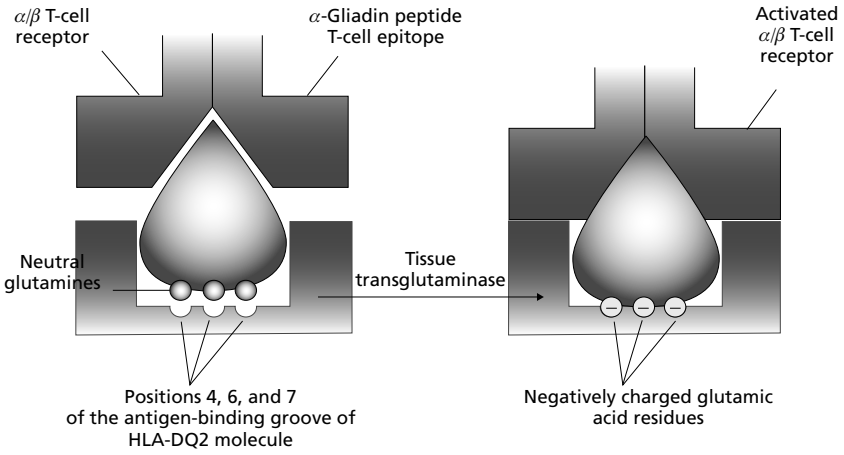
Genetic epidemiology of CD

Multiple lines of evidence favoring a genetic contribution to the pathogenesis of CD have been suggested by epidemiologic data (table 1). A familial aggregation is found in 5-15% of CD patients and a striking 83-86% concordance rate was observed among monozygotic twin pairs.[19;20] CD incidence and prevalence have been found to vary significantly, depending on geographic location and racial or ethnic background; these differences might be reflected by either genetic or environmental susceptibility factors.[21]

Table 1 Evidence for genetic susceptibility to celiac disease

Ethnic differences in disease incidence/prevalence	[21]
Familial aggregation	5-15% first-degree relatives of affected individuals are also affected 30% HLA identical sibs
Twin studies concordance rates	Monozygotic twins 83-86% Dizygotic twins 11%
Identification of susceptibility loci by genome screening	Chromosomes 2, 5, 6, 9, 15, 19
Genetic association studies of functional candidate genes	CTLA4
Association found with genetic syndromes	Down syndrome[57;58] Turner syndrome[59-62] Williams syndrome[63]

Figure 1 HLA-DQ2 and tissue transglutaminase with permission of *Farrell RJ, Kelly CP in N Engl J Med.* 2002 Jan 17;346(3):180-8.



Pathogenesis of Celiac Disease

Gliadin is absorbed into the lamina propria and presented in conjunction with HLA-DQ2 or DQ8 cell-surface antigens by antigen presenting cells, probably dendritic cells, to sensitized T cells expressing the α / ß-cell receptor. Tissue transglutaminase deamidates gliadin peptides, generating acidic, negatively charged residues of glutamic acid from neutral glutamines. Because negatively charged residues are preferred in positions 4, 6, and 7 of the antigen-binding groove of HLA-DQ2, deamidated gliadin elicits a stronger T-cell response.

Gene identification in CD

Two complementary approaches are used in the search for genetic susceptibility genes in CD: genetic linkage and genetic association studies. Genetic linkage studies make use of families with affected sibling pairs to identify chromosomal regions shared between the affected siblings above the mean of what is statistically expected. To identify the actual susceptibility locus we use genetic markers (SNPs; single nucleotide polymorphisms). Linkage regions usually encompass 10 to 100 genes. Once linkage is identified, the next step is a genetic association study to identify the specific disease gene from the candidate gene locus.

Candidate gene association studies search for differences in frequencies of genetic variants in patients compared to control individuals. Such association studies can focus on positional candidate genes from a linkage region, or on functional candidate genes selected from hypothesized disease pathology. More recently, it has become feasible to perform genome-wide association studies – a hypothesis-free approach which can test thousands of SNPs across the whole genome for association.

Genetic linkage studies in CD

CELIAC1 locus

The CELIAC1 locus on chromosome 6p21 contains HLA class II molecules.[22] It is unequivocal that CD is strongly associated with specific HLA class II genes known as HLA-DQ2 and HLA-DQ8.[23] HLA-DQ molecules are heterodimers consisting of an α and β chain. Particularly the combination of alleles encoding for the α chain DQA1*05 and β chain DQB1*02 of the HLA-DQ2 heterodimer are associated with CD. Most CD patients (~95%) express HLA-DQ2 and the remaining patients are usually HLA-DQ8 positive. The HLA-DQ2 allele is common and is carried by approximately 30% of Whites.[23] However, only ~3% of individuals in the general population who carry HLA-DQ2 will develop CD.[24] It is noteworthy that individuals homozygous for the DQ2 molecule comprise approximately 2% of the European population but make up approximately 25% of all CD patients.[5] Thus, HLA-DQ2 or HLA-DQ8 is necessary for disease development but not sufficient as its estimated risk effect is only 36%-53%.[25] Thus, non-HLA genes may well contribute more than HLA. The importance of non-HLA genes is supported by the difference in concordance rates seen among HLA identical siblings (~30%).[19;26;27] However, linkage peaks observed in non-HLA regions are much lower and not consistent compared to HLA. This might be because many non-HLA genes contribute to the pathogenesis of CD. Hence, the contribution of a single predisposing non-HLA gene might be modest.

CELIAC2 locus

The support for linkage to the CELIAC2 locus on chromosome 5q31-33 was first identified by Greco *et al*.[7;8] Replication of this locus has not been universal and no disease causing functional gene could be identified. This region contains a cytokine gene cluster and it might play a role in immune regulation and inflammation.

CELIAC3 locus

The *CELIAC3* locus on chromosome 2q33 has shown linkage to CD and was replicated by a few but not all the studies performed.[9;28-33] The *CELIAC3* locus contains the T lymphocyte regulatory genes *CD28, CTLA4 and ICOS*.

CTLA4 (Cytotoxic T lymphocyte-associated antigen 4) is a negative costimulatory molecule of the T cell response and *CTLA4* was already pinpointed as a candidate gene based on this known function before the era of genome-wide linkage studies. No evidence for a single mutation in *CTLA4* specific to CD has been found but a strong association was suggested at the haplotype level.[34] The genetic variants in the *CTLA4* gene in CD were extensively studied in several populations but with opposite results.[35]

The CELIAC4 locus

A genome-wide scan by our group identified a region of significant linkage at chromosome 19p13.1.[11] Further association analysis showed association to the myosin IXB gene (*MYO9B)*. Interestingly, *MYO9B* is a good candidate gene for CD because of its function; it encodes an unconventional myosin molecule that may have a role in actin remodeling of epithelial enterocytes. It is hypothesized that this genetic variant might lead to an impaired intestinal barrier, which might allow the passage of immunogenic gluten peptides. This could be a factor involved in the early mucosal events preceding the inflammatory response in CD. However, in CD populations in the United Kingdom, Spain, Italy and Scandinavia this association could not be replicated.[36-39] Furthermore, it is unlikely that the SNP itself is the causing mutation as it is located deep in the intron, which means that it has no function in coding for protein synthesis. Most probably the SNP is rather a disease marker, an allele 'hitchhiking' (in linkage disequilibrium) with the true causative variant.

Chromosome 19p13 was also shown to have significant linkage to inflammatory bowel disease (*IBD6*) and recently our group showed an association of ulcerative colitis with *MYO9B* and Morbus Crohn, albeit weaker.[40;41] This suggests that *MYO9B* might also promote susceptibility to other intestinal inflammatory diseases, although the precise mechanism of how gene variants in *MYO9B* can lead to altered gut function is unclear.

A strong association to *MYO9B* was also reported in patients with a complicated form of CD, known as refractory CD type II (RCD II). In this group the enteropathy persists despite adherence to a gluten-free diet or it recurs after an initially good response to the diet. RCD II patients are characterized by the presence of aberrant intra epithelial lymphocytes in the small bowel mucosa.

Genetic association studies

CTLA4

Although increasing data pinpoint *CTLA4* as a candidate gene, results of association studies did not support certain polymorphisms in *CTLA4* being the major susceptibility locus for CD.[14;28;29;32;34;42-44] A recent study analyzing all common SNPs in *CTLA4* suggested an association on

haplotype level rather than on single SNP variant level.[34] In figure 2 the suggested genetic loci of CD are shown.

Other candidate genes

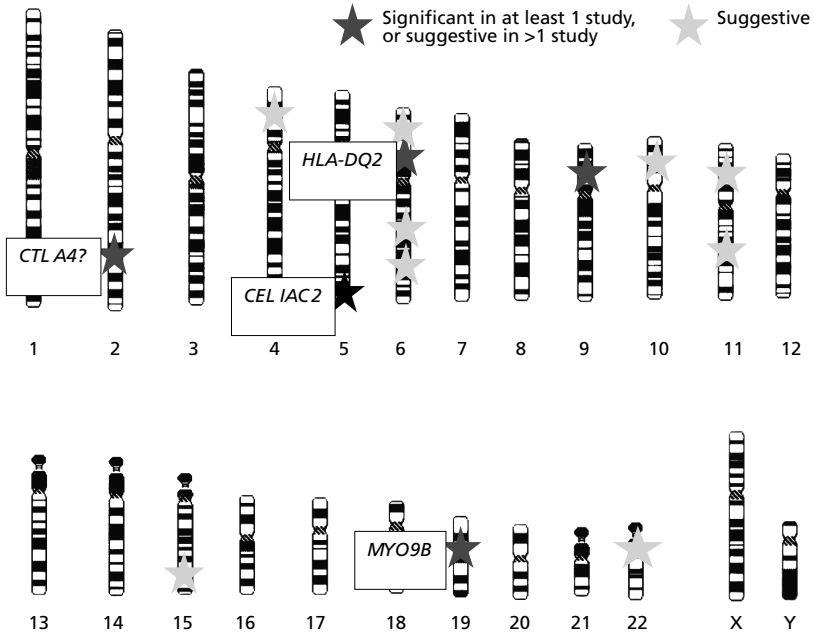
Other studies did not confirm association for promising genes like *tTG, FAS, MMP-1* and *3, TCR αβγδ, IL12β, CD28, CD80, CD86, KIR, LILR, STAT 1, PGPEP1, IRF1, DPPIV, TGM2, NOS2* or *IFNγ*.[35]

Other new (genetic) developments in CD

Factor V Leiden

Recently a genetic association between factor V Leiden (FVL) and CD was discovered.[45] A family was described in which the two diseases segregated in all cases as no sibling was affected by only one of the diseases, suggesting that the genetic mutation responsible for the development of CD in this family occurs in a gene very close to FVL on chromosome 1q. Further assessment of this association is needed to unravel the pathogenesis of CD.

Figure 2 Genetic loci in CD



Possibly causal factors

Breastfeeding, amount of gluten, and age of gluten introduction

Sweden experienced a three-fold increase in incidence of CD in children younger than 2-yr old in the mid-1980s.[27] This was partly explained by changing recommendations on gluten introduction in infants (in 1982) from 4 to 6 months of age; as a result more infants were introduced to gluten without ongoing breastfeeding. At the same time the content of gluten in baby food was increased. In 1996, the Swedish authorities recommended a gradual introduction of gluten from the age of 4 months while breastfeeding, and the incidence of CD soon dropped dramatically. The current European recommendation states that breast-feeding during gluten introduction might be beneficial to children at high risk for CD, although it is unclear if breastfeeding prevents CD or simply delays the onset of disease.[46]

Infectious episodes

Interestingly, in the Swedish epidemic of CD, children born during the summer had a greater risk for CD, which might be because gluten was introduced during the winter when infections are more common.[47] Two explanations for this increased CD risk were suggested: infections might change gut permeability leading to the passage of immunogenic gluten peptides through the epithelial barrier. The other possibility implies that sequence similarities exist between proteins produced during adenovirus infections and proteins of gluten. In 1987 Kagnoff suggested a role for human intestinal adenovirus.[48] Recently, a prospective study showed that multiple rotavirus infections predicted a higher risk of CD.[49]

Medication

The onset of CD has been reported during a course of treatment of hepatitis C with interferon (IFN).[50-52] This medication might increase epithelial permeability and proinflammatory cytokine production.[53;54] In hepatitis C patients, the activation of silent CD during IFN treatment should be suspected although symptoms subsided in almost all patients after IFN was withdrawn and without a gluten-free diet.[55] However, the histological abnormalities were still seen several months after discontinuing IFN, which means a timely diagnosis of CD after IFN.

Animal model

So far there is no good animal model for CD although some experience is available with gluten-sensitive enteropathy (GSE) in Irish setter dogs.[56]

Genes and syndromes

Several genetic syndromes are associated with CD, *e.g*. Down syndrome (DS), Turner syndrome (TS) and Williams syndrome (WS) and it is tempting to speculate that possible chromosomal derangements might influence a disturbed immune response in these syndromes.

Clinical relevance

To date, only HLA-DQ2 or HLA-DQ8 typing is clinically relevant, as all the other promising genetic loci could not be replicated consistently. The main role of HLA typing lies in its high

negative predictive value to exclude CD (close to 100%). CD can be virtually excluded in non-biopsy proven white individuals on a gluten-free diet who are non-HLA-DQ2 and non-HLA-DQ8. HLA typing can be useful to help exclude the possibility of the future development of CD in patients at high risk and can provide additional information if the clinical picture is unclear. First degree relatives of CD patients should be HLA typed and if CD cannot be excluded (i.e. HLA-DQ2 or -DQ8 positive) serologic tests might be performed in asymptomatic patients with a frequency of ~ every five to ten years years when patients have still growing potential (< 20 years). In asymptomatic HLA DQ2/DQ8 positive first degree relatives > 20 years one single screening at the age of ~ 50 years might be indicated as complications of CD can develop. When a first degree relative is symptomatic a low threshold for biopsy is indicated.

Future prospects

We assume an etiology model for CD comprising a major gene (HLA) and several low risk genes with a function in the intestinal barrier and immune system. It is therefore important to identify susceptibility genes as well as protective genes in CD.
In the coming years identifying other target genes and understanding the pathways they influence will lead to a better understanding of CD pathogenesis. Ultimately we might be able to define genetic risk profiles for more precise diagnoses and for predicting disease progression, and they may lead to novel therapies.

Clinically relevant conclusions

Genetic susceptibility is a prerequisite for developing CD with HLA class II as the most important genetic factor identified so far.

The absence of HLA-DQ2/-DQ8 can exclude the possibility or future development of CD with a certainty close to 100%.

In asymptomatic HLA-DQ2/-DQ8 positive first degree relatives < 20 years serologic screening is indicated approximately every 5-10 years. In asymptomatic HLA-DQ2/-DQ8 positive first degree relatives > 20 years one single screening at the age of approximately 50 years is indicated as complications of CD can develop.

The presence of HLA-DQ2/-DQ8 is clinically irrelevant since 30-40% of the general population is positive for one or both of these factors and only a fraction (0.5-1%) of these individuals have CD.

Considerable advances in the genetics in CD will identify more genetic determinants for CD development and disease progression and to establish clinically relevant genetic risk profiles. CD might serve as a model for other auto-immune diseases.

Cisca Wijmenga is the guarantor of the paper. Each of the two authors contributed equally to the paper. Victorien Wolters received funding from The Wilhelmina Research Fund and Cisca Wijmenga received funding from the Netherlands Organization of Scientific Research, the Dutch Digestive Diseases Foundation, and the Celiac Disease Consortium, an Innovative Cluster approved by the Netherlands Genomics Initiative and partially funded by the Dutch Government (BSIK03009). There are no competing interests.

Acknowledgements

We thank Chris Mulder, Jackie Senior and Alexandra Zhernakova for commenting on the manuscript.

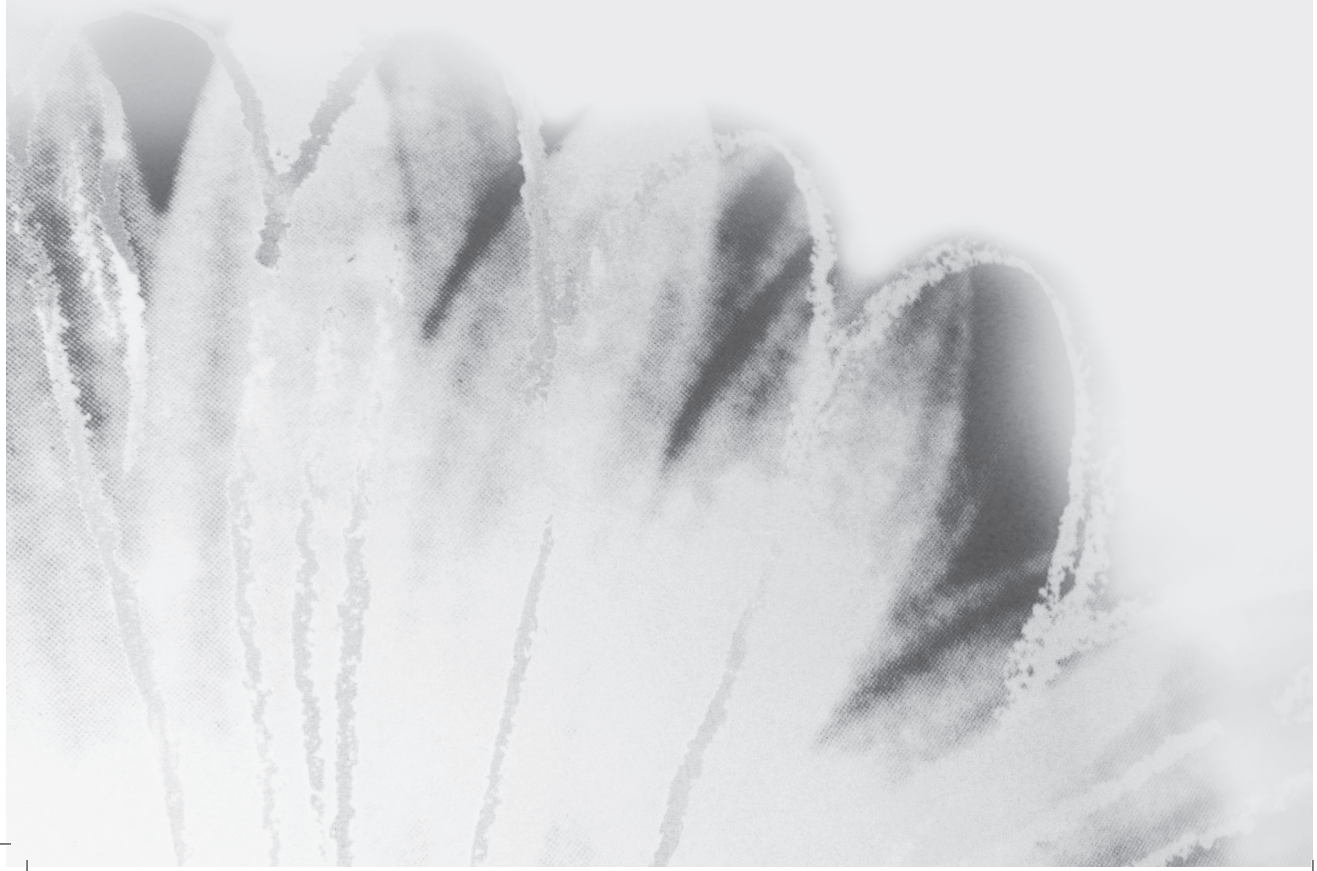
References

1. Catassi C, Ratsch IM, Fabiani E et al. Coeliac disease in the year 2000: exploring the iceberg. *Lancet* 1994;**343**:200-203.
2. Fasano A, Berti I, Gerarduzzi T et al. Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. *Arch Intern Med* 2003;**163**:286-292.
3. Gomez JC, Selvaggio GS, Viola M et al. Prevalence of celiac disease in Argentina: screening of an adult population in the La Plata area. *Am J Gastroenterol* 2001;**96**:2700-2704.
4. Catassi C, Fabiani E, Ratsch IM et al. The coeliac iceberg in Italy. A multicentre antigliadin antibodies screening for coeliac disease in school-age subjects. *Acta Paediatr Suppl* 1996;**412**:29-35.
5. Karell K, Louka AS, Moodie SJ et al. HLA types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the European Genetics Cluster on Celiac Disease. *Hum Immunol* 2003;**64**:469-477.
6. Margaritte-Jeannin P, Babron MC, Bourgey M et al. HLA-DQ relative risks for coeliac disease in European populations: a study of the European Genetics Cluster on Coeliac Disease. *Tissue Antigens* 2004;**63**:562-567.
7. Greco L, Corazza G, Babron MC et al. Genome search in celiac disease. *Am J Hum Genet* 1998;**62**:669-675.
8. Greco L, Babron MC, Corazza GR et al. Existence of a genetic risk factor on chromosome 5q in Italian coeliac disease families. *Ann Hum Genet* 2001;**65**:35-41.
9. Holopainen P, Naluai AT, Moodie S et al. Candidate gene region 2q33 in European families with coeliac disease. *Tissue Antigens* 2004;**63**:212-222.
10. Liu J, Juo SH, Holopainen P et al. Genomewide linkage analysis of celiac disease in Finnish families. *Am J Hum Genet* 2002;**70**:51-59.
11. van Belzen MJ, Meijer JW, Sandkuijl LA et al. A major non-HLA locus in celiac disease maps to chromosome 19. *Gastroenterology* 2003;**125**:1032-1041.
12. van Belzen MJ, Vrolijk MM, Meijer JW et al. A genomewide screen in a four-generation Dutch family with celiac disease: evidence for linkage to chromosomes 6 and 9. *Am J Gastroenterol* 2004;**99**:466-471.
13. Woolley N, Holopainen P, Ollikainen V et al. A new locus for coeliac disease mapped to chromosome 15 in a population isolate. *Hum Genet* 2002;**111**:40-45.
14. King AL, Moodie SJ, Fraser JS et al. CTLA-4/CD28 gene region is associated with genetic susceptibility to coeliac disease in UK families. *J Med Genet* 2002;**39**:51-54.
15. Babron MC, Nilsson S, Adamovic S et al. Meta and pooled analysis of European coeliac disease data. *Eur J Hum Genet* 2003;**11**:828-834.
16. Percopo S, Babron MC, Whalen M et al. Saturation of the 5q31-q33 candidate region for coeliac disease. *Ann Hum Genet* 2003;**67**:265-268.
17. Molberg O, Uhlen AK, Jensen T et al. Mapping of gluten T-cell epitopes in the bread wheat ancestors: implications for celiac disease. *Gastroenterology* 2005;**128**:393-401.
18. van de WY, Kooy YM, van VP et al. Glutenin is involved in the gluten-driven mucosal T cell response. *Eur J Immunol* 1999;**29**:3133-3139.
19. Greco L, Romino R, Coto I et al. The first large population based twin study of coeliac disease. *Gut* 2002;**50**:624-628.

20. Nistico L, Fagnani C, Coto I et al. Concordance, disease progression, and heritability of coeliac disease in Italian twins. *Gut* 2006;55:803-808.
21. Greco L, Maki M, Di Donato F, Visakorpi JK. Epidemiology of coeliac disease in Europe and the Mediterranean Area. In: Auricchio S, Visakorpi JK, eds. Common food tolerances I: epidemiology of coeliac disease. Basel: Karger; 1992:25-44.
22. Liu E. Genetic testing for celiac disease. *MLO Med Lab Obs* 2006;**38**:10-13.
23. Sollid LM, Markussen G, Ek J et al. Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. *J Exp Med* 1989;**169**:345-350.
24. Liu E, Rewers M, Eisenbarth GS. Genetic testing: who should do the testing and what is the role of genetic testing in the setting of celiac disease? *Gastroenterology* 2005;**128**:S33-S37.
25. Petronzelli F, Bonamico M, Ferrante P et al. Genetic contribution of the HLA region to the familial clustering of coeliac disease. *Ann Hum Genet* 1997;**61**:307-317.
26. Ciacci C, Cirillo M, Sollazzo R et al. Gender and clinical presentation in adult celiac disease. *Scand J Gastroenterol* 1995;**30**:1077-1081.
27. Ivarsson A, Persson LA, Nystrom L, Hernell O. The Swedish coeliac disease epidemic with a prevailing twofold higher risk in girls compared to boys may reflect gender specific risk factors. *Eur J Epidemiol* 2003;**18**:677-684.
28. Djilali-Saiah I, Schmitz J, Harfouch-Hammoud E et al. CTLA-4 gene polymorphism is associated with predisposition to coeliac disease. *Gut* 1998;43:187-189.
29. Holopainen P, Arvas M, Sistonen P et al. CD28/CTLA4 gene region on chromosome 2q33 confers genetic susceptibility to celiac disease. A linkage and family-based association study. *Tissue Antigens* 1999;**53**:470-475.
30. Holopainen P, Mustalahti K, Uimari P et al. Candidate gene regions and genetic heterogeneity in gluten sensitivity. *Gut* 2001;**48**:696-701.
31. King AL, Fraser JS, Moodie SJ et al. Coeliac disease: follow-up linkage study provides further support for existence of a susceptibility locus on chromosome 11p11. *Ann Hum Genet* 2001;**65**:377-386.
32. Naluai AT, Nilsson S, Samuelsson L et al. The CTLA4/CD28 gene region on chromosome 2q33 confers susceptibility to celiac disease in a way possibly distinct from that of type 1 diabetes and other chronic inflammatory disorders. *Tissue Antigens* 2000;**56**:350-355.
33. Neuhausen SL, Feolo M, Farnham J, Book L, Zone JJ. Linkage analysis of HLA and candidate genes for celiac disease in a North American family-based study. *BMC Med Genet* 2001;**2**:12.
34. Hunt KA, McGovern DP, Kumar PJ et al. A common CTLA4 haplotype associated with coeliac disease. *Eur J Hum Genet* 2005;**13**:440-444.
35. van Heel DA, Hunt K, Greco L, Wijmenga C. Genetics in coeliac disease. *Best Pract Res Clin Gastroenterol* 2005;**19**:323-339.
36. Amundsen SS, Monsuur AJ, Wapenaar MC et al. Association analysis of MYO9B gene polymorphisms with celiac disease in a Swedish/Norwegian cohort. *Hum Immunol* 2006;**67**:341-345.
37. Giordano M, Marano C, Mellai M et al. A family-based study does not confirm the association of MYO9B with celiac disease in the Italian population. *Genes Immunol* 2006;**7**:606-608.

38. Nunez C, Marquez A, Varade J et al. No evidence of association of the MYO9B polymorphisms with celiac disease in the Spanish population. *Tissue Antigens* 2006;**68**:489-492.
39. Ogura Y, Bonen DK, Inohara N et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001;**411**:603-606.
40. Rioux JD, Silverberg MS, Daly MJ et al. Genomewide search in Canadian families with inflammatory bowel disease reveals two novel susceptibility loci. *Am J Hum Genet* 2000;**66**:1863-1870.
41. van Bodegraven AA, Curley CR, Hunt KA et al. Genetic variation in myosin IXB is associated with ulcerative colitis. *Gastroenterology* 2006;**131**:1768-1774.
42. Popat S, Hearle N, Hogberg L et al. Variation in the CTLA4/CD28 gene region confers an increased risk of coeliac disease. *Ann Hum Genet* 2002;**66**:125-137.
43. Rioux JD, Karinen H, Kocher K et al. Genomewide search and association studies in a Finnish celiac disease population: Identification of a novel locus and replication of the HLA and CTLA4 loci. *Am J Med Genet* 2004;**130**:345-350.
44. van Belzen MJ, Mulder CJ, Zhernakova A et al. CTLA4 +49 A/G and CT60 polymorphisms in Dutch coeliac disease patients. *Eur J Hum Genet* 2004;**12**:782-785.
45. Mari T, Zullo A, Hassan C, Di GL, Morini S. Genetic association between factor V Leiden and coeliac disease. *Gut* 2006;**55**:1677-1678.
46. Ivarsson A, Hernell O, Stenlund H, Persson LA. Breast-feeding protects against celiac disease. *Am J Clin Nutr* 2002;**75**:914-921.
47. Ivarsson A, Hernell O, Nystrom L, Persson LA. Children born in the summer have increased risk for coeliac disease. *J Epidemiol Community Health* 2003;**57**:36-39.
48. Kagnoff MF, Paterson YJ, Kumar PJ et al. Evidence for the role of a human intestinal adenovirus in the pathogenesis of coeliac disease. *Gut* 1987;**28**:995-1001.
49. Stene LC, Honeyman MC, Hoffenberg EJ et al. Rotavirus infection frequency and risk of celiac disease autoimmunity in early childhood: a longitudinal study. *Am J Gastroenterol* 2006;**101**:2333-2340.
50. Bardella MT, Marino R, Meroni PL. Celiac disease during interferon treatment. *Ann Intern Med* 1999;**131**:157-158.
51. Adinolfi LE, Durante ME, Andreana A. Interferon and ribavirin treatment for chronic hepatitis C may activate celiac disease. *Am J Gastroenterol* 2001;**96**:607-608.
52. Cammarota G, Cuoco L, Cianci R, Pandolfi F, Gasbarrini G. Onset of coeliac disease during treatment with interferon for chronic hepatitis C. *Lancet* 2000;**356**:1494-1495.
53. Watson CJ, Hoare CJ, Garrod DR, Carlson GL, Warhurst G. Interferon-gamma selectively increases epithelial permeability to large molecules by activating different populations of paracellular pores. *J Cell Sci* 2005;**118**:5221-5230.
54. Kamal SM, Fehr J, Roesler B, Peters T, Rasenack JW. Peginterferon alone or with ribavirin enhances HCV-specific CD4 T-helper 1 responses in patients with chronic hepatitis C. *Gastroenterology* 2002;**123**:1070-1083.
55. Durante-Mangoni E, Iardino P, Resse M et al. Silent celiac disease in chronic hepatitis C: impact of interferon treatment on the disease onset and clinical outcome. *J Clin Gastroenterol* 2004;**38**:901-905.

56. Polvi A, Garden OA, Houlston RS et al. Genetic susceptibility to gluten sensitive enteropathy in Irish setter dogs is not linked to the major histocompatibility complex. *Tissue Antigens* 1998;**52**:543-549.
57. Bonamico M, Mariani P, Danesi HM et al. Prevalence and clinical picture of celiac disease in italian down syndrome patients: a multicenter study. *J Pediatr Gastroenterol Nutr* 2001;**33**:139-143.
58. No authors. American Gastroenterological Association (AGA) Institute Technical Review on the diagnosis and management of celiac disease. *Gastroenterology* 2007;**131**:1981-2002.
59. Bonamico M, Bottaro G, Pasquino AM et al. Celiac disease and Turner syndrome. *J Pediatr Gastroenterol Nutr* 1998;**26**:496-499.
60. Bonamico M, Pasquino AM, Mariani P et al. Prevalence and clinical picture of celiac disease in Turner syndrome. *J Clin Endocrinol Metab* 2002;**87**:5495-5498.
61. Gillett PM, Gillett HR, Israel DM et al. Increased prevalence of celiac disease in girls with Turner syndrome detected using antibodies to endomysium and tissue transglutaminase. *Can J Gastroenterol* 2000;**14**:915-918.
62. Ivarsson SA, Carlsson A, Bredberg A et al. Prevalence of coeliac disease in Turner syndrome. *Acta Paediatr* 1999;**88**:933-936.
63. Giannotti A, Tiberio G, Castro M et al. Coeliac disease in Williams syndrome. *J Med Genet* 2001;**38**:767-768.



Chapter 7

The *MYO9B* gene is a strong risk factor for developing refractory celiac disease

Wolters VM
Verbeek WHM
Zhernakova A
Onland-Moret C
Schreurs MWJ
Monsuur AJ
Verduijn W
Wijmenga C
Mulder CJJ

Clin Gastroenterol Hepatol 2007;5(12):1399-405

Abstract

Background & Aims

Celiac disease (CD) is associated with HLA-DQ2 and HLA-DQ8, and has been linked to genetic variants in the *MYO9B* gene on chromosome 19. HLA-DQ2 homozygosity is associated with complications of CD such as refractory celiac disease type II (RCD II) and enteropathy-associated T-cell lymphoma (EATL). We investigated whether *MYO9B* also predisposes to RCD II and EATL.

Methods

Genotyping of *MYO9B* and molecular HLA-DQ2 typing was performed on 62 RCD II and EATL patients, 421 uncomplicated CD patients, and 1624 controls.

Results

One SNP in *MYO9B* showed a significantly different allele distribution in RCD II and EATL patients compared to controls (p=0.00002). The rs7259292 T allele was significantly more frequent in RCD II and EATL patients compared to CD patients (p=0.0003, OR 3.61 (95% CI 1.78-7.31)). The frequency of the haplotype carrying the T allele of this SNP was significantly increased in RCD II and EATL patients (11%), compared to controls (2%) and CD patients (3%) (OR 6.76 (95% CI 3.40–13.46), p=2.27E-09 and OR 4.22 (95% CI 1.95–9.11) p=0.0001, respectively). Both *MYO9B* rs7259292 and HLA-DQ2 homozygosity increase the risk for RCD II and EATL to a similar extent when compared to uncomplicated CD patients (OR 4.3 (95% CI 1.9–9.8) and 5.4 (95% CI 3.0–9.6), respectively), but there was no evidence for any interaction between these two risk factors.

Conclusion

We show that both *MYO9B* and HLA-DQ2 homozygosity might be involved in the prognosis of CD and the chance of developing RCD II and EATL.

Introduction

Celiac disease (CD) is an immune-mediated enteropathy following the ingestion of gluten. It is characterized by a permanent intolerance for the gluten proteins present in dietary wheat, rye and barley. The pathogenesis of disease involves interactions between environmental, genetic and immunologic factors.[1;2]

Genes play a key role in the pathogenesis of CD. The class II human leucocyte antigen (HLA)-DQ2 and HLA-DQ8 loci are the most important genetic contributors identified so far. The HLA-DQ2 heterodimer is encoded by the DQA1*0501 and DQB1*02 alleles and is present in 95% of all individuals diagnosed correctly with CD. Almost all the remaining patients express HLA-DQ8.[2-7] These HLA molecules are necessary to develop the disease, but are not sufficient for the phenotypic expression. HLA explains only 40% of the heritable risk, so other, non-HLA genes must also be involved in CD.[5;8;9] Several studies found evidence for linkage to regions on different chromosomes, including chromosomes 5, 6 and 19.[10-14] Our group found strong evidence for linkage to genetic variants on chromosome 19, in the myosin IXB gene (*MYO9B*), which might lead to an impaired intestinal barrier and thereby play a role in the pathogenesis of CD.[15] Individuals homozygous for the at-risk *MYO9B* allele had a 2.3 times higher risk of CD.

So far, the only treatment for CD is a life-long gluten-free diet (GFD). However, a small subgroup (2-5%) of celiac patients diagnosed at adult age fails to improve on a gluten-free diet.[16] In this group of patients the enteropathy persists despite adherence to the diet or recurs after an initially good response to the diet. These patients are regarded as suffering from refractory celiac disease (RCD), defined as persisting villous atrophy with crypt hyperplasia and increased intraepithelial T-lymphocytes (IELs) despite maintaining a strict GFD for more than 12 months, or when severe symptoms necessitate intervention independent of the duration of the GFD.[17-21] Using immune phenotypical analysis, two types of RCD can be recognized; RCD I and RCD II. RCD II patients are characterized by the presence of aberrant (CD7+CD3-CD4-CD8- cytoplasmic CD3+) IELs in the small bowel mucosa, whereas these lymphocytes are not detected in RCD I patients.[18;21;22] Approximately half of the RCD II patients develop an enteropathy-associated T-cell lymphoma (EATL) within 5 years, whereas RCD I patients seldom develop an EATL.[23;24]

In the RCD II and EATL group, significantly more patients were seen to be HLA-DQ2 homozygous compared to uncomplicated celiac patients. This difference was even more pronounced when compared to healthy controls.[25]

Our aim was to investigate whether the *MYO9B* gene is an additional risk factor for the development of RCD II and EATL, similar to HLA-DQ2 homozygosity. We also investigated a possible interaction between *MYO9B* and HLA.

Patients and methods

Subjects

RCD II patients: A total of 62 Dutch Caucasian RCD II and EATL patients were included in the study (50 RCD II patients and 12 EATL patients). All patients were ill and suffered from severe malabsorption; RCD II is difficult to treat. More than half of the RCD II patients have since died. Patients were treated with Cladribine therapy[23] and 8 patients underwent autologous stem cell transplantation with good results.[26] However, 25 (52%) of the RCD II patients went on to develop EATL. The two-year survival rate for EATL patients is 15-20% despite aggressive treatment with CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone), alemtuzumab and 4 patients even had autologous stem cell transplantation, but with disappointing results in contrast to the 8 RCD II patients. All except one of our patients were also included in a former study by Al Toma *et al.*[25] Since our main interest was to look for genetic association in RCD II patients, we will refer to our cohort of RCD II and EATL patients as 'RCD II' from here on. The diagnosis of CD was confirmed by histological examination with a documented histological response to gluten withdrawal.[21;27] All patients and their adherence to GFD were regularly checked in the outpatient clinic by a dietician. Patients with CD were considered to be refractory if their symptoms of malabsorption due to villous atrophy persisted despite strict adherence to a GFD or recurred after an initially good response to the diet. Their histopathology showed at least partial villous atrophy (Marsh IIIA) according to the modified Marsh criteria and after excluding other possible causes of villous atrophy.[18;21]

Additional information about the diagnosis, evaluation, HLA typing and IEL phenotyping is given in table 1 and in the supplementary information on RCD II patients.

Table 1 Baseline demographic characteristics of refractory celiac disease (RCD II) groups

Patients / Characteristic	Total group	RCD II Total	EATL after RCD II	EATL only
Total (male:female)	62 (29:33)	50 (19:31)	25 (10:15)	12 (10:2)
Age in years at diagnosis of CD (± SD) (range)	56 (± 10.2) (33-74)	54 (± 10.6) (33-74)	55 (± 9.4) (34-71)	63 (± 3.8) (56-70)
Age in years at diagnosis of RCD II /EATL (± SD) (range)	62 (± 7.4) (39-79)	58 (± 8.5) (39-74)	61 (± 8.6) (52-79)	64 (± 4.0) (56-70)
DQ2 Total	60 (96%)	49 (98%)	24 (96%)	11 (92%)
-Heterozygosity	22 (35%)	19 (38%)	5 (20%)	3 (25%)
-Homozygosity	38 (61%)	30 (60%)	19 (76%)	8 (67%)
Myosin IXB				
-Heterozygosity	12 (19%)	10 (20%)	4 (16%)	2 (16%)

SD standard deviation, EATL enteropathy-associated T-cell lymphoma

Celiac patients: A total of 421 Dutch Caucasians with uncomplicated celiac disease were included in the study. They are a subgroup of CD cases described previously[15], but from which we excluded all individuals with RCD I (n=22), RCD II (n=9), RCD II and EATL (n=10), RCD II and ulcerative jejunitis (n=1) and EATL (n=1). HLA data was available for 407 individuals.

Controls: A total of 1624 Dutch Caucasian controls were included in the study. The control group comprised the controls used in the paper by Monsuur *et al*[15] expanded with 938 extra blood bank controls.[28] The total control cohort (n=1624) comprised blood donors from Amsterdam (n=429), Leiden (n=475) and Utrecht (n=500) and healthy spouses from different, non-autoimmune projects (n=220). HLA data was available for 477 controls.

HLA typing

Whole blood was obtained from CD and RCD II patients for typing of HLA-DQA1* and DQB1* alleles, performed with a combined single-stranded conformation polymorphism (SSCP)/heteroduplex (HD) method by a semi-automated electrophoresis and gel staining method on the Phastsystem™ (Amersham Pharmacia Biotech, Sweden). Individuals were designated HLA-DQ2 if alleles DQA1*0501 and DQB1*02 were present, and HLA-DQ8 if alleles DQA1*03 and DQB1*0302 were present.[29-31] We did not examine the compound heterozygote of DQA1*05 and DQA1*0201 with DQB1*0201 and DQB1*0202 and we have not classified this combination.
In controls DQA1, DQB1 and DRB1 typing was performed by PCR using sequence-specific biotin-labeled oligonucleotides (PCR-SSO) as described.[32] HLA data of a part of the control patients was collected from the ITItwo panel (the ITI panel is a DNA panel from the Immunogenetics and Transplantation Immunology Section and consists of 477 unrelated, randomly selected, Dutch blood donors).

SNP selection

Eight tag SNPs (see table 2) completely tagging the 3' region of *MYO9B* (from rs7259292-rs388484) were typed.[28] Three of these SNPs (rs2305767, rs2305764 and rs1457092) were also used by Monsuur *et al*.[15;28]
SNPs were genotyped using Taqman assays (Applied Biosystems, Foster City, California, USA) and were performed according to the manufacturer's specifications. Genotypes were analyzed using a TaqMan 7900HT (Applied Biosystems). In the controls, the frequency of all SNPs were in Hardy-Weinberg equilibrium (HWE) ($p>0.05$).

Statistical analysis

The haplotype structure and linkage disequilibrium in the region was investigated using HAPMAP data (a haplotype map of the human genome, www.hapmap.org)[33] with the Haploview application.[34] Allele and genotype distributions in cases and controls were compared using the COCAPHASE module of the UNPHASED statistical package.[35] Haplotype association was estimated using the same package. Hardy-Weinberg equilibrium was tested by comparing the expected and observed genotypes in 2 x 3 χ^2 table. Odds ratios (OR) were calculated and the confidence intervals were approximated using Woolf's method with Haldane's correction.[36]

To investigate the added value of *MYO9B* to HLA-DQ2 homozygosity, we used a logistic regression model. First HLA-DQ2 homozygosity (no/yes) was added to the model in which we compared RCD II patients to CD patients. Then we added the *MYO9B* variant (combining the heterozygous and the homozygous variants) which was most strongly associated to the disease and tested whether the model improved using a likelihood ratio test. We also evaluated whether the *MYO9B* variant was independently associated to the risk of RCD II compared to CD patients, by evaluating the OR and 95% CI. We tested for possible interaction between HLA-DQ2 homozygosity and the *MYO9B* variant by including the interaction term in the logistic regression model. We used Stata (Stata/SE 8.2 for Windows, StataCorp LP, College Station, TX, USA) for these analyses.

Table 2

a. Allele distribution of MYO9b SNPs in RCD II patients (n=62) compared to healthy controls (n=1624)

		Healthy controls		RCD II patients				
SNP	*Maj/min allele*	*Maj.allele (freq)*	*Min.allele (freq)*	*Maj.allele (freq)*	*Min.allele (freq)*	*P=value*	*OR*	*95% CI*
rs7259292	C_T	*3119 (0.97)*	*91 (0.03)*	*112 (0.90)*	*12 (0.10)*	0.00002	3.90	2.10 - 7.25
rs2305767	A_G	*1764 (0.56)*	*1392 (0.44)*	*79 (0.65)*	*43 (0.35)*	0.05	1.43	0.99 - 2.09
rs1545620	A_C	*2056 (0.64)*	*1148 (0.36)*	*67 (0.55)*	*55 (0.45)*	0.04	1.47	1.03 - 2.12
rs1457092	C_A	*2120 (0.67)*	*1042 (0.33)*	*77 (0.63)*	*45 (0.37)*	0.37	1.20	0.83 - 1.74
rs8107108	C_T	*2959 (0.92)*	*245 (0.08)*	*110 (0.90)*	*12 (0.10)*	0.37	1.41	0.77 - 2.57
rs2305766	G_C	*2114 (0.67)*	*1050 (0.33)*	*76 (0.62)*	*46 (0.38)*	0.30	1.23	0.85 - 1.78
rs2305764	G_A	*1958 (0.62)*	*1204 (0.38)*	*71 (0.60)*	*47 (0.40)*	0.70	1.08	0.75 - 1.58
rs2279002	A_G	*2263 (0.71)*	*933 (0.29)*	*81 (0.66)*	*41 (0.34)*	0.30	1.24	0.85 - 1.82

b. Allele distribution of MYO9b SNPs in RCD II patients (n=62) compared to uncomplicated CD patients (n=421)

		CD patients		RCD II patients				
SNP	*Maj/min allele*	*Maj.allele (freq)*	*Min.allele (freq)*	*Maj.allele (freq)*	*Min.allele (freq)*	*P=value*	*OR*	*95% CI*
rs7259292	C_T	*815 (0.97)*	*25 (0.03)*	*112 (0.90)*	*12 (0.10)*	0.0003	3.61	1.78 - 7.31
rs2305767	A_G	*508 (0.62)*	*316 (0.38)*	*79 (0.65)*	*43 (0.35)*	0.51	0.88	0.59 - 1.31
rs1545620	A_C	*485 (0.58)*	*349 (0.42)*	*67 (0.55)*	*55 (0.45)*	0.50	1.14	0.78 - 1.67
rs1457092	C_A	*510 (0.61)*	*328 (0.39)*	*77 (0.63)*	*45 (0.37)*	0.63	0.92	0.62 - 1.35
rs8107108	C_T	*774 (0.93)*	*58 (0.07)*	*110 (0.90)*	*12 (0.10)*	0.26	1.54	0.81 - 2.92
rs2305766	G_C	*508 (0.62)*	*314 (0.38)*	*76 (0.62)*	*46 (0.38)*	0.92	0.99	0.67 - 1.46
rs2305764	G_A	*445 (0.54)*	*381 (0.46)*	*71 (0.60)*	*47 (0.40)*	0.20	0.78	0.53 - 1.15
rs2279002	A_G	*546 (0.66)*	*282 (0.34)*	*81 (0.66)*	*41 (0.34)*	0.92	0.99	0.66 - 1.48

Results

We observed that SNPs rs7259292 showed a different allele distribution in RCD II patients compared to the control group, with minor allele frequencies of 10% in RCD II patients, compared to 3% in controls (p=0.00002, OR 3.90, 95% CI: 2.10 – 7.25) (Table 2a). The association of rs1545620 with RCD II compared to controls was borderline statistically significant (OR 1.47; 95% CI 1.03 – 2.12; p=0.04) (Table 2a).

All tested SNPs were located in one haploblock and were in strong linkage disequilibrium with each other. We therefore continued by constructing 8-SNP haplotypes. Five haplotypes occurred with a frequency of more than 5% in RCD II cases or controls. Haplotype h5, which carries the associated rs7259292*T allele, occurred significantly more frequently in RCD II patients than controls (11% vs 2%, p=2.27E-09, OR 6.76, 95% CI 3.40 – 13.46) (Table 3).

On comparing the allele frequencies of the eight tested SNPs in *MYO9B* between RCD II patients and CD patients, we observed a statistically significant difference in the frequency of rs7259292, as well as in the frequency of haplotype h5 in RCD II patients compared with CD patients (p=0.0003, OR 3.61, 95% CI 1.78 – 7.31 and p=0.0001, OR 4.22, 95% CI 1.95 – 9.11 on a single SNP and haplotype h5, respectively) (Tables 2b and 3).

Strong association of RCD II and EATL with homozygosity for the HLA-DQ2 allele (DQA1*0501 and DQB1*0201) was recently reported by Al-Toma *et al*.[25] In the cohort of RCD II patients included in our study, 38 out of 61 HLA-typed patients (62%) were DQ2 homozygous (DQA1*0501 and DQB1*0201), whereas only 97 out of the 407 HLA-typed CD patients (24%) and 10 out of the 477 HLA typed controls (2%) carried two copies of the DQ2 allele. The OR for RCD II patients compared with uncomplicated CD was 5.2; CI (2.9 – 9.0), p=6.27E-10. Compared to controls the OR was 69.1; CI (31.1 – 153.6), p=2.16E-54. The frequencies of individuals who carry both HLA-DQ2 homozygosity and rs7259292*T are higher in our patient cohort (figure 1). Both *MYO9B* rs7259292 and HLA-DQ2 homozygosity increase the risk for RCD II and EATL to a similar extent when compared to uncomplicated CD patients (OR 4.3 (95% CI 1.9–9.8) and 5.4 (95% CI 3.0–9.6), respectively) and we found no evidence for interaction between these two risk factors.

We performed a logistic regression model including both HLA-DQ2 homozygosity and the presence of rs7259292*T allele, comparing RCD II patients to CD cases and observed that both HLA-DQ2 homozygosity and the presence of rs7259292*T allele are independently associated with the development of RCD II (Table 4, 5). In the likelihood ratio test, the model fit improved when the rs7259292*T allele was included in the model subsequent to HLA-DQ2 homozygosity (p<0.0009).

To test for possible interaction between HLA-DQ2 homozygosity and the rs7259292 genotype, we included an interaction term in the model. It was not statistically significant and we therefore concluded that there was no significant interaction.

Table 3 Haplotype analysis of myosin IXB SNPs in RCD II patients (n=62) compared to healthy controls (n=1624) and uncomplicated CD patients (n=421)

		RCD II patients	*Controls*	*RCD II patients compared to controls*			*CD patients*	*RCD II patients compared to CD*		
	Haplotype*	count (freq)	Count (freq)	p-value	OR	95% CI	count (freq)	p-single haplotype	OR	95% CI
h1	C-A-A-C-C-G-G-A	10 (0.09)	223.9 (0.08)	0.26	1.58	0.79 - 3.18	52.56 (0.07)	0.33	1.53	0.73 - 3.22
h2	C-A-A-C-T-G-G-A	9.33 (0.08)	228.2 (0.08)	0.41	1.46	0.71 - 2.98	50.37 (0.07)	0.38	1.50	0.70 - 3.21
h3	C-A-C-A-C-C-A-G	32.91 (0.29)	824.7 (0.29)	0.27	1.33	0.83 - 2.13	251.4 (0.33)	0.99	1.01	0.61 - 1.64
h4	C-G-A-C-C-G-G-A	37.65 (0.34)	1249 (0.43)	ref	1	-	287.3 (0.37)	ref	1	-
h5	T-A-C-C-C-G-G-A	12 (0.11)	61.2 (0.02)	2.27E-09	6.76	3.40 - 13.46	21.99 (0.03)	0.0001	4.22	1.95 - 9.11
	overall p-value			0.0004				0.002		

* the order of SNPs is as follows: rs7259292, rs2305767, rs1545620, rs1457092, rs8107108, rs2305766, rs2305764, rs2279002

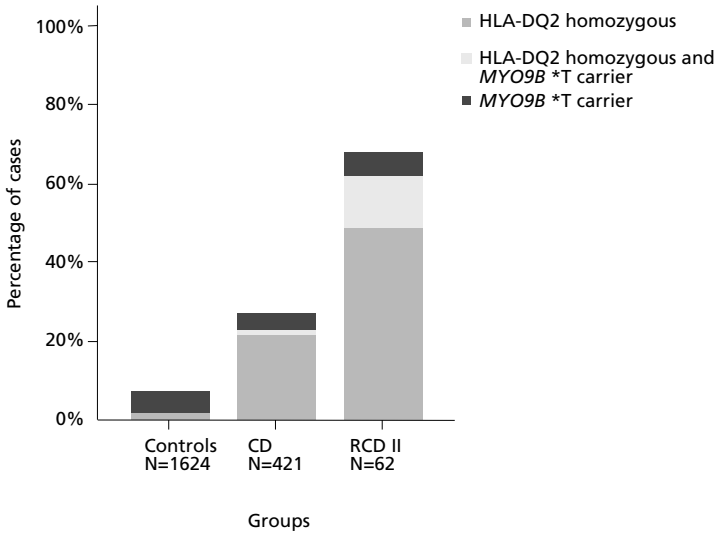


Figure 1 *MYO9B* and HLA-DQ2 homozygosity

Table 4 Distribution of genotypes and alleles of rs7259292 SNP in groups studied

Groups studied	CC (freq)	CT (freq)	TT (freq)	C(freq)	T(freq)
Controls (n=1624)	1517 (0.95)	85 (0.05)	3 (0.00)	3119 (0.97)	91 (0.03)
Controls HLA-DQ2 homoz (n=10)	10 (1.00)	0 (0.00)	0 (0.00)	20 (1.00)	0 (0.00)
CD (n=421)	396 (0.94)	23 (0.05)	1 (0.00)	815 (0.97)	25 (0.03)
CD HLA-DQ2 homozygous (n=97)	93 (0.96)	4 (0.04)	0 (0.00)	190 (0.98)	4 (0.02)
RCD II (n=62)	50 (0.81)	12 (0.19)	0 (0.00)	112 (0.90)	12 (0.10)
RCD II HLA-DQ2 homozygous (n=38)	30 (0.79)	8 (0.21)	0 (0.00)	68 (0.89)	8 (0.11)
RCD II non-HLA-DQ2 homozygous (n=23)	19 (0.83)	4 (0.17)	0 (0.00)	42 (0.91)	4 (0.09)

Table 5 Logistic regression model for HLA-DQ2 homozygosity and the presence of rs7259292*T allele in RCD II patients compared to uncomplicated CD patients

Risk factors	P>|z|	OR	95% CI
HLA-DQ2 homozygous	<0.000	5.4	3.0 – 9.6
rs7259292*T allele	<0.000	4.3	1.9 – 9.8

Discussion

We found a strong association of myosin IXB rs7259292 SNP with the RCD II and EATL patients, referred to as 'RCD II' here. The frequency of the minor allele was increased to 10% in RCD II patients, compared to 3% in controls and 3% in uncomplicated CD patients. Although the risk associated with the minor allele is high, the impact of these alleles at the population level is relatively small because of the low allele frequency (population attributable risk (PAR) = 7%). The frequency of the associated but rare rs7259292*T allele was also higher compared to uncomplicated CD patients and this was also observed at the haplotype level. We found that the presence of the susceptible rs7259292*T allele did not interact with the HLA risk.

RCD is usually considered as a complication of CD, affecting only a small proportion (2-5%) of CD patients, almost all of whom were diagnosed above the age of 50.[16] RCD II is characterized by the development of aberrant T-cells, a high frequency of EATL, and poor prognosis.[17-20] Finding genetic markers that could predict predisposition to RCD II among the CD population is of great interest: the subgroup of CD patients identified with a high risk for RCD II could undergo regular endoscopy with the aim of detecting T-cell lymphoma at an early stage and providing early treatment. The most important risk factor for the development of RCD II known to date is the presence of two copies of the HLA-DQ2 haplotype.[23;24] By comparing RCD II patients with a group of uncomplicated CD patients, we have discovered that the presence of the susceptible *MYO9B* rs7259292*T allele is another genetic risk factor for the development of RCD II, independent and comparable to HLA. Nearly 70% of RCD II patients carry either 2 copies of the HLA-DQ2, or the *MYO9B* rs7259292*T allele, or both, compared to 7.5% in controls and 27.7% in uncomplicated CD patients (figure 1). We do not have access to RCD groups from other populations and cannot determine whether this observation is specific to our Dutch cohort.

We can only speculate why *MYO9B* might underlie the mechanisms of refractory celiac disease. The RCD-associated SNP lies in a different part of the gene (intron 14) when compared to the variant associated to uncomplicated celiac disease (intron 28). The possible differences of association may point to differences in disease mechanisms. We speculate that the RCD-associated variant changes the cytoskeleton, for example, specifically into T cells that might result in abnormal T cell responses and ultimately in clonal expansion of T cells. Much functional work is required to determine the answer.

Rs7259292 is a rare SNP, with a frequency of 3% in the control population and in uncomplicated CD patients. As the frequency of this SNP is 4.2% in the HAPMAP-CEU population, we do not think that the Dutch population is at especially high risk of RCD II. The SNP rs7259292 is located deep in the intron, not in a conserved sequence. According to the splicing prediction program (http://www.tigr.org/tdb/GeneSplicer/gene_spl.html), it does not create any alternative splicing sites. It is therefore unlikely that the SNP is the causal mutation, but rather that it is a marker of disease in linkage disequilibrium with a causative variant.

We therefore investigated which SNPs in the myosin IXB region were in strong linkage disequilibrium (i.e. have a high correlation, i.e. r^2) with rs7259292. We found two SNPs with an r^2 greater than 0.8: rs16981660 and rs11879844. Both are located in the introns and also do not potentially influence splicing. However, since the HAPMAP data is far from complete, we believe that other SNPs located on the same haplotype might be the causative variants. Fine-mapping this region in the course of our study was not possible due to the strong linkage disequilibrium in the region and our low number of patients. Rs7259292 could also be in linkage disequilibrium with polymorphisms other than SNPs, such as deletions, insertions or repeats. Sequence analysis of the *MYO9B* gene in RCD II patients who carry the susceptible h5 haplotype could lead to identifying the functional variant.

Another potentially interesting SNP that showed significant, albeit weaker, association with RCD II is rs1545620. This observation does not survive the correction for multiple testing; interestingly however, the same allele of this SNP is strongly associated with inflammatory bowel disease (IBD) in multiple populations.[37]

Previously, the rs2305764 SNP was shown to be strongly associated with uncomplicated CD in the Dutch population.[15] Excluding the 42 RCD patients from the original study by Monsuur *et al* did not change these results (data not shown), but this could not be confirmed in CD populations in the UK, Spain, Italy and Scandinavia.[38-40] This discrepancy raises some doubts about the influence of this gene on predisposition to CD. The limited replication of *MYO9B* association with CD in other populations reduced the validity of this gene and makes it difficult to interpret the true role of *MYO9B* in celiac disease pathogenesis, although a positive association was recently found between *MYO*9B gene polymorphisms and CD and two other auto-immune diseases in a Spanish population.[41]
Furthermore, the replication studies mentioned included only the 3 most associated SNPs from the original study in a Dutch population, whereas our results show an association of RCD II with a rare MYO9B haplotype, different from the CD-associated haplotype. This might imply genetic heterogeneity between CD and RCD II in the Dutch population and might suggest that different Myo9B variants could be associated with CD in other populations.

In other auto-immune mediated intestinal diseases, such as Crohn's disease, genetic association studies have also reported conflicting results. Allelic variants in NOD2/CARD15 and DLG5 have been found to be associated with Crohn's disease susceptibility. [42-45] Both associations differed among various populations and could not be confirmed in all populations.[46-53]

We can speculate that certain loci might be specific to a population and only relate to a specific phenotype of the disease, for example, as in the development of complicated CD. It is tempting to speculate that rs2305764 is associated with the development of RCD II or EATL. For example, in inflammatory bowel disease, the *IBD5* locus has been identified as a determinant of disease susceptibility as well as of disease severity.[54]

We realize that our study has limitations, the most important of which is the relatively small number of patients (n=62). However, RCD II and EATL are rare complications seen in only 2-5% of CD patients and there are, as yet, no large cohorts of these complicated CD patients. The control group is proportionally extremely high (n=1624). The MYO9B genotypes for 938 extra controls were already available from previous studies.[28] Frequencies of all MYO9B SNPs were similar in both the initial control group[15] and in the 938 extra controls. We investigated the frequency of rs7259292 SNP in subgroups of our control cohorts but observed similar frequencies in all the groups. The differences in frequency of rs7259292*T were significant when the RCD II group was compared to any particular subgroup of controls (table 1 in supplementary information).

Transmission disequilibrium test (TDT) analysis would be relevant to confirm the results of the case-control analysis, however, typing parents of RCD patients is almost impossible given that the mean age of these patients is 56 years. Besides, the patients were diagnosed several years ago (since 1992).

Our study shows that not only HLA-DQ2 homozygosity, but also the SNP rs7259292 in the *MYO9B* gene, is associated with an increased risk of developing RCD II and EATL. It would be interesting to design a prospective study to investigate the positive predictive value of both HLA-DQ2 homozygosity and *MYO9B* rs7259292*T carriership for RCD II and EATL prognosis and subsequent disease management.

Acknowledgements

We thank Jackie Senior for editing the manuscript. The study was supported by the Celiac Disease Consortium, an Innovative Cluster approved by the Netherlands Genomics Initiative and partially funded by the Dutch Government (BSIK03009), the Wilhelmina Research Fund The study was supported by an unrestricted grant from Astra Zeneca The Netherlands.

References

1. Kagnoff MF. Celiac disease. A gastrointestinal disease with environmental, genetic, and immunologic components. *Gastroenterol Clin North Am* 1992;**21**(2):405-425.
2. Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F, Thorsby E. Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. *J Exp Med* 1989;**169**(1):345-350.
3. Kagnoff MF. Overview and pathogenesis of celiac disease. *Gastroenterology* 2005;**128**(4 Suppl 1):S10-S18.
4. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992;**102**(1):330-354.
5. Pena AS, Wijmenga C. Genetic factors underlying gluten-sensitive enteropathy. *Curr Allergy Asthma Rep* 2001;**1**(6):526-533.
6. Spurkland A, Sollid LM, Polanco I, Vartdal F, Thorsby E. HLA-DR and -DQ genotypes of celiac disease patients serologically typed to be non-DR3 or non-DR5/7. *Hum Immunol* 1992;**35**(3):188-192.
7. Zubillaga P, Vidales MC, Zubillaga I, Ormaechea V, Garcia-Urkia N, Vitoria JC. HLA-DQA1 and HLA-DQB1 genetic markers and clinical presentation in celiac disease. *J Pediatr Gastroenterol Nutr* 2002;**34**(5):548-554.
8. Bevan S, Popat S, Braegger CP et al. Contribution of the MHC region to the familial risk of coeliac disease. *J Med Genet* 1999; **36**(9):687-690.
9. Sollid LM, Thorsby E. HLA susceptibility genes in celiac disease: genetic mapping and role in pathogenesis. *Gastroenterology* 1993;**105**(3):910-922.
10. Greco L, Corazza G, Babron MC et al. Genome search in celiac disease. *Am J Hum Genet* 1998;**62**(3):669-675.
11. Greco L, Babron MC, Corazza GR et al. Existence of a genetic risk factor on chromosome 5q in Italian coeliac disease families. *Ann Hum Genet* 2001;**65**:35-41.
12. Percopo S, Babron MC, Whalen M et al. Saturation of the 5q31-q33 candidate region for coeliac disease. *Ann Hum Genet* 2003;**67**:265-268.
13. van Belzen MJ, Meijer JW, Sandkuijl LA et al. A major non-HLA locus in celiac disease maps to chromosome 19. *Gastroenterology* 2003;**125**(4):1032-1041.
14. van Belzen MJ, Vrolijk MM, Meijer JW et al. A genomewide screen in a four-generation Dutch family with celiac disease: evidence for linkage to chromosomes 6 and 9. *Am J Gastroenterol* 2004;**99**(3):466-471.
15. Monsuur AJ, de Bakker PI, Alizadeh BZ et al. Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat Genet* 2005;**37**(12):1341-1344.
16. Wahab PJ, Meijer JW, Mulder CJ. Histologic follow-up of people with celiac disease on a gluten-free diet: slow and incomplete recovery. *Am J Clin Pathol* 2002; **118**(3):459-463.
17. Abdulkarim AS, Burgart LJ, See J, Murray JA. Etiology of nonresponsive celiac disease: results of a systematic approach. *Am J Gastroenterol* 2002;**97**(8):2016-2021.
18. Biagi F, Corazza GR. Defining gluten refractory enteropathy. *Eur J Gastroenterol Hepatol* 2001;**13**(5):561-565.
19. Daum S, Cellier C, Mulder CJ. Refractory coeliac disease. *Best Pract Res Clin Gastroenterol* 2005;**19**(3):413-424.

20. Mulder CJ, Wahab PJ, Moshaver B, Meijer JW. Refractory coeliac disease: a window between coeliac disease and enteropathy associated T cell lymphoma. *Scand J Gastroenterol Suppl* 2000;**232**:32-37.
21. Wahab PJ, Meijer JW, Goerres MS, Mulder CJ. Coeliac disease: changing views on gluten-sensitive enteropathy. *Scand J Gastroenterol Suppl* 2002;**236**:60-65.
22. Cellier C, Delabesse E, Helmer C et al. Refractory sprue, coeliac disease, and enteropathy-associated T-cell lymphoma. French Coeliac Disease Study Group. *Lancet* 2000; **356**(9225):203-208.
23. Al Toma A, Verbeek WHM, Mulder CJ. Survival in patients with Refractory Coeliac Disease and Enteropathy associated T cell Lymphoma. *Gut* 2007;**56**(10):1373-8.
24. Meijer JW, Mulder CJ, Goerres MG, Boot H, Schweizer JJ. Coeliac disease and (extra) intestinal T-cell lymphomas: definition, diagnosis and treatment. *Scand J Gastroenterol Suppl* 2004;**241**:78-84.
25. Al Toma A, Goerres MS, Meijer JW, Pena AS, Crusius JB, Mulder CJ. Human leukocyte antigen-DQ2 homozygosity and the development of refractory celiac disease and enteropathy-associated T-cell lymphoma. *Clin Gastroenterol Hepatol* 2006; **4**(3):315-319.
26. Al Toma A, Verbeek WHM, Visser OJ et al. Disappointing outcome of Autologous Stem Cell Transplantation for Enteropathy-Associated T-cell Lymphoma. *Dig Liver Dis* 2007;**39**(7): 634-41.
27. When is a coeliac a coeliac? Report of a working group of the United European Gastroenterology Week in Amsterdam, 2001. *Eur J Gastroenterol Hepatol* 2001; **13**(9): 1123-1128.
28. van Bodegraven AA, Curley CR, Hunt KA et al. Genetic variation in myosin IXB is associated with ulcerative colitis. *Gastroenterology* 2006;**131**(6):1768-1774.
29. O'Mahony S, Howdle PD, Losowsky MS. Review article: management of patients with non-responsive coeliac disease. *Aliment Pharmacol Ther* 1996;**10**(5):671-680.
30. Carrington M, Miller T, White M et al. Typing of HLA-DQA1 and DQB1 using DNA single-strand conformation polymorphism. *Hum Immunol* 1992;**33**(3):208-212.
31. Csizmadia CG, Mearin ML, Oren A et al. Accuracy and cost-effectiveness of a new strategy to screen for celiac disease in children with Down syndrome. *J Pediatr* 2000; **137**(6): 756-761.
32. Verduyn W, Doxiadis II, Anholts J et al. Biotinylated DRB sequence-specific oligonucleotides. Comparison to serologic HLA-DR typing of organ donors in eurotransplant. *Hum Immunol* 1993;**37**(1):59-67.
33. The International HapMap Project. *Nature* 2003;**426**(6968):789-796.
34. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;**21**(2):263-265.
35. Dudbridge F. Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol* 2003;**25**(2):115-121.
36. HALDANE JB. The estimation and significance of the logarithm of a ratio of frequencies. *Ann Hum Genet* 1956;**20**(4):309-311.
37. Bodegraven AA, Curley CR, Hunt K, Monsuur AJ, van Heel DA. Genetic variation in Myosin IXB is associated with ulcerative colitis. *Gastroenterology* 2006;**131**(6):1768-74.
38. Nunez C, Marquez A, Varade J et al. No evidence of association of the MYO9B polymorphisms with celiac disease in the Spanish population. *Tissue Antigens* 2006; 68(6):489-492.
39. Giordano M, Marano C, Mellai M et al. A family-based study does not confirm the association of MYO9B with celiac disease in the Italian population. Genes Immun 2006; **7**(7):606-608.

40. Amundsen SS, Monsuur AJ, Wapenaar MC et al. Association analysis of MYO9B gene polymorphisms with celiac disease in a Swedish/Norwegian cohort. *Hum Immunol* 2006;**67**(4-5):341-345.
41. Sanchez E, Alizadeh BZ, Valdigem G et al. MYO9B gene polymorphisms are associated with autoimmune diseases in Spanish population. *Hum Immunol* 2007;**68**(7):610-615.
42. Ogura Y, Bonen DK, Inohara N et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001; **411**(6837):603-606.
43. Hugot JP, Chamaillard M, Zouali H et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001;**411**(6837):599-603.
44. Hampe J, Cuthbert A, Croucher PJ et al. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet* 2001; **357**(9272): 1925-1928.
45. Stoll M, Corneliussen B, Costello CM et al. Genetic variation in DLG5 is associated with inflammatory bowel disease. *Nat Genet* 2004;**36**(5):476-480.
46. Gao M, Cao Q, Luo LH, Wu ML, Hu WL, Si JM. [NOD2/CARD15 gene polymorphisms and susceptibility to Crohn's disease in Chinese Han population]. *Zhonghua Nei Ke Za Zhi* 2005;**44**(3):210-212.
47. Karban A, Waterman M, Panhuysen CI et al. NOD2/CARD15 genotype and phenotype differences between Ashkenazi and Sephardic Jews with Crohn's disease. *Am J Gastroenterol* 2004; **99**(6):1134-1140.
48. Nagy Z, Karadi O, Rumi G et al. Crohn's disease is associated with polymorphism of CARD15/NOD2 gene in a Hungarian population. *Ann N Y Acad Sci* 2005;**1051**:45-51.:45-51.
49. Buning C, Molnar T, Nagy F et al. NOD2/CARD15 gene polymorphism in patients with inflammatory bowel disease: is Hungary different? *World J Gastroenterol* 2005;**11**(3): 407-411.
50. Cavanaugh J. NOD2: ethnic and geographic differences. *World J Gastroenterol* 2006;**12**(23):3673-3677.
51. Pearce AV, Fisher SA, Prescott NJ et al. Investigation of association of the DLG5 gene with phenotypes of inflammatory bowel disease in the British population. *Int J Colorectal Dis* 2007;**22**:419–424.
52. Noble CL, Nimmo ER, Drummond H, Smith L, Arnott ID, Satsangi J. DLG5 variants do not influence susceptibility to inflammatory bowel disease in the Scottish population. *Gut* 2005;**54**(10):1416-1420.
53. Buning C, Geerdts L, Fiedler T et al. DLG5 variants in inflammatory bowel disease. *Am J Gastroenterol* 2006;**101**(4):786-792.
54. Noble CL, Nimmo ER, Drummond H et al. The contribution of OCTN1/2 variants within the IBD5 locus to disease susceptibility and severity in Crohn's disease. *Gastroenterology* 2005;**129**(6):1854-1864.
55. Patey-Mariaud De SN, Cellier C, Jabri B et al. Distinction between coeliac disease and refractory sprue: a simple immunohistochemical method. *Histopathology* 2000;**37**(1):70-77.
56. Bagdi E, Diss TC, Munson P, Isaacson PG. Mucosal intra-epithelial lymphocytes in enteropathy-associated T-cell lymphoma, ulcerative jejunitis, and refractory celiac disease constitute a neoplastic population. *Blood* 1999;**94**(1):260-264.
57. Murray A, Cuevas EC, Jones DB, Wright DH. Study of the immunohistochemistry and T cell clonality of enteropathy-associated T cell lymphoma. *Am J Pathol* 1995;**146**(2):509-519.

Supplementary information on RCD II patients

Diagnostic criteria

The diagnosis of CD was confirmed by histological examination with a documented histological response to gluten withdrawal.(21;27) Patients with CD were considered to be refractory (RCD) when (1) their symptoms of malabsorption due to villous atrophy persisted despite strict adherence to a gluten-free diet (GFD) or recurred after an initially good response to the diet; and (2) the histopathology showed at least partial villous atrophy (Marsh IIIA) according to the modified Marsh criteria and after other possible causes of villous atrophy had been excluded.(18;21;27)

Two types of RCD can be distinguished immunologically in the small bowel mucosal, depending on the presence or absence of T-lymphocytes with an abnormal phenotype. In RCD I, the intra-epithelial lymphocyte (IEL) phenotype is normal with the expression of surface CD3, CD8 and T-cell receptors (TCR). In RCD II, the IELs have normal morphological features, but they exhibit an aberrant IEL phenotype despite the normal expression of CD103 and CD7. They also show downregulation of surface CD3 to intracytoplasmic CD3, and lack of classical surface T-cell markers such as CD4, CD8 and, as a consequence of the CD3 down-regulation, lack of surface TCR expression.(22;55) Using immunophenotyping flow-cytometric analysis of the intestinal mucosal, we arbitrarily chose to regard ≤10% of aberrant cells as normal, and >20% as abnormal, for lack of solid data in the literature. We included only patients with RCD II in our study as they are a well-defined group.

Evaluation

Clinical, laboratory (hematology, biochemistry and serology), endoscopic and histological examination of the small intestine was performed at regular 6-month intervals. Patients were checked at the outpatient clinic at regular intervals (3-6 months) and their adherence to the GFD was monitored. Particular attention was paid to symptoms and signs of malabsorption, body mass index and performance status. Antiendomysium antibodies and anti-tissue transglutaminase antibodies were tested at diagnosis and at follow-up in all patients. Immunophenotyping of IELs was performed on all patients. Endoscopy using upper gastrointestinal endoscopy, videocapsule endoscopy and/ or double balloon endoscopy with small intestinal biopsies, CT scan, magnetic resonance enteroclysis, PET scan and dual energy X-absorptiometry were performed to check for and exclude EATL development.[23]

Small intestinal biopsies

Upper gastrointestinal endoscopy was performed in all RCD II patients. At least 10 duodenal biopsies were taken for histological, immunohistochemical and flow cytometric examination. Four to six biopsies were fixed and preserved in 10% formalin for histopathological and immunohistochemical evaluation. Three or four biopsies for TCR gene rearrangement studies were taken separately, preserved on histocon and frozen at –20°C. Another three or four biopsies were taken for immunophenotypical evaluation and preserved in RPMI medium.

Isolation of IEL and cell-staining for immunophenotyping

Lymphocytes and enterocytes were isolated from three or four small intestinal biopsies (SIBs) by homogenizing tissue samples and passing fragments through a 100μm nylon cell strainer (Becton Dickinson®, Cell strainer) in RPMI medium supplemented with 1% FCS. The released cells were subsequently washed and labeled by 4-color staining for 30 minutes on ice with various combinations of fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein and allophycocyanin labeled monoclonal antibodies against CD3, CD4, CD8, CD7, CD103, CD19, CD45, CD16/56, $\gamma\delta$TCR and cytoplasmic CD3. The FACS method used in this study followed the manufacturer's guidelines.

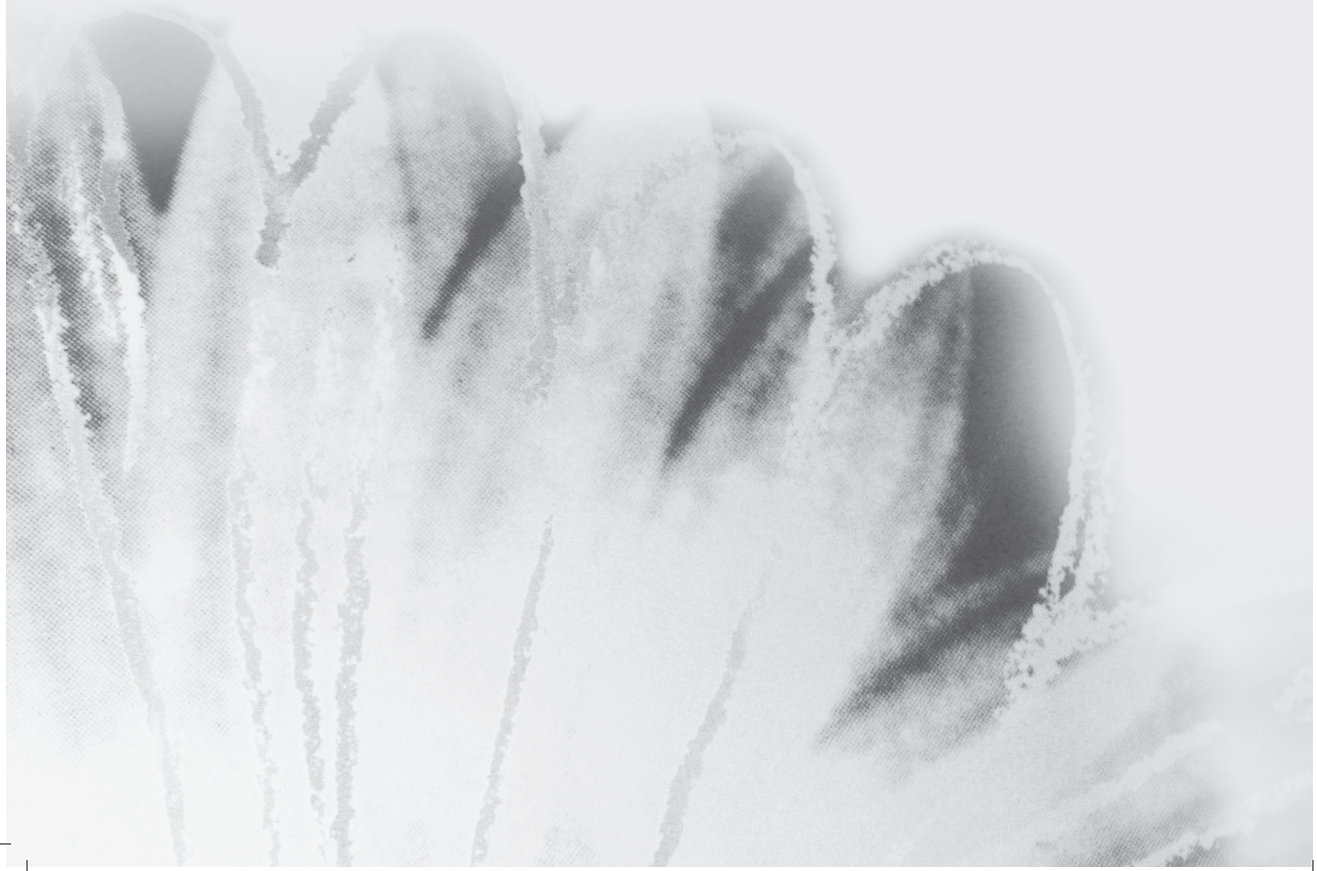
Cell surface immunophenotyping of IEL was performed on a 4-color FACS Calibur flow cytometer (Becton Dickinson, BD, immunocytometry systems, San Jose, CA). Non-viable cells and debris were excluded based on forward and sideways light scatter properties and a gate on CD45 positive cells was used for selecting lymphocytes. Intraepithelial localization of lymphocytes was confirmed by surface expression of CD103 (αEβ7 integrin, a gut homing receptor for E-cadherin). IELs were analyzed, using CellQuest^{tm} (KS Stat) based on their expression of cell markers: cytoplasmic CD3, surface CD3, CD4, CD7, CD8, CD16/56, CD19, CD103 and TCR$\gamma\delta$ on CD45+gated IELs. A level of aberrant cells (Cytoplasmic CD3$^+$ surface CD3$^-$ % of lymphocytes) of ≤10% was regarded as normal, and >20% as abnormal.

Assessment of TCR gene rearrangement by Polymerase Chain Reaction (PCR)

DNA was extracted from cryosections of duodenal biopsies by a standard procedure using proteinase-K digestion and ethanol precipitation of the genomic DNA. T-cell receptor (TCR)-gamma (TCR-γ) gene rearrangements were analyzed by multiplex PCR amplification under standardized conditions. A monoclonal and polyclonal control were included in each experiment. Clonality assessment for TCR-γ gene rearrangements was done using the BIOMED-2 multiplex TCR PCR protocol.(56;57)

Supplementary Table 1 Association of RCD II with rs7259292*T allele when compared to 4 subgroups of controls used in the study.

Group	Total no	frq rs7259292*T	p-value	OR	CI
RCD II	62	0.097	n/a	1	-
Lab controls	220	0.026	5.00E-04	3.99	1.74 - 9.12
Amsterdam controls	429	0.028	1.00E-04	3.82	1.88 - 7.77
Leiden controls	475	0.027	6.90E-05	4.02	1.99 - 8.13
Utrecht controls	500	0.03	2.00E-04	3.58	1.80 - 7.12



Chapter 8

Tight junction gene variants may not explain the increased levels of antigliadin antibodies, suggesting other mechanisms than altered permeability

Wolters VM
Alizadeh BZ
Weijerman ME
Zhernakova A
van Hoogstraten IMW
Mearin ML
Wapenaar MC
Wijmenga C
Schreurs MWJ

Submitted

Abstract

Introduction

Various genes that may influence intestinal barrier function have been identified, including *PARD3, MYO9B* and *MAGI2*, which are associated to coeliac disease (CD) and to ulcerative colitis (UC). Since direct measurement of intestinal permeability is difficult, an indirect test can be based on measuring antibodies against food antigens including gliadin (AGA) and baker's yeast (ASCA).

Objective

To investigate correlations between permeability as measured by AGA and ASCA and genetic variants (*PARD3, MYO9B, MAGI2*) in Down syndrome (DS).

Methods

In 126 children with DS the correlation between AGA and ASCA was studied. Then all patients were genotyped for six SNPs: rs1457092 and rs2305764 in *MYO9B*, rs10763976 and rs6962966 in *MAGI2* and rs9640699 and rs1496770 in *PARD3*. An allele dosage association of genetic variations in these tight junction genes and the levels of AGA antibodies was performed.

Results

There was a strong correlation between AGA and ASCA ($p<0.01$). The group of patients with one or more risk genotypes had lower mean AGA levels (trend test $P=0.007$). This group also consisted of a significantly larger number of patients with normal AGA levels ($P=9.3x10^{-5}$). We consistently found that AGA production was suppressed instead of increased for a CD associated risk genotype.

Conclusion

Tight junction CD associated risk genotypes are associated with lower AGA values instead of elevated ones. This might be due to different immunological mechanisms in DS patients as a result of chromosomal aberrations, possibly involving altered induction and/or maintenance of tolerance.

Introduction

Autoimmune diseases result from dysregulation of the immune response leading to tissue damage. They have a complex pathogenesis in which external environmental, genetic and microbial factors and the immune system are involved. Independent studies have suggested that alterations in intestinal permeability are involved in the pathogenesis of different autoimmune diseases.[1] For example, recent animal studies indicate that in type 1 diabetes, coeliac disease (CD) and inflammatory bowel disease (IBD), increased permeability was present before disease onset but was also required for the development of disease, suggesting it plays a role in pathogenesis.[1;2] Tight junctions play a major role in regulating intestinal permeability and seal the route between the intestinal epithelial cells.[1] Therefore, genes encoding tight junction proteins could be highly relevant candidates for autoimmune diseases of the intestine, such as CD and IBD.
In previous studies, we found several genetic associations in CD patients for candidate tight junction (TJ) genes at chromosomes 7, 10 and 19.[3;4] For example, *PARD3* on chromosome 10 encodes the protein PAR-3 that has a role in regulating epithelial cell polarity and facilitating tight junction formation.[5] Likewise, *MAGI2* on chromosome 7 encodes the protein MAGI-2 that localizes to the tight junction, where it acts as a scaffold to directly interact with the lipid phosphatase tumour suppressor PTEN (phosphatase and tensin homolog).[6] *MAGI2* was also found to be associated with ulcerative colitis (UC). Likewise, *MYO9B* at chromosome 19 [3;4] encodes a single motor protein[7] belonging to the class IX myosin molecules, which are involved in remodelling of the cytoskeleton and thought to influence tight junction assembly, both of which can result in enhanced epithelial paracellular permeability[8;9]. These observations suggest that autoimmune diseases such as CD and UC share part of their aetiology through tight-junction-mediated intestinal barrier defects.[4]

An indirect test to study intestinal permeability is to measure the level of food antigen (FA) antibodies, such as gliadin antibodies (AGA) and baker's yeast or anti-*Saccharomyces Cerevisiae* antibodies (ASCA). It has been suggested that mild intestinal permeability alteration can cause elevated AGA antibody levels.[10;11] These antibodies are produced when one of the above antigens crosses the impaired intestinal barrier and becomes trapped by the intolerant immune system.

Down syndrome (DS) patients have a high prevalence of CD (4.6%) compared to the general population (1%) and are therefore routinely screened for CD.[12-15] However, up to 20% of DS patients show increased levels of AGA without manifesting CD.[12;16-19] This may imply that DS patients have an enhanced intestinal permeability. Thus, DS patients are an interesting group to study a possible correlation between gene variants thought to be involved in intestinal permeability and levels of AGA.

The aim of this study was to investigate correlations between AGA and ASCA levels, and between genetic variants (*PARD3, MYO9B, MAGI2*) and permeability, as measured by AGA in DS patients.

Methods

Study design and population

We included 126 consecutive children with DS, aged 19 years or younger and registered at the VU University Medical Centre in the period 2001-2008. The patients were examined for possible CD, since it is a common condition in DS. The levels of AGA (IgA and IgG) and ASCA (IgA and IgG) antibodies were also measured. The diagnosis of DS was confirmed by karyotyping in all cases except one. DS patients with positive tissue transglutaminase antibodies (tTG) and/or endomysium antibodies (EMA) were excluded. We did not perform intestinal biopsy in the AGA-positive patients for ethical reasons as the recent literature shows that most CD patients with DS have been detected among EMA and tTG-positive DS patients.[20] This study was approved by the Medical Ethical Committee of the VU University Medical Centre.

Measurements

Serologic markers

tTG and EMA were determined by a standard in-house enzyme-linked immunosorbent assay (ELISA) using recombinant human tissue transglutaminase (Diarect AG, Freiburg, Germany) as substrate and an in-house indirect immunofluorescence test according to Lerner using monkey oesophagus as substrate, respectively.[21] IgA- and IgG-AGA were determined by Standard in-house ELISA using gliadin extract (Sigma-Aldrich, Zwijndrecht, the Netherlands) as substrate (IgA cut-off value 4 U/ml, IgG cut-off value 21 U/ml). IgA- and IgG-ASCA were determined using a commercial ELISA (Inova Diagnostics, San Diego, California), according to the manufacturer's instructions (IgA and IgG cut-off 25 U/ml). IgA-AGA was analysed in 119 patients, IgG-AGA in 119, IgA-ASCA in 74, and IgG-ASCA in 74 (supplementary table 1).

Genotyping

All 126 DS patients were genotyped for two CD- and IBD-associated SNPs in *MYO9B* (rs1457092 and rs2305764), two SNPs in *MAGI2* (rs10763976 and rs6962966), and two SNPs in *PARD3* (rs9640699, and rs1496770). The genotyping was successful in 124 patients (call rate = 98.4%) for rs1457092, in 122 (96.8%) for rs2305764, in 124 (98.4%) for rs10763976, in 126 (100%) for rs6962966, in 126 (100%) for rs9640699 and in 123 (96.2%) for rs1496770. Genotype frequency for SNP rs6962966 was not in Hardy-Weinberg equilibrium (HWE)[22] and this SNP was excluded from further analysis. Genotyping of all polymorphisms was performed using TaqMan, as described elsewhere.[3;4]

Data analysis

First we studied whether the levels of AGA and ASCA were correlated with each other. We normalized the distribution of the levels of AGA and ASCA by taking their natural logarithms (ln). The correlation analysis was performed using Pearson's correlation test.

Next we checked whether the accumulative load of genetic risk variation associated to permeability was associated to the levels of AGA. For this analysis we focused on AGA, since it showed the highest sensitivity to alterations in intestinal permeability, i.e. even a mild

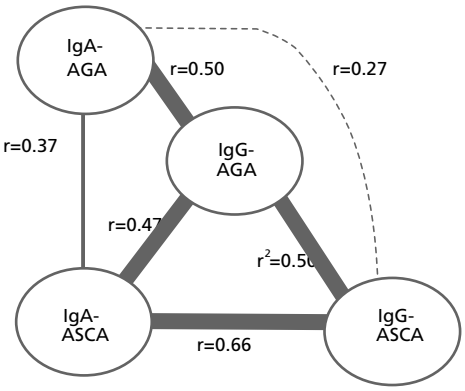
intestinal permeability alteration can cause elevated levels of AGA.[10;11] Furthermore, the elevation in AGA was most pronounced. Since the study size was limited, and the alterations in the intestinal permeability were determined by the net effect of all the genetic variation involved, we decided to analyse the collective effect of genetic risk variants on permeability. Thus, a genetic risk load was calculated as the sum of the number of times a participant was a carrier of the recessive genotype of the studied risk variants. We studied the relation between the genetic risk load and the levels of AGA (see supplementary online methods). We first analysed the association of the genetic risk load to quantitative levels of AGA, then we studied the association of genetic risk load to qualitative levels of AGA. Patients with no genetic risk factors were considered as the reference group to which other patients with a genetic risk load of at least 1 were compared. We used the ANOVA, linear regression, CHI^2 test and logistic regression to analyse the quantitative and qualitative data, implemented in SPSS version 15.0.

Results

Serologic study IgA-AGA was elevated in 17 patients (14%, 10 patients aged < 6 years). The mean of IgA-AGA level was 2.7 U/ml (range 0.3-27.1). IgG-AGA was elevated in 34 patients (29%, 21 patients aged < 6 years). The mean of IgG-AGA levels was 23.3 U/ml (range 0.6- 441.0). IgA-ASCA was elevated in 2 patients (3%, values 25.2 U/ml en 25.6 U/ml, cut off 25 U/ml). IgG-ASCA was elevated in 4 patients (5%, values 28-28-41-66 U/ml, cut off 25 U/ml). The levels of FA autoantibodies were significantly correlated with each other, suggesting a common pathophysiological pathway for all tested FA antibodies (figure 1, supplementary tables 1 and 2). Our data suggest that there is a strong correlation between IgG-AGA, IgG-ASCA and IgA-ASCA, and to a lesser extent to IgA-AGA.

Figure 1 Correlation between the levels of different serologic FA antibodies

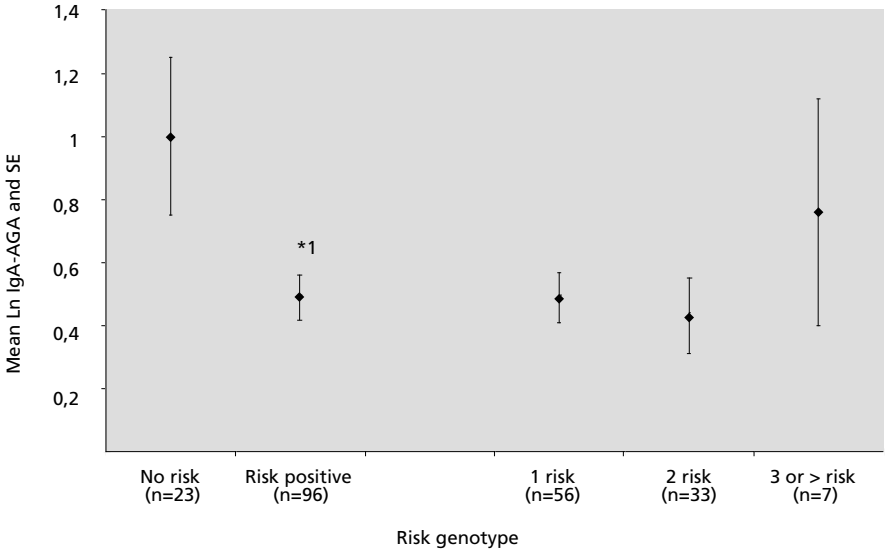
r^2 Pearson correlation coefficient, IgA-AGA: IgA antigliadin antibody, IgG-AGA: IgG antigliadin antibody, IgA-ASCA: IgA antisaccharomyces antibody, IgG-ASCA: IgG antisaccharomyces antibody. ---------- Pearson correlation significant at p< 0.05, ——— Pearson correlation significant at p< 0.01, ▬▬ Pearson correlation significant at p< 0.0001



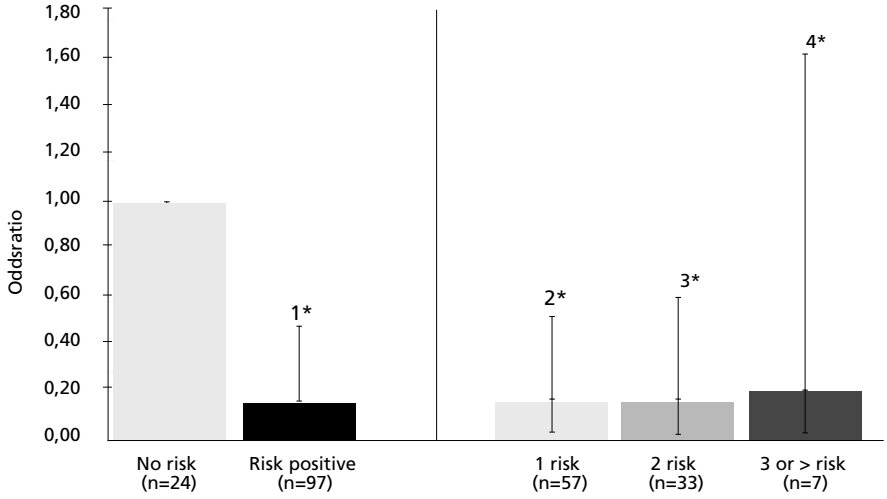
Genetic study There were 24 patients with no genetic risk factor, 57 patients had one recessive risk genotype, 33 patients had two, 7 patients had three or more risk genotypes, of whom one patient had a recessive risk genotype for all four studied genetic variants. The group of patients (n=97) with one or more risk genotypes showed lower mean ln IgA-AGA levels (0.49 versus 1.01 U/ml, P trend=0.007, figure 2a). When we correlated the genetic risk load to normal or elevated levels of IgA-AGA, we found patients with normal levels of IgA-AGA were significantly more often a carrier of one or more genetic variants associated to permeability, yielding an odds ratio of 0.16 (95% CI 0.06-0.47) (figure 2b, 2c, P=9.3 x 10^{-5}). Interestingly, all recessive genotypes or haplotypes which have been associated with CD showed a lower level of AGA.[3;4] (See supplementary tables 4 and 5).

Figure 2 Association of genotype risk dosage with permeability measured by AGA (a) Qualitative. Mean IgA-AGA with standard error of mean (b) Quantitative. Odds ratio with 95% confidence interval of IgA-AGA levels (elevated vs normal AGA) (c) Frequency of quantitative levels of IgA-AGA levels (elevated vs normal AGA; high vs low)

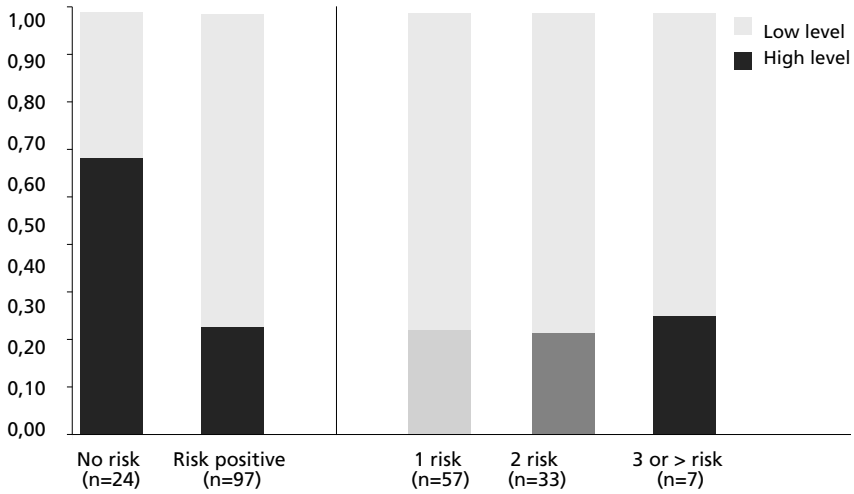
(a) *1 significance: Trend test P=0.007



(b) Significance: 1*p= 9.3 x 10 -5; 2*p= 0.003; 3*p=0.001; 4*p=0.07; P overall 2,3,4 = 0.008.



(c)



Discussion

This is the first study reporting a correlation between genetic variants in genes that might be involved in intestinal permeability and FA antibodies. In DS patients, we found that the different FA antibodies are strongly correlated with each other and that AGA levels were significantly lower in carriers of one or more variants of CD-associated risk genotypes [3;4]
We hypothesized that suspected CD-associated permeability genes are associated with an increased intestinal permeability that would be marked by elevated FA antibodies. Hence, genetic variants in these genes might play a role in intestinal permeability and precede the development of CD. These results are in contrast with our hypothesis that suggested the presence of alternative mechanisms for the elevated levels of FA antibody in Down patients.

In other words, our findings suggest that the levels of AGA may not be explained by the presences of only *MYO9B*, *MAGI2*, *PARD3* genes. It suggests that there are other factors which influence the penetrance of these genetic risk variants in permeability. Our findings raise the question of what other phenomena might play a role in the increased levels of FA antibodies in DS patients. This is not yet clear, though there might be several explanations for our findings. First, in our study we excluded all patients who had CD. Therefore the genetic structure underlying the increased permeability in CD autoimmune diseases, may have been exuded from this analysis. This is in contrast with those studies which have reported the association between TJ genes and CD,[3;4] where CD patients were compared with controls. CD might overrule permeability function whenever it develops. Therefore, the normal function of TJ genes might be difficult to study. Secondly, one may speculate that the elevation of AGA may be mainly determined by an immunological disturbance rather than by solely alterations in the genetics of TJ, possibly at the level of tolerance induction and/or maintenance, as a result of the chromosomal aberration causing DS. The increased prevalence of autoimmune disease and susceptibility to infections in DS suggests support for this hypothesis. Derangements of immunoglobulin levels, phytohemagglutinins responsiveness and T and B cell markers are also reported in DS patients. Both thymus-dependent and thymus-independent functions are impaired in DS patients with a characteristic age distribution. IgA and IgG immunoglobulin levels in DS patients older than 6 years are definitely increased.[23] This elevation is particularly found for FA antibodies. This immunological disorder might also explain why DS patients have an increased prevalence of elevated AGA.

There are some limitations to our study. First, although it was an effort to collect 126 patients with DS, the interpretation of our data was difficult because of the relatively small number of patients with elevated FA antibody levels. Secondly, we did not perform sugar absorption tests with lactulose-manitol, the other marker that, besides AGA, can be used to study intestinal permeability. However, this tests is too sensitive and frequently influenced by environmental factors.[24-27] Thirdly, we may not have investigated all the relevant genetic variations, as many more genes might be involved in intestinal permeability.

We found that DS patients had elevated levels of FA antibodies, in agreement with earlier reports.[12] However, these publications showed that the hyperglobulinemia of IgA and IgG type was observed in DS patients older than 6 years and that serum immunoglobulin levels were normal in patients less than 5 years.[23;28] Our study could not support this finding; the majority of our patients with elevated immunoglobulins were younger than six years, except for IgG-ASCA (all four patients with elevated IgG-ASCA were older than seven years). A correlation between different serological markers was found in the large majority of patients. Furthermore, we found no elevation in levels of ASCA, which might be explained by the fact that this is a very large FA and it might be too large to penetrate the intestinal barrier. This is supported by earlier studies showing that even in young CD patients with a disturbed gut barrier ASCA could only be detected in 18% of patients.[29]

Conclusion

DS patients had increased levels of FA autoantibodies, though this increase cannot be explained by the presence of genetic variations in TJ genes that are associated with CD and possibly with intestinal permeability. Our findings suggest that other mechanisms might be involved in the production of elevated FA antibody levels. More understanding of the factors involved in the levels of FA antibodies in DS might be useful in unravelling the multi-factorial causes of permeability, and the mechanisms underlying FA antibodies, CD, and other auto-immune diseases. Future studies on more (DS) patients and with more sophisticated markers for intestinal permeability are needed to unravel the pathogenesis of autoimmune diseases.

Grant support

This work was supported by the Celiac Disease Consortium, an Innovative Cluster approved by the Netherlands Genomics Initiative and partially funded by the Dutch Government (BSIK03009), and the Wilhelmina Research Fund, Utrecht. Dr. B.Z. Alizadeh was supported by a grant from the Netherlands Organization for Health Research and Development (*ZonMw*, grant number 016.096.121).

Acknowledgements

We thank the participants for their cooperation and Jackie Senior for critically reading the manuscript.

References

1. Meddings J. The significance of the gut barrier in disease. *Gut* 2008;**57**(4):438-40.
2. Vaarala O. Leaking gut in type 1 diabetes. *Curr Opin Gastroenterol* 2008;**24**(6):701-6.
3. Monsuur AJ, de Bakker PI, Alizadeh BZ *et al*. Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat Genet* 2005;**37**(12):1341-4.
4. Wapenaar MC, Monsuur AJ, van Bodegraven AA *et al*. Associations with tight junction genes PARD3 and MAGI2 in Dutch patients point to a common barrier defect for coeliac disease and ulcerative colitis. *Gut* 2008;**57**(4):463-7.
5. Izumi Y, Hirose T, Tamai Y *et al*. An atypical PKC directly associates and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of Caenorhabditis elegans polarity protein PAR-3. *J Cell Biol* 1998;**143**(1):95-106.
6. Wu X, Hepner K, Castelino-Prabhu S *et al*. Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2. *Proc Natl Acad Sci U S A* 2000;**97**(8):4233-8.
7. Post PL, Tyska MJ, O'Connell CB *et al*. Myosin-IXb is a single-headed and processive motor. *J Biol Chem* 2002;**277**(14):11679-83.
8. Bruewer M, Hopkins AM, Hobert ME *et al*. RhoA, Rac1, and Cdc42 exert distinct effects on epithelial barrier via selective structural and biochemical modulation of junctional proteins and F-actin. *Am J Physiol Cell Physiol* 2004;**287**(2):C327-C335.
9. Matter K, Balda MS. Signalling to and from tight junctions. *Nat Rev Mol Cell Biol* 2003;**4**(3):225-36.
10. Lerner A, Lebenthal E. The controversy of the use of anti-gluten antibody (AGA) as a diagnostic tool in celiac disease. *J Pediatr Gastroenterol Nutr* 1991;**12**(4):407-9.
11. Savilahti E, Viander M, Perkkio M *et al*. IgA antigliadin antibodies: a marker of mucosal damage in childhood coeliac disease. *Lancet* 1983;**1**(8320):320-2.
12. Bonamico M, Rasore-Quartino A, Mariani P *et al*. Down syndrome and coeliac disease: usefulness of antigliadin and antiendomysium antibodies. *Acta Paediatr* 1996;**85**(12):1503-5.
13. Catassi C, Fasano A. Celiac disease. *Curr Opin Gastroenterol* 2008;**24**(6):687-91.
14. Bonamico M, Mariani P, Danesi HM *et al*. Prevalence and clinical picture of celiac disease in italian down syndrome patients: a multicenter study. *J Pediatr Gastroenterol Nutr* 2001;**33**(2):139-43.
15. Wouters J, Weijerman ME, van Furth AM *et al*. Prospective human leukocyte antigen, endomysium immunoglobulin A antibodies, and transglutaminase antibodies testing for celiac disease in children with Down syndrome. *J Pediatr* 2009;**154**(2):239-42.
16. Ruch W, Schurmann K, Gordon P *et al*. Coexistent coeliac disease, Graves' disease and diabetes mellitus type 1 in a patient with Down syndrome. *Eur J Pediatr* 1985;**144**(1):89-90.
17. Failla P, Ruberto C, Pagano MC *et al*. Celiac disease in Down's syndrome with HLA serological and molecular studies. *J Pediatr Gastroenterol Nutr* 1996;**23**(3):303-6.
18. Gale L, Wimalaratna H, Brotodiharjo A *et al*. Down's syndrome is strongly associated with coeliac disease. *Gut* 1997;**40**(4):492-6.
19. Rumbo M, Chirdo FG, Ben R *et al*. Evaluation of coeliac disease serological markers in Down syndrome patients. *Dig Liver Dis* 2002;**34**(2):116-21.

20. Shamaly H, Hartman C, Pollack S *et al*. Tissue transglutaminase antibodies are a useful serological marker for the diagnosis of celiac disease in patients with Down syndrome. *J Pediatr Gastroenterol Nutr* 2007;**44**(5):583-6.
21. Lerner A, Kumar V, Iancu TC. Immunological diagnosis of childhood coeliac disease: comparison between antigliadin, antireticulin and antiendomysial antibodies. *Clin Exp Immunol* 1994;**95**(1):78-82.
22. Terwilliger JD. Handbook of Human Genetic Linkage. New York: John Hopkins University Press, 1994.
23. Burgio GR, Ugazio AG, Nespoli L *et al*. Derangements of immunoglobulin levels, phytohemagglutinin responsiveness and T and B cell markers in Down's syndrome at different ages. *Eur J Immunol* 1975;**5**(9):600-3.
24. Bjarnason I, Maxton D, Reynolds AP *et al*. Comparison of four markers of intestinal permeability in control subjects and patients with coeliac disease. *Scand J Gastroenterol* 1994;**29**(7):630-9.
25. Bjarnason I, MacPherson A, Hollander D. Intestinal permeability: an overview. *Gastroenterology* 1995;**108**(5):1566-81.
26. Smecuol E, Bai JC, Vazquez H *et al*. Gastrointestinal permeability in celiac disease. *Gastroenterology* 1997;**112**(4):1129-36.
27. Smecuol E, Bai JC, Sugai E *et al*. Acute gastrointestinal permeability responses to different non-steroidal anti-inflammatory drugs. *Gut* 2001;**49**(5):650-5.
28. Reichelt KL, Lindback T, Scott H. Increased levels of antibodies to food proteins in Down syndrome. *Acta Paediatr Jpn* 1994;**36**(5):489-92.
29. Mallant-Hent RC, Mary B, von BE *et al*. Disappearance of anti-Saccharomyces cerevisiae antibodies in coeliac disease during a gluten-free diet. *Eur J Gastroenterol Hepatol* 2006;**18**(1):75-8.
30. Croes EA, Alizadeh BZ, Bertoli-Avella AM *et al*. Polymorphisms in the prion protein gene and in the doppel gene increase susceptibility for Creutzfeldt-Jakob disease. *Eur J Hum Genet* 2004;**12**(5):389-94.

Supplementary information

Collective association of all genetic variations in three tight junction genes and levels of AGA

First, we performed a genetic risk profile. From earlier studies we knew that in *MAGI2* recessive genotype AA (rs1496770, rs9640699) was associated with CD, and in *PARD3* (rs10763976) recessive genotype AA was associated with CD and UC (supplementary table 2).[3;4] We continued making a genetic risk profile by performing a haplotype analysis (allelic) on two *MYO9B* SNPs which were in LD (rs 1457092 and rs 2305764).[3] From earlier studies we knew that the AA haplotype was associated with CD (p=0.000018, supplementary table 3) and we defined the haplotype as the risk haplotype described by Croes *et al*.[30] Patients were defined by whether they were recessive for the AA haplotype, i.e. carrier of risk variants in *MYO9B*. One haplotype group (AC) consisted of only 1 patient and was therefore excluded from further analysis. We calculated the risk genetic load. We summed the risk genotypes for the above 4 SNPs in each patients. Patients showed as having no (n=24), 1 (n=57), 2 (n=33), or 3 or more recessive (n=7) risk genotypes in the tested genes. As only one subject was included in the '3 recessive' category, it was excluded from the analysis because it could not make a meaningful statistical comparison. We analysed the levels of AGA antibodies and the presence of 0, 1, 2, 3 or 4 recessive genetic risk variants (table 3). We used ANOVA, linear regression, chi-squared- and logistic regression analysis, when applicable. SPSS statistical software (version 15.0; SPSS Inc, Chicago, Illinois) was used for statistical modelling.

Supplementary Table 1 Levels of antibodies against food antigens in DS patients

Antibody	N=126	Elevated N (%)	Mean (Std. Deviation)	Range
IgA-AGA	119	17 (14%)	2.68 (3.36)	0.30-27.00
IgG-AGA	119	34 (29%)	23.26 (47.46)	0.60-441.00
IgA-ASCA	74	2 (3%)	6.24 (5.58)	0.20-25.60
IgG-ASCA	74	4 (5%)	8.69 (10.57)	0.0-66.00

Supplementary Table 2 Pearson's correlations among serologic values (n given in brackets)

	Ln IgA-AGA	Ln IgG-AGA	Ln IgA-ASCA
Ln IgG-AGA (n)	0.502 *** (117)		
Ln IgA-ASCA (n)	0.366 ** (82)	0.472 *** (81)	
Ln IgG-ASCA (n)	0.272 * (80)	0.501 ** (79)	0.663 *** (83)

Ln IgA-AGA: logarithm IgA antigliadin antibody, Ln IgG-AGA: logarithm IgG antigliadin antibody, ln IgA-ASCA: IgA antisaccharomyces antibody, ln IgG-ASCA: logarithm IgG; *Significant at $p < 0.05$; ** Significant at $p < 0.001$; *** Significant at $p < 0.000001$

Supplementary Table 3 Relation between IgA-AGA/ IgG-AGA and SNPs in three intestinal barrier genes

Polymorphism	Elevated IgA or IgG-AGA (%)	No elevated IgA or IgG-AGA (%)	P value Chi-Squares	OR (95% CI)	IgA-AGA median (range)	P value (Mann Whitney)	IgG-AGA median (range)	P value (Mann Whitney)
rs1496770	N=79	N=39						
gg	27.85	30.77	Ref *1		1.55 (0.50-10.00)	Ref	11.00 (0.40-441.00)	Ref
ag	53.16	58.97	0.99		1.60 (0.50-27.00)	0.55	11.00 (0.80-120.00)	0.33
aa	18.98	10.26	0.23		1.90 (0.30-10.00)	0.96	8.40 (0.60-95.00)	0.91
ag/ gg	81.02	89.74			1.60 (0.50-27.00)		11.00 (0.40-441.0)	
rs9640699	N=81	N=40						
cc	25.93	40.00	Ref *2		1.40 (0.30-8.00)	Ref	15.00 (0.60-210.00)	Ref
ac	51.86	47.50	0.025	0.59 (0.23- 1.51)	1.60 (0.50-27.00)	0.67	7.90 (0.40-110.00)	0.19
aa	22.22	12.50	0.20		1.80 (0.60- 7.00)	0.84	9.35 (2.00-441.00)	0.89
ac/ cc	77.78	87.50			1.60 (0.30-27.00)		10.80 (0.40-210.00)	
rs10763976	N=80	N=39						
gg	27.50	25.64	Ref *3		1.40 (0.50-27.00)	Ref	10.20 (1.20-120.00)	Ref
ag	50.00	56.41	0.68		1.80 (0.30-16.00)	0.32	10.60 (0.40-441.00)	0.80
aa	22.50	17.95	0.56		1.30 (0.40-10.0)	0.26	8.30 (0.60-110.0)	0.34
ag/ gg	77.50	82.05			1.75 (0.30- 27.0)		11.30 (0.40-441.00)	
Haplo-genotype MYO9B	**Elevated IgA or IgG-AGA (%)**	**No elevated IgA or IgG-AGA (%)**	**P value Chi-Squares**	**OR (95% CI)**	**IgA-AGA median (range)**	**P value (Mann Whitney)**	**IgG-AGA median (range)**	**P value (Mann Whitney)**
rs1457092 and rs2305764	N=23	N=34						
CGCG	78.26	61.76	Ref		1.80 (0.40-27.00)	Ref	10.60 (0.60-441.00)	Ref
AAAA	21.74	38.24	0.19		1.40 (0.70-5.00)	0.91	10.05 (1.70-61.00)	0.78

Significance for genotype *1 p=0.48, *2 p=0.21 *3 p=0.78

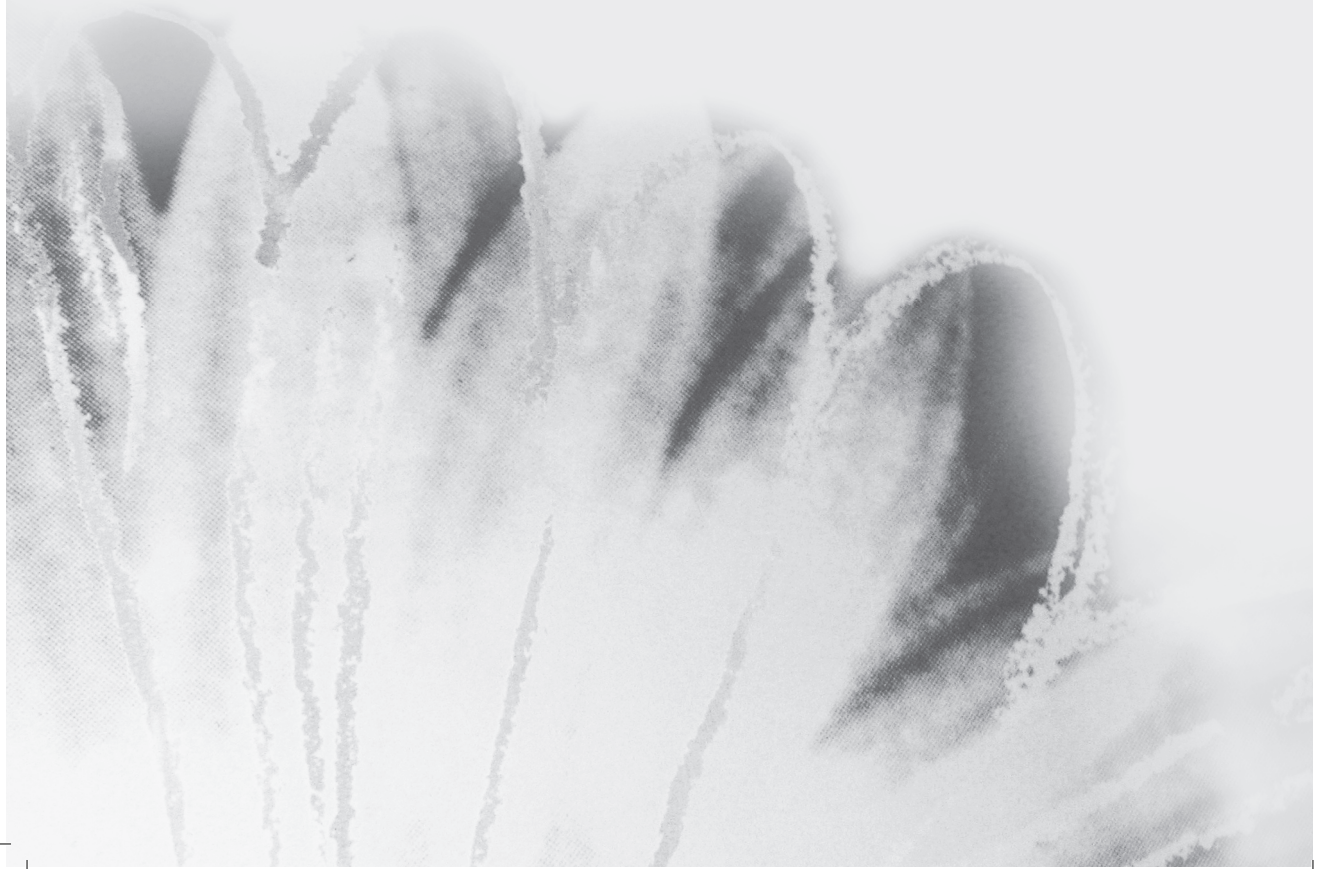
Supplementary Table 4 Genotypes of three SNPs (*PARD3* and *MAGI2*) associated with coeliac disease in two earlier studies (3;4)

SNP	CD associated genotype	Not associated CD genotype	P value
rs1496770	AA	GG/ AG	P< 0.04
rs9640699	AA	CC/ AC	P<0.01
rs10763976	AA	GG/ AG	P<0.000

Supplementary Table 5 Allelic haplotype of two SNPs (*MYO9B*) associated with coeliac disease (3)

Rs 1457092	Rs 2305764	P value
C	G	Ref OR 1.0
A	G	
A	A	P=0.000018
C	A	

8



Chapter 9

General discussion
Summary
Nederlandse samenvatting
List of publications
Curriculum Vitae
Dankwoord

General Discussion

Part 1 Diagnostics in coeliac disease

Serologic tests and diagnosis

The diagnostic criteria for CD were established for the first time in 1969 at the Interlaken symposium.[1;2] CD was defined as a permanent disorder which required three criteria: an abnormal jejunal mucosa on a gluten containing diet, clear improvement of villous structure when taking a gluten free diet, and deterioration of the mucosa during gluten challenge. In 1990 these criteria were revised as the need for routine gluten challenge had been questioned.[3] The revision was partially based on a large study including patients of all ages; only in approximately 5% of patients another diagnosis was made following gluten challenge.[4] As the mean age at initial biopsy of these non confirmed coeliac patients was 8 months, the revised criteria recommended gluten challenge only for children aged two years or less at diagnosis. In this thesis we now show that even in these young children a gluten challenge is not necessary anymore to confirm the diagnosis of CD, provided that clinical symptoms and the characteristic histological intestinal changes (Marsh III[5]) are indeed compatible with CD and endomysium antibodies (EMA) and/or raised IgA tissue transglutaminase antibodies (tTG) are present (chapter 5).

Confirmation of the diagnosis of CD still requires an intestinal biopsy in all cases and is considered the 'gold standard'. Traditionally, the (typical) diagnosis of CD is made in patients with clinical symptoms, positive serology and total villous atrophy (Marsh III) on intestinal biopsy.[6] However, our understanding of the histological spectrum of CD has changed in recent years and new insights and recommendations were published.[7] The disease may gradually develop from lymphocytic enteritis (Marsh I) to lymphocytic enteritis with crypt hyperplasia (Marsh II) and finally to villous atrophy, subdivided in partial, subtotal and total (Marsh IIIA, B and C respectively).[8] This is supported by gluten challenges performed in CD patients which showed that it sometimes took years before the mucosa relapsed to Marsh III suggesting that there might indeed be a continuum.[9-11] The guidelines have changed accordingly. Marsh III on intestinal mucosa in the presence of tTG was traditionally diagnostic for CD.[12] The presence of infiltrative changes with crypt hyperplasia (Marsh II) on intestinal biopsy now is also compatible with CD. Diagnosis in these cases is strengthened by the presence of positive serological tests (tTG or EMA) for CD. The presence of infiltrative changes alone (Marsh I) on intestinal biopsy is not specific but concomitant positive serological tests for CD (tTG or EMA) increases the likelihood that such an individual has CD. Furthermore, the new recommendation states that gluten challenges are only indicated in case of diagnostic uncertainty. The study described in chapter 5 of this thesis supports this change in recommendation. This change in the diagnostic criteria for CD was partly possible because of our better understanding of the different histological manifestations of CD, and partly because of the better performance of serologic tests, which are now an essential part of the diagnostic algorithm. In this thesis (chapter 3) we showed that the sensitivities and specificities of EMA and especially tTG nowadays are indeed very high (over 95% for sensitivity and close to

100% for specificity) as are their positive and negative predictive values. The high reliability of these tests was recently also summarized by Rostom *et al*.[13] However some patients with a positive tTG and/or EMA do have a normal mucosa, while others, with a clear villous atrophy due to CD, do have negative serology, even when serum IgA is normal. Therefore the search is on for an optimal test that can better discriminate between CD and non-CD, either alone, or in combination with tTG/ EMA.

Recently, the first results for a new serologic test were published, deamidated anti-gliadine antibodies, which seems to be promising but not outperforming tTG.[14;15] A combined method, detecting both antibodies (IgA and IgG) against deamidated anti-gliadine and tTG even showed a sensitivity of 100% (119 of 119) compared to 97% for IgA tTG. However the specificity of 89% for disease controls and 97% for blood donors was lower than the specificity for tTG; 96% and 98% respectively.[16] Thus this new serologic test seems promising in the identification of childhood CD, but obviously these results need to be confirmed in larger and independent samples. In addition the reported sensitivities and specificities were calculated in children with gastro-intestinal symptomatology, and therefore the prevalence of CD was probably higher than seen in the general population. This might bias the performance of the test outside the population described.

In addition, adding the determination of serum intestinal fatty acid binding protein (I-FABP) to the current serological arsenal might prove to be very valuable. I-FABP is a newly discovered small cytosolic protein present in enterocytes, of which the serum level correlates very well with intestinal mucosal damage.[17] In chapter 4 we now report that combining the serum level of I-FABP with the results of tTG and EMA does indeed increase the sensitivity and specificity of these conventional serological parameters. It might also be useful in the follow-up of patients with CD. Usually patients become symptom free after initiating a gluten free diet, and tTG falls to normal levels within a few months. However, tTG does not seem effective to assess mucosal recovery in the follow-up of CD patients after they have started a gluten free diet, due to its poor correlation with histological damage.[18] The response of serum I-FAPB seems to be a better parameter as it directly reflects mucosal damage (chapter 4). Although tTG is also widely used for the follow up of CD patients after starting a gluten free diet, tTG is a poor predictor of dietary transgressions in adults. A negative tTG in adults correlates with a strict compliance to the diet, but the proportion of correct recognition of dietary gluten ingestion was only 52%. Thus, tTG negativity is not a good marker for diet compliance.[19] In this respect I-FABP might be better suited, as it is a direct marker of intestinal damage and not an immunologic marker like tTG.

Future directions

In the near future, it should be investigated whether an intestinal biopsy can be omitted in selected patients. The group of patients with the combination of classical symptoms, a positive tTG (> 100 IE/ ml), I-FABP above cut-off value, HLA-DQ2 and/or -DQ8 and a good clinical and serological response (both tTG and I-FABP) to a gluten free diet might be the first candidates for such an approach. A biopsy or a gluten challenge might only be necessary whenever there is doubt regarding the diagnosis.

Obviously with this approach the number of biopsies and its associated burden will be reduced and many patients can start a gluten free diet without this invasive procedure. However with such a regimen some patients with a positive tTG but a normal mucosa will start a gluten free diet. It can be argued that these patients have in fact CD in an early phase, as many patients have been described with positive serology and a normal duodenal mucosa in whom later on the mucosa deteriorated and CD was diagnosed.[20-26] Supporting this are data that suggest that CD might exist even in the presence of a normal mucosa. Kaukinen *et al* [27] observed tTG 2 specific IgA deposits on intestinal mucosa, suggestive of mucosal auto-immunity, without villous atrophy. Interestingly, the clinical and serological response to dietary intervention of these tTG 2 specific IgA deposits was similar in overt and mild enteropathy CD.[28] These recent findings again questions the meaning of a 'normal mucosa'.

Although some patients with a positive tTG might indeed have CD in an early phase, it is also known that tTG levels can become normal again during follow up, despite a gluten containing diet.[29] The reason for this remains unknown but may be related to the multifactorial pathogenesis of CD and more specific to the threshold of tolerance in relation to environmental factors. This is supported by recent findings suggesting that infectious diseases might be associated with a temporary 'false' positive tTG, as four out of five patients with positive tTG turned out to have a normal tTG after three months. However these patients were HLA-DQ2 and -DQ8 negative, virtually ruling out CD.[30] Positive tTG in absence of EMA and with negative HLA-DQ2 and -DQ8 was also found in patients with Non-Hodgkin lymphoma.[31] More importantly a 'false' positive tTG also seems to exist in patients with HLA-DQ2 and/or -DQ8, as in a group of predominantly asymptomatic children with HLA-DQ2 and/or -DQ8, a positive tTG became normal in 51% of cases without gluten exclusion.[32] Thus, abandoning biopsy will result in a false diagnosis of CD in some tTG positive children, and the decrease of tTG in this group of patients will be falsely attributed to the gluten free diet.

In future studies the natural history of symptomatic and asymptomatic patients with a positive serology and a normal intestinal biopsy has to be elucidated. It is unknown whether there is an increased health risk in these groups of patients. However, the possible increased health risk, if any, has to outweigh the burden of a gluten free diet. An additional aim for this research is to analyze whether symptomatic and asymptomatic tTG positive patients with a normal intestinal mucosa also benefit from a gluten free diet by reducing the incidence of other auto-immune diseases as was recently suggested.[33] In a group of asymptomatic tTG positive patients with a normal mucosa a total of 5.1% developed other auto-immune diseases during follow up which is higher than the 3% risk in the general population.[34]

All in all, there is much debate about the biopsy as the gold standard for diagnosing CD. The reliable serologic tests and recent broadening of the histological definition questions the need for biopsy in case of a 'typical' CD patient. It is tempting to speculate that in a few years in a selected group of patients the biopsy will not be necessary anymore if studies will

show that symptomatic patients with tTG > 100 IE, I-FABP above the cut-off value and a good clinical and serological response will benefit from a gluten free diet.
In the (near?) future we might even diagnose CD with a simple blood test assessing gluten-specific T cells as was suggested by Raki *et al*.[35] In addition, within ten years, all genetic variants associated with CD might be known and a genetic risk profile might be calculated. As a result, small intestinal biopsies might not be needed anymore.

Part 2 Genetics in coeliac disease

The strongest genetic factor associated with CD is HLA-DQ2 and -DQ8 and is found in virtually all CD patients.[36;37] HLA-DQ is expressed on antigen presenting cells and presents exogenous antigen to T cells.[38;39] Gluten proteins provoke CD in genetically susceptible individuals as the high proline content of gluten is relatively resistant to proteolytic digestion in the intestinal tract.[40;41] The undigested gluten peptides are deamidated by tTG, which results in a perfect "fit" in the pocket of HLA-DQ2 or HLA-DQ8 molecules on antigen-presenting cells.[42] This complex is presented to CD4+ T cells and the ensuing immune response causes inflammation and intestinal tissue damage.

Twin and family studies indicate that non-HLA genes also contribute to the development of CD.[43;44] Several genetic linkage studies have identified susceptibility loci on various chromosomes, such as 2, 5, 6, 7, 9, 10, 15 and 19, revealing the complexity of CD as we also described in chapter 6.[45-56] However, so far replication studies could only confirm a few of these associations, e.g. 5q31-q33[57-62] and 2q33 (CTLA4).[63-66]. A large European meta-analysis showed evidence of linkage to 5q31 but only marginally to 2q33.[67] It could be that some of the non-replicated loci were initially identified due to chance effects, i.e. are not involved in CD, are population specific or that the replication studies were underpowered. Recently, the first large Genome Wide Association Study (GWAS) was performed and identified eight new genomic regions. Seven of these eight genomic regions contain genes with a suspected immunologic function.[68;69] None of these genomic regions (except HLA) were already identified in previous studies. The strongest association outside HLA was found in genomic region 4q27 and encodes several genes including cytokines interleukin 2 (*IL2*) and interleukin 21 (*IL 21*).[70;71] Interestingly, the same 4q27 region was also found to be associated with type 1 diabetes and rheumatoid arthritis.[72;73] Furthermore, very recently two novel CD risk regions were identified making a total of ten non-HLA risk factors at this moment.[74]
In this thesis we focused on three genes (*PARD3, MYO9B, MAGI2*) associated with CD.[75;76] These genes might have a role in intestinal permeability and more specifically in tight junction function.[77-79] These three 'permeability' genes were shown to be associated with CD, but also with other autoimmune diseases, suggesting a shared pathogenesis. This is an intriguing field and might give more insight into the complex and overlapping pathogenesis between CD and other autoimmune disorders.

Intestinal permeability

We experience our environment through the small intestine. The intestinal barrier controls

trafficking of macromolecules between the environment and the host and depends on the interplay of paracellular tight junctions (TJ) as well as on the activity of transcellular transport via endocytosis. Particular attention has been placed on TJ dysfunction in the pathogenesis of autoimmune diseases after the discovering of *MYO9B* and subsequently *PARD3* and *MAGI2*.[80;81] All three genetic variants might have a role in TJ function and might be linked to a defect in barrier function that appears to be required before the development of disease. In this thesis we focussed on these three TJ genes and we tried to unravel whether TJ risk genotypes were associated with an increased intestinal permeability, as measured by anti-gliadin antibodies (AGA) in Down syndrome (chapter 8). However we found lower instead of higher AGA serum levels, thus other immunologic phenomena than permeability seem to play a role in the development of auto-immune diseases such as CD and thyroid dysfunction in patients with Down syndrome. However, in patients without chromosomal aberrations, increased intestinal permeability might promote the development of autoimmune diseases. Apart from CD current genetic evidence also suggests a role for risk alleles in *PARD3* and *MAGI2* in ulcerative colitis[82] and for *MYO9B* in Crohns disease[83;84] and diabetes type I.[85] Moreover, we obtained indications that *MYO9B* is involved in the development of refractory CD and progression to EATL (chapter 7).

Other evidence also points to an increased intestinal permeability as a risk factor for the development of specific auto-immune diseases. In Irish setter dogs with gluten sensitive enteropathy intestinal permeability turned out be increased, even in the absence of gluten exposure.[57] One might hypothesize that peptides larger than normal, or in greater quantities than normal, can cross the 'leaky' intestinal epithelium and initiate disease. In individuals who are at increased risk for the development of IBD increased gastrointestinal permeability has been demonstrated in absence of disease as well.[86;87] This phenomenon is also extensively studied in diabetes type 1, where normalization of the increased intestinal permeability in the BB rat, prone to develop diabetes type I, largely prevents the development of diabetes.[61] An abnormally elevated secretion of the luminal hormone zonulin was found in these rats, which increased epithelial permeability.[61] Antagonizing the effect of zonulin normalised the increased permeability and prevented the appearance of diabetes. Potential new targets for therapy, like influencing the intestinal barrier by blocking the zonulin receptor, are future directions for study. Interestingly, feeding these rats a hydrolysed diet, reducing exposure to larger peptide fragments, also prevented the development of diabetes type 1.[88] It is tempting to speculate that an increased intestinal permeability is also a pathogenetic factor in other autoimmune diseases. In fact in rheumatic disease too an increased intestinal permeability was found.[89] As autoimmune diseases have strong genetic components one might speculate that these diseases all share common genetic defects in the control of intestinal barrier function, apart from shared immunological pathways. This hypothesis is the focus of research now and in the future.

Shared non permeability genetic loci for different autoimmune diseases

Unravelling the genes involved in the balance between tolerance and the development of autoimmune diseases is an important subject of research. As discussed above, part of the

genes associated with CD and IBD are suspected permeability genes, which fits perfectly well in the hypothesis that autoimmunity might develop whenever there is an increased intestinal permeability. Not only suspected permeability genes were found to be associated with CD, but other genes as well. In fact almost all found associated genes are suspected to play a role in the immune system.[90]

The first round of genome-wide association scans (GWAS) is already completed in different autoimmune diseases. In 2007 the first GWAS on CD was undertaken and 310 606 single nucleotide polymorphisms (SNPs) were tested.[91] An association was found on chromosome 4 in region 4q27. The minor allele of a SNP (rs13119723) was found to be present at higher frequency in controls compared to cases. The associated 4q27 region encodes several genes such as interleukin2 (*IL2*) and interleukin21 (*IL21*). Both are cytokines involved in T cell activation and proliferation. However, this 4q27 region harbours a lot of genes and due to high levels of correlation between the markers, it is unknown yet which marker is the true causal variant. Interestingly, other auto-immune mediated diseases have also been found to be associated with this 4q27 region, like diabetes type 1 and rheumatic arthritis, again suggesting a shared genetic mechanism between these diseases.[92;93] One might suggest that, given these GWAS results, alterations in the immune response might be more important than an increased intestinal barrier. However, we do not know yet in which way the intestinal permeability and the immune response are precisely connected and how the interplay contributes to disease and to what extent. It could be that a change in intestinal barrier results in an unregulated immune response in susceptible individuals, followed by the development of auto-immunity. A next step in unravelling the hypothesis of shared genetic loci is to perform a GWAS in different autoimmune diseases. It might enable us to obtain more insight in the shared genetic susceptibility variants in all known autoimmune diseases. However, as the associated variants (SNPs) are not necessarily localized within the causative gene, it is necessary to subsequently investigate which gene(s) really are involved in the development of an autoimmune disease. This may require newly developed techniques.

Problems and disadvantages of genetic studies

Disadvantages to this 'genetic' approach become apparent as hundreds and maybe thousands of regions associated with genetic risk factors for CD and other autoimmune diseases are being identified. Finding the causative gene and attributing the amount of risk is a formidable task. In addition, genetic heterogeneity might complicate matters as well. Another problem is the low penetrance of individual disease alleles, resulting in a large proportion of patients with a normal phenotype in presence of the disease causing alleles. Epigenetics, semi-permanent changes in the methylation pattern of the DNA might be very important too for the activation and function of genes, but will not be identified by a genetic approach that solely searches for differences in DNA sequence. Resolving all these puzzles, if possible, require large collaborations, which are however already taking shape, as recent papers show.

Future directions

Currently HLA genes have a high negative predictive value and are often used to exclude the diagnosis of CD.[94] Creating non-HLA genetic risk profiles for CD and other autoimmune disease is interesting but will only be clinically relevant when they have a clear positive and negative predictive value. In this thesis we did a first step in creating a genetic risk profile for complicated forms of CD such as refractory coeliac disease type II (RCD II) and enteropathy-associated T-cell lymphoma (EATL). We found that both non-HLA (*MYO9B* rs7259292) and HLA-DQ2 homozygosity increased the risk for the development of RCD II and EATL when compared to CD patients without this complication (OR 4.3 (95% CI 1.9–9.8) and 5.4 (95% CI 3.0–9.6), respectively (chapter 7).

It may take a considerable amount of time before we can perform truly informative genetic risk profiles for CD. However a first step was already set by Romanos *et al* who reported about HLA and non-HLA risk alleles that might identify individuals at high risk for CD.[95] Individuals carrying 13 or more risk alleles had a higher CD risk (OR = 6.2; 95% CI 4.1-9.3) compared to those carrying zero to five risk alleles. Also in Crohn's disease the first papers regarding clinical relevance of genetic risk profiles have been published. Weersma *et al* showed that an increase in the number of risk alleles is associated with an increased risk for the development of Crohn's disease and with a more severe disease course.[96] Clinicians might predict the course of Crohn's disease by combining the information from the known risk genotypes. This might result in a therapeutic intervention that is tailored to a specific patient (group).

At present in CD the associated non-HLA risk factors add such a small risk (OR 0.7-1.4, ~3-4% of heritability)[97] that it is not yet clinically relevant. However, in this 'GWAS' era more and more non-HLA genes will be discovered and might help us to identify combinations of genetic factors influencing the risk to develop CD. Analyzing genotype-phenotype correlations for (non-) HLA genes might become very important to identify patient (groups) who might benefit from tailored interventions. For example in CD we hypothesize that babies with a positive family history and 'high risk' HLA- and 'high risk' non-HLA-genetic profile might be advised to have the introduction of gluten in a lower amount and at an earlier age, in order to prevent the occurrence of CD.[98] Furthermore, patient selection based on information like age, biomarkers, and family history can improve the performance of genetic tests. In a study on the risk of developing prostate cancer the positive predictive value of genetic profiling increased in patients with a positive family history.[99].

Thus, when the combination of these non-HLA genes will have high positive and negative predictive value, this will become clinically useful. For now only HLA genes are clinically very useful as the absence of HLA-DQ2 and -DQ8 virtually excludes diagnosis of CD.[100] For non-HLA statistical significant associations in CD are found, however without clinical relevance yet

Any genetic test, if necessary in combination with relevant accurate phenotypic information, needs to be clinically useful (high positive/ negative predictive value) and to outweigh the costs. Discovering genetic markers reliably associated with CD is the first step and creating genetic risk profiles is the next challenging step before we can move on. The ultimate goal is to discover clinical relevant genetic profiles for CD that can be applied clinically and have a good costs/benefit ratio.

Conclusion

The field of genetics in CD and other autoimmune diseases is moving very rapid and new genetic findings being reported at an astounding pace. Multiple genes seem to contribute to each of the major autoimmune disorders, with significant genetic overlap between them. However we are only beginning to unveil the (patho)genetics of these diseases. Future studies will extend our knowledge both of the role of intestinal permeability and of associated genetic risk factors in autoimmune diseases in general and CD in particular. Clinical relevance for these studies might be a long way. However, since big steps forward are being made, it might also be just around the corner.

The main findings of this thesis

- IgA-tTG is the best serologic test for diagnosis of coeliac disease and follow up after introduction of a gluten free diet.
- I-FABP can be additive in the follow up of coeliac disease but cannot be used for identifying individuals who require a biopsy to confirm the diagnosis of coeliac disease.
- Routine gluten challenge is not indicated anymore in children younger than 2 years at diagnosis, if clinical symptoms and histological changes (Marsh III[101]) are compatible with coeliac disease in combination with EMA and/or tTG.

- Genetic factors (HLA and suspected permeability genes associated with CD) might be associated with RCD.
- Genetic factors (suspected permeability genes associated with CD) are associated with lower gliadin values instead of elevation thus other immunologic phenomena play a role in Down Syndrome patients.

References

1. McNeish AS, Harms HK, Rey J *et al*. The diagnosis of coeliac disease. A commentary on the current practices of members of the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN). *Arch Dis Child* 1979;**54**(10):783-6.
2. Meeuwisse G.W. Diagnostic criteria in coeliac disease. *Acta Paediatr Scand* 70 A.D.;**59**:461.
3. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. *Arch Dis Child* 1990;**65**(8):909-11.
4. Guandalini S, Ventura A, Ansaldi N *et al*. Diagnosis of coeliac disease: time for a change? *Arch Dis Child* 1989;**64**(9):1320-4.
5. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992;**102**(1):330-54.
6. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. *Arch Dis Child* 1990;**65**(8):909-11.
7. Hill ID, Dirks MH, Liptak GS *et al*. Guideline for the diagnosis and treatment of celiac disease in children: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. *J Pediatr Gastroenterol Nutr* 2005;**40**(1):1-19.
8. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992;**102**(1):330-54.
9. Danielsson L, Stenhammar L, Astrom E. Is gluten challenge necessary for the diagnosis of coeliac disease in young children? *Scand J Gastroenterol* 1990;**25**(9):957-60.
10. Kuitunen P, Savilahti E, Verkasalo M. Late mucosal relapse in a boy with coeliac disease and cow's milk allergy. *Acta Paediatr Scand* 1986;**75**(2):340-2.
11. Shmerling DH, Franckx J. Childhood celiac disease: a long-term analysis of relapses in 91 patients. *J Pediatr Gastroenterol Nutr* 1986;**5**(4):565-9.
12. Hill ID, Dirks MH, Liptak GS *et al*. Guideline for the diagnosis and treatment of celiac disease in children: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. *J Pediatr Gastroenterol Nutr* 2005;**40**(1):1-19.
13. Rostom A, Dube C, Cranney A *et al*. The diagnostic accuracy of serologic tests for celiac disease: a systematic review. *Gastroenterology* 2005;**128**(4 Suppl 1):S38-S46.
14. Volta U, Granito A, Fiorini E *et al*. Usefulness of antibodies to deamidated gliadin peptides in celiac disease diagnosis and follow-up. *Dig Dis Sci* 2008;**53**(6):1582-8.
15. Basso D, Guariso G, Fogar P *et al*. Antibodies against synthetic deamidated gliadin peptides for celiac disease diagnosis and follow-up in children. *Clin Chem* 2009;**55**(1):150-7.
16. Agardh D. Antibodies against synthetic deamidated gliadin peptides and tissue transglutaminase for the identification of childhood celiac disease. *Clin Gastroenterol Hepatol* 2007;**5**(11):1276-81.
17. Derikx JP, Vreugdenhil AC, Van den Neucker AM *et al*. A Pilot Study on the Noninvasive Evaluation of Intestinal Damage in Celiac Disease Using I-FABP and L-FABP. *J Clin Gastroenterol* 2009.

18. Tursi A, Brandimarte G, Giorgetti GM. Lack of usefulness of anti-transglutaminase antibodies in assessing histologic recovery after gluten-free diet in celiac disease. *J Clin Gastroenterol* 2003;**37**(5):387-91.
19. Vahedi K, Mascart F, Mary JY *et al*. Reliability of antitransglutaminase antibodies as predictors of gluten-free diet compliance in adult celiac disease. *Am J Gastroenterol* 2003;**98**(5):1079-87.
20. Collin P, Helin H, Maki M *et al*. Follow-up of patients positive in reticulin and gliadin antibody tests with normal small-bowel biopsy findings. *Scand J Gastroenterol* 1993;**28**(7):595-8.
21. Corazza GR, Andreani ML, Biagi F *et al*. Clinical, pathological, and antibody pattern of latent celiac disease: report of three adult cases. *Am J Gastroenterol* 1996;**91**(10):2203-7.
22. Dickey W, Hughes DF, McMillan SA. Patients with serum IgA endomysial antibodies and intact duodenal villi: clinical characteristics and management options. *Scand J Gastroenterol* 2005;**40**(10):1240-3.
23. Mohamed BM, Feighery C, Coates C *et al*. The absence of a mucosal lesion on standard histological examination does not exclude diagnosis of celiac disease. *Dig Dis Sci* 2008;**53**(1):52-61.
24. Paparo F, Petrone E, Tosco A *et al*. Clinical, HLA, and small bowel immunohistochemical features of children with positive serum antiendomysium antibodies and architecturally normal small intestinal mucosa. *Am J Gastroenterol* 2005;**100**(10):2294-8.
25. Salmi TT, Collin P, Jarvinen O *et al*. Immunoglobulin A autoantibodies against transglutaminase 2 in the small intestinal mucosa predict forthcoming coeliac disease. *Aliment Pharmacol Ther* 2006;**24**(3):541-52.
26. Westerlund A, Ankelo M, Simell S *et al*. Affinity maturation of immunoglobulin A anti-tissue transglutaminase autoantibodies during development of coeliac disease. *Clin Exp Immunol* 2007;**148**(2):230-40.
27. Kaukinen K, Peraaho M, Collin P *et al*. Small-bowel mucosal transglutaminase 2-specific IgA deposits in coeliac disease without villous atrophy: a prospective and randomized clinical study. *Scand J Gastroenterol* 2005;**40**(5):564-72.
28. Koskinen O, Collin P, Korponay-Szabo I *et al*. Gluten-dependent small bowel mucosal transglutaminase 2-specific IgA deposits in overt and mild enteropathy coeliac disease. *J Pediatr Gastroenterol Nutr* 2008;**47**(4):436-42.
29. Westerlund A, Ankelo M, Simell S *et al*. Affinity maturation of immunoglobulin A anti-tissue transglutaminase autoantibodies during development of coeliac disease. *Clin Exp Immunol* 2007;**148**(2):230-40.
30. Quaglia S, Chinello M, Ferrara F *et al*. Non celiac patients anti-tissue Transglutaminase antibodies: immunological and functional characterization. *ICDS Amsterdam* 2009;116.
31. Carroccio A, Iannitto E, Di PL *et al*. Screening for celiac disease in non-Hodgkin's lymphoma patients: a serum anti-transglutaminase-based approach. *Dig Dis Sci* 2003;**48**(8):1530-6.
32. Simell S, Kupila A, Hoppu S *et al*. Natural history of transglutaminase autoantibodies and mucosal changes in children carrying HLA-conferred celiac disease susceptibility. *Scand J Gastroenterol* 2005;**40**(10):1182-91.

33. Salvati VM, Tosco A, Indolfi V. *et al*. Natural history of children with potential celiac disease. *ICDS Amsterdam* 2009;48.
34. Cooper GS, Stroehla BC. The epidemiology of autoimmune diseases. *Autoimmun Rev* 2003;**2**(3):119-25.
35. Raki M, Fallang LE, Brottveit M *et al*. Tetramer visualization of gut-homing gluten-specific T cells in the peripheral blood of celiac disease patients. *Proc Natl Acad Sci U S A* 2007;**%20 ;104**(8):2831-6.
36. Karell K, Louka AS, Moodie SJ *et al*. HLA types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the European Genetics Cluster on Celiac Disease. *Hum Immunol* 2003;**64**(4):469-77.
37. Margaritte-Jeannin P, Babron MC, Bourgey M *et al*. HLA-DQ relative risks for coeliac disease in European populations: a study of the European Genetics Cluster on Coeliac Disease. *Tissue Antigens* 2004;**63**(6):562-7.
38. van Heel DA, Hunt K, Greco L *et al*. Genetics in coeliac disease. Best Pract Res Clin *Gastroenterol* 2005;**19**(3):323-39.
39. Jones EY, Fugger L, Strominger JL *et al*. MHC class II proteins and disease: a structural perspective. *Nat Rev Immunol* 2006;**6**(4):271-82.
40. Molberg O, Uhlen AK, Jensen T *et al*. Mapping of gluten T-cell epitopes in the bread wheat ancestors: implications for celiac disease. *Gastroenterology* 2005;**128**(2):393-401.
41. van de WY, Kooy YM, van VP *et al*. Glutenin is involved in the gluten-driven mucosal T cell response. *Eur J Immunol* 1999;**29**(10):3133-9.
42. van de WY, Kooy YM, Drijfhout JW *et al*. Peptide binding characteristics of the coeliac disease-associated DQ(alpha1*0501, beta1*0201) molecule. *Immunogenetics* 1996;**44**(4):246-53.
43. Greco L, Romino R, Coto I *et al*. The first large population based twin study of coeliac disease. *Gut* 2002;**50**(5):624-8.
44. Nistico L, Fagnani C, Coto I *et al*. Concordance, disease progression, and heritability of coeliac disease in Italian twins. *Gut* 2006;**55**(6):803-8.
45. Greco L, Corazza G, Babron MC *et al*. Genome search in celiac disease. *Am J Hum Genet* 1998;**62**(3):669-75.
46. Greco L, Babron MC, Corazza GR *et al*. Existence of a genetic risk factor on chromosome 5q in Italian coeliac disease families. *Ann Hum Genet* 2001;**65**(Pt 1):35-41.
47. Holopainen P, Naluai AT, Moodie S *et al*. Candidate gene region 2q33 in European families with coeliac disease. *Tissue Antigens* 2004;**63**(3):212-22.
48. Liu J, Juo SH, Holopainen P *et al*. Genomewide linkage analysis of celiac disease in Finnish families. *Am J Hum Genet* 2002;**70**(1):51-9.
49. van Belzen MJ, Meijer JW, Sandkuijl LA *et al*. A major non-HLA locus in celiac disease maps to chromosome 19. *Gastroenterology* 2003;**125**(4):1032-41.
50. van Belzen MJ, Vrolijk MM, Meijer JW *et al*. A genomewide screen in a four-generation Dutch family with celiac disease: evidence for linkage to chromosomes 6 and 9. *Am J Gastroenterol* 2004;**99**(3):466-71.
51. Woolley N, Holopainen P, Ollikainen V *et al*. A new locus for coeliac disease mapped to chromosome 15 in a population isolate. *Hum Genet* 2002;**111**(1):40-5.

52. King AL, Moodie SJ, Fraser JS *et al*. CTLA-4/CD28 gene region is associated with genetic susceptibility to coeliac disease in UK families. *J Med Genet* 2002;**39**(1):51-4.
53. Babron MC, Nilsson S, Adamovic S *et al*. Meta and pooled analysis of European coeliac disease data. *Eur J Hum Genet* 2003;**11**(11):828-34.
54. Percopo S, Babron MC, Whalen M *et al*. Saturation of the 5q31-q33 candidate region for coeliac disease. *Ann Hum Genet* 2003;**67**(Pt 3):265-8.
55. Wapenaar MC, Monsuur AJ, van Bodegraven AA *et al*. Associations with tight junction genes PARD3 and MAGI2 in Dutch patients point to a common barrier defect for coeliac disease and ulcerative colitis. *Gut* 2008;**57**(4):463-7.
56. Monsuur AJ, de Bakker PI, Alizadeh BZ *et al*. Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat Genet* 2005;**37**(12):1341-4.
57. Babron MC, Nilsson S, Adamovic S *et al*. Meta and pooled analysis of European coeliac disease data. *Eur J Hum Genet* 2003;**11**(11):828-34.
58. Greco L, Corazza G, Babron MC *et al*. Genome search in celiac disease. *Am J Hum Genet* 1998;**62**(3):669-75.
59. Greco L, Babron MC, Corazza GR *et al*. Existence of a genetic risk factor on chromosome 5q in Italian coeliac disease families. *Ann Hum Genet* 2001;**65**(Pt 1):35-41.
60. Liu J, Juo SH, Holopainen P *et al*. Genomewide linkage analysis of celiac disease in Finnish families. *Am J Hum Genet* 2002;**70**(1):51-9.
61. Naluai AT, Nilsson S, Gudjonsdottir AH *et al*. Genome-wide linkage analysis of Scandinavian affected sib-pairs supports presence of susceptibility loci for celiac disease on chromosomes 5 and 11. *Eur J Hum Genet* 2001;**9**(12):938-44.
62. Percopo S, Babron MC, Whalen M *et al*. Saturation of the 5q31-q33 candidate region for coeliac disease. *Ann Hum Genet* 2003;**67**(Pt 3):265-8.
63. Holopainen P, Naluai AT, Moodie S *et al*. Candidate gene region 2q33 in European families with coeliac disease. *Tissue Antigens* 2004;**63**(3):212-22.
64. Naluai AT, Nilsson S, Samuelsson L *et al*. The CTLA4/CD28 gene region on chromosome 2q33 confers susceptibility to celiac disease in a way possibly distinct from that of type 1 diabetes and other chronic inflammatory disorders. *Tissue Antigens* 2000;**56**(4):350-5.
65. Popat S, Hearle N, Hogberg L *et al*. Variation in the CTLA4/CD28 gene region confers an increased risk of coeliac disease. *Ann Hum Genet* 2002;**66**(Pt 2):125-37.
66. Rioux JD, Karinen H, Kocher K *et al*. Genomewide search and association studies in a Finnish celiac disease population: Identification of a novel locus and replication of the HLA and CTLA4 loci. *Am J Med Genet A* 2004;**130**(4):345-50.
67. Babron MC, Nilsson S, Adamovic S *et al*. Meta and pooled analysis of European coeliac disease data. *Eur J Hum Genet* 2003;**11**(11):828-34.
68. Hunt KA, Zhernakova A, Turner G *et al*. Newly identified genetic risk variants for celiac disease related to the immune response. *Nat Genet* 2008;**40**(4):395-402.
69. van Heel DA, Franke L, Hunt KA *et al*. A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. *Nat Genet* 2007;**39**(7):827-9.
70. Adamovic S, Amundsen SS, Lie BA *et al*. Association study of IL2/IL21 and FcgRIIa: significant association with the IL2/IL21 region in Scandinavian coeliac disease families. *Genes Immun* 2008;**9**(4):364-7.

71. Hunt KA, Zhernakova A, Turner G *et al*. Newly identified genetic risk variants for celiac disease related to the immune response. *Nat Genet* 2008;**40**(4):395-402.
72. Todd JA, Walker NM, Cooper JD *et al*. Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. *Nat Genet* 2007;**39**(7):857-64.
73. Zhernakova A, Alizadeh BZ, Bevova M *et al*. Novel association in chromosome 4q27 region with rheumatoid arthritis and confirmation of type 1 diabetes point to a general risk locus for autoimmune diseases. *Am J Hum Genet* 2007;**81**(6):1284-8.
74. Trynka G, Zhernakova A, Romanos J *et al*. Coeliac disease associated risk variants in TNFAIP3 and REL implicate altered NF-{kappa}B signalling. *Gut* 2009, in press.
75. Monsuur AJ, de Bakker PI, Alizadeh BZ *et al*. Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat Genet* 2005;**37**(12):1341-4.
76. Wapenaar MC, Monsuur AJ, van Bodegraven AA *et al*. Associations with tight junction genes PARD3 and MAGI2 in Dutch patients point to a common barrier defect for coeliac disease and ulcerative colitis. *Gut* 2008;**57**(4):463-7.
77. Izumi Y, Hirose T, Tamai Y *et al*. An atypical PKC directly associates and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of Caenorhabditis elegans polarity protein PAR-3. *J Cell Biol* 1998;**143**(1):95-106.
78. Post PL, Tyska MJ, O'Connell CB *et al*. Myosin-IXb is a single-headed and processive motor. *J Biol Chem* 2002;**277**(14):11679-83.
79. Wu X, Hepner K, Castelino-Prabhu S *et al*. Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2. *Proc Natl Acad Sci U S A* 2000;**97**(8):4233-8.
80. Monsuur AJ, de Bakker PI, Alizadeh BZ *et al*. Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat Genet* 2005;**37**(12):1341-4.
81. Wapenaar MC, Monsuur AJ, van Bodegraven AA *et al*. Associations with tight junction genes PARD3 and MAGI2 in Dutch patients point to a common barrier defect for coeliac disease and ulcerative colitis. *Gut* 2008;**57**(4):463-7.
82. Wapenaar MC, Monsuur AJ, van Bodegraven AA *et al*. Associations with tight junction genes PARD3 and MAGI2 in Dutch patients point to a common barrier defect for coeliac disease and ulcerative colitis. *Gut* 2008;**57**(4):463-7.
83. Rioux JD, Silverberg MS, Daly MJ *et al*. Genomewide search in Canadian families with inflammatory bowel disease reveals two novel susceptibility loci. *Am J Hum Genet* 2000;**66**(6):1863-70.
84. van Heel DA, Dechairo BM, Dawson G *et al*. The IBD6 Crohn's disease locus demonstrates complex interactions with CARD15 and IBD5 disease-associated variants. *Hum Mol Genet* 2003;**12**(20):2569-75.
85. Concannon P, Chen WM, Julier C *et al*. Genome-wide scan for linkage to type 1 diabetes in 2,496 multiplex families from the Type 1 Diabetes Genetics Consortium. *Diabetes* 2009;**58**(4):1018-22.
86. Hollander D, Vadheim CM, Brettholz E *et al*. Increased intestinal permeability in patients with Crohn's disease and their relatives. A possible etiologic factor. *Ann Intern Med* 1986;**105**(6):883-5.

87. May GR, Sutherland LR, Meddings JB. Is small intestinal permeability really increased in relatives of patients with Crohn's disease? *Gastroenterology* 1993;**104**(6):1627-32.
88. Scott FW. Food-induced type 1 diabetes in the BB rat. *Diabetes Metab Rev* 1996;**12**(4):341-59.
89. Picco P, Gattorno M, Marchese N *et al*. Increased gut permeability in juvenile chronic arthritides. A multivariate analysis of the diagnostic parameters. *Clin Exp Rheumatol* 2000;**18**(6):773-8.
90. Hunt KA, Zhernakova A, Turner G *et al*. Newly identified genetic risk variants for celiac disease related to the immune response. *Nat Genet* 2008;**40**(4):395-402.
91. van Heel DA, Franke L, Hunt KA *et al*. A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. *Nat Genet* 2007;**39**(7):827-9.
92. Todd JA, Walker NM, Cooper JD *et al*. Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. *Nat Genet* 2007;**39**(7):857-64.
93. Zhernakova A, Alizadeh BZ, Bevova M *et al*. Novel association in chromosome 4q27 region with rheumatoid arthritis and confirmation of type 1 diabetes point to a general risk locus for autoimmune diseases. *Am J Hum Genet* 2007;**81**(6):1284-8.
94. Hill ID, Dirks MH, Liptak GS *et al*. Guideline for the diagnosis and treatment of celiac disease in children: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. *J Pediatr Gastroenterol Nutr* 2005;**40**(1):1-19.
95. Romanos J, van Diemen CC, Nolte IM *et al*. Analysis of HLA and non-HLA alleles can identify individuals at high risk for celiac disease. *Gastroenterology* 2009.
96. Weersma RK, Stokkers PC, van Bodegraven AA *et al*. Molecular prediction of disease risk and severity in a large Dutch Crohn's disease cohort. *Gut* 2009;**58**(3):388-95.
97. Hunt KA, van Heel DA. Recent advances in coeliac disease genetics. *Gut* 2009;**58**(4):473-6.
98. Troncone R, Ivarsson A, Szajewska H *et al*. Review article: future research on coeliac disease - a position report from the European multistakeholder platform on coeliac disease (CDEUSSA). *Aliment Pharmacol Ther* 2008;**27**(11):1030-43.
99. Kraft P, Wacholder S, Cornelis MC *et al*. Beyond odds ratios--communicating disease risk based on genetic profiles. *Nat Rev Genet* 2009;**10**(4):264-9.
100. Hill ID, Dirks MH, Liptak GS *et al*. Guideline for the diagnosis and treatment of celiac disease in children: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. *J Pediatr Gastroenterol Nutr* 2005;**40**(1):1-19.
101. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992;**102**(1):330-54.

Chapter 9

Summary

Coeliac disease (CD) is an auto-immune disease triggered by ingestion of gluten, a major protein in wheat, rye and barley. It is the most common food intolerance in the Western population with a prevalence of 1%. In susceptible individuals the ingestion of gluten triggers an auto-immune reaction, giving chronic inflammation of the small intestinal mucosa, and generally resulting in villous atrophy. This causes malabsorption of nutrients and subsequently leads to symptoms such as diarrhea, growth retardation, osteoporosis, and fatigue. There is a strong genetic predisposition. CD patients have inherited a mix of genes that results in an increased likelihood of an aberrant immune response to gluten. The most important genetic factor identified is HLA-DQ2 and /or DQ8, which is present in almost all CD patients. However it is not the sole cause of the immune response to gluten in CD patients as 35% of the general population also carries this HLA genotype. Treatment consists of a lifelong gluten free diet (GFD). After its introduction almost all patients experience remission.

Although CD is a disease that generally can be treated successfully, coming to a correct diagnosis is not straightforward. Traditionally, the (typical) diagnosis of CD is made in patients with clinical symptoms, positive serology and total villous atrophy (Marsh III) on intestinal biopsy. Confirmation of the diagnosis with an intestinal biopsy is required in all cases and is considered the 'gold standard'. In the international guidelines a subsequent gluten challenge with additional intestinal biopsies was required for patients younger than 2 years of age at initial diagnosis. Since intestinal biopsy in children requires anaesthesia and is an invasive procedure, the ideal situation would be to improve the sensitivity and specificity of serological tests so that intestinal biopsy is no longer necessary. Therefore, in the first part of this thesis the reliability of new serological tests are investigated, as well as the yield of the gluten challenge in young children.

The second part of this thesis focuses on the role of genetics in the pathogenesis of CD. The strongest genetic factor associated with CD is HLA-DQ2 and -DQ8 and is found in virtually all CD patients. Twin and family studies indicate that non-HLA genes also contribute to the development of CD. Several genetic linkage studies have identified different susceptibility loci on various chromosomes revealing the complexity of CD. Recently, three genetic risk variants for CD (*MYO9B*, *MAGI2* and *PARD3*) were identified that might have a role in intestinal permeability and more specifically in tight junction function. These three 'permeability' genes were shown to be associated with CD, but also with other autoimmune diseases, suggesting a shared pathogenesis. This is an intriguing field and might give more insight into the complex and overlapping pathogenesis between CD and other autoimmune disorders. This thesis focused on these three ´permeability´ genes and investigated their role in different populations and tried to elucidate whether these factors might indeed influence intestinal permeability.

This thesis starts with an overview of CD **(chapter 2),** including the history of CD, pathogenesis, clinical characteristics, diagnosis and genetic factors. We highlight the development of

a treatment for CD by the Dutch paediatrician Willem Karel Dicke. He showed that exclusion of gluten from wheat and rye resulted in a dramatic improve of the condition of children with CD. Nowadays this gluten free diet (GFD) is still the only and well-working treatment for CD. On a GFD the symptoms of CD and the histological changes in the intestinal mucosa disappear in the vast majority of patients. We subsequently describe that in the presentation of CD the classical gastrointestinal malabsorption syndrome is still prevalent but that nowadays more patients are diagnosed with non-specific symptoms such as anemia, osteoporosis, or abdominal discomfort. IgA-anti-endomysium (IgA-EMA) and IgA-anti tissue transglutaminase (IgA-tTG) antibodies are the first diagnostic modalities in the screening for CD. However a small bowel biopsy remains the gold standard in the diagnosis of CD. Our understanding of the key steps underlying the intestinal inflammatory response in CD has increased dramatically in recent years. Gluten proteins provoke the disease in susceptible individuals as the high proline content of gluten makes this protein relatively resistant to proteolytic digestion in the intestinal tract. The undigested gluten peptides are deamidated by tissue transglutaminase, which results in a better binding capacity to HLA-DQ2 or HLA-DQ8 molecules on antigen-presenting cells. The gluten peptides are subsequently presented to CD4+ T cells and the ensuing immune response causes inflammation and intestinal tissue damage. A direct response of the epithelium via the innate immune system also plays a role. The disease is strongly linked to HLA-DQ2 and HLA-DQ8 and absence of this HLA type virtually rules out CD. Non-HLA genes are involved as well in the pathogenesis of CD. As the contribution of each of these non-HLA genes might be quite modest, it is quite difficult to identify these factors.

Part 1 Diagnostics

In **chapter 3** the sensitivity and specificity of serum antibodies against tissue transglutaminase (IgA-tTG) were compared with serum antigliadin antibodies (IgA-AGA and IgG-AGA) and anti-endomysium antibodies (IgA-EMA) in the diagnosis of CD in a group of patients and controls who were all biopsied. A total of 52 patients with CD and 49 patients without CD were investigated. Human IgA-tTG had a sensitivity of 96% and a specificity of 100%. Guinea pig IgA-tTG had a sensitivity of 96% and a specificity of 92%. IgA-EMA had a sensitivity of 92% and a specificity of 90%. Both IgA-AGA and IgG-AGA had a sensitivity of 83% and a specificity of 86% and 80% respectively. Thus, human IgA-tTG is currently the serological method of choice in identifying patients with CD in the absence of IgA deficiency.

Subsequently in **chapter 4** it was investigated whether the serum level of intestinal fatty acid binding protein (I-FABP) can be of additional value in the diagnosis and follow-up of CD. I-FABP is a small cytosolic enterocyte protein and a newly developed non-invasive marker for intestinal mucosal damage. Serum I-FABP levels were analyzed in 49 children with biopsy proven CD and in 19 patients without the histological characteristics matching CD. All had elevated serum IgA-tTG and IgA-EMA antibodies. Serum I-FABP levels were significantly elevated ($p<0.001$) in children with biopsy proven CD compared to controls and I-FABP concentrations correlated significantly ($p<0.003$) with the severity of villous atrophy. In the group of patients with villous atrophy 40/49 (82%) patients had a serum I-FABP level above

the upper level of normal, while in the control group only 2 of 19 children were found to have elevated I-FABP levels, in whom one subsequently was diagnosed with CD after a gluten challenge. So in this group of patients in only 1/68 patients an erroneous diagnosis of CD would have been made when using serum levels of IgA-tTG/IgA-EMA in combination with I-FABP.
In all CD patients I-FABP levels decreased quickly after gluten free diet (GFD) and normalized in 80% of patients within 12 weeks, which was faster than the normalization of serum IgA-tTG. Thus, I-FABP improves the reliability of the current serologic parameters (IgA-tTG and IgA-EMA) in the diagnosis of CD and in monitoring the response to GFD.

In **chapter 5** the diagnostic yield of routine gluten challenges in young children was investigated. The ESPGHAN criteria state that a gluten challenge is needed to confirm the diagnosis in every child diagnosed under the age of two years. This might be an unnecessary burden to this group of children so we investigated 100 children in whom CD was diagnosed under the age of 2 years, and in whom a gluten challenge was performed. In 97 patients the diagnosis was confirmed, so looking back, the gluten challenge had not been necessary in these patients. Retrospectively, in two of the three children without mucosal relapse, data available before gluten challenge did not justify the initial diagnosis of CD. In the third patient transient gluten intolerance could not be excluded. At first biopsy, the two children without mucosal relapse had negative serologic parameters, while the third patient had IgA anti-gliadin antibodies, but no IgA anti-endomysium antibodies (IgA-EMA). Indeed all patients with a raised serum IgA-EMA at diagnosis had a mucosal relapse at gluten challenge. We could conclude that routine gluten challenge in the group of patients diagnosed under the age of 2 years is not necessary when they have villous atrophy at intestinal biopsy in combination with a raised serum IgA-EMA. However, gluten challenge remains useful in children with equivocal histological changes, conflicting serological test results, incomplete disappearance of symptoms or no improvement of serology during gluten-free diet.

Part 2 Genetics

CD is a complex genetic disorder with multiple contributing genes. In **chapter 6** an overview of the recent genetic developments is given with its implications for clinical practice. The most important genetic factors identified are HLA-DQ2 and HLA-DQ8, which are necessary but not sufficient for the development of CD. The main role of HLA typing in clinical practice lies in its high negative predictive value to exclude CD (close to 100%). The associations found in non-HLA genome-wide linkage and association studies are much weaker. This might be caused by a large number of non-HLA genes contributing to the pathogenesis of CD. Hence, the contribution of a single predisposing non-HLA gene might be quite modest. In the future, genetic risk profiles for CD, using multiple non-HLA genes in addition to HLA, might be helpful in clinical practice for predicting disease susceptibility and progression. However, it may take a considerable amount of time before we can perform truly informative genetic risk profiles for CD.

In **chapter 7** it was investigated whether the newly found *MYO9B* gene is a risk factor for the development of refractory coeliac disease (RCD) type II and enteropathy-associated T-cell lymphoma (EATL). CD has been linked to genetic variants in the *MYO9B* gene on chromosome 19. Genotyping of *MYO9B* and molecular HLA-DQ2 typing was performed on 62 RCD II and EATL patients, 421 uncomplicated CD patients, and 1624 controls. One SNP in *MYO9B* (rs7259292) showed a significantly different allele distribution in RCD II and EATL patients compared to controls (p=0.00002). The rs7259292 T allele was significantly more frequent in RCD II and EATL patients compared to CD patients (p=0.0003, OR 3.61 (95% CI 1.78-7.31)). The frequency of the haplotype carrying the T allele of this SNP was significantly increased in RCD II and EATL patients (11%), compared to controls (2%) and CD patients (3%) (OR 6.76 (95% CI 3.40–13.46), p=2.27E-09 and OR 4.22 (95% CI 1.95–9.11) p=0.0001, respectively). Both *MYO9B* rs7259292 and HLA-DQ2 homozygosity increased the risk for RCD II and EATL to a similar extent when compared to uncomplicated CD patients (OR 4.3 (95% CI 1.9–9.8) and 5.4 (95% CI 3.0–9.6), respectively), but there was no evidence for any interaction between these two risk factors. Both *MYO9B* and HLA-DQ2 homozygosity might be involved in the prognosis of CD and the chance of developing RCD II and EATL.

In **chapter 8** three CD associated genes (*MYO9B*, *MAGI2* and *PARD3*) were studied. These genes might encode tight junction (TJ) proteins and thus have a role in regulating intestinal permeability, but evidence for this function is far from complete. In selected populations with a supposed increased intestinal permeability this hypothesis might be tested further, e.g. Down syndrome (DS) patients. Up to 20% of DS patients have increased levels of AGA without CD, far more than the general population. These elevated AGA levels might be the consequence of an increased intestinal permeability. It was hypothesized that the suspected CD-associated permeability genes are associated with an increased intestinal permeability as revealed by elevated antibodies against food allergens such as gliadin (AGA) and baker's yeast (ASCA). Furthermore, correlations between different food allergens (AGA and baker´s yeast (ASCA)) were analyzed. A total of 126 patients were genotyped for six SNPs: rs1457092 and rs2305764 in *MYO9B*; rs10763976 and rs6962966 in *MAGI2;* and rs9640699 and rs1496770 in *PARD3*, all associated with the development of CD. An allele dosage association of genetic variations in these tight junction genes and the levels of AGA antibodies was performed. There was a strong correlation between AGA and ASCA (p<0.01). The group of patients with one or more risk genotypes had lower mean AGA levels (trend test P=0.007). This group also consisted of a significantly larger number of patients with normal AGA levels (P=9.3x10-5). We consistently found that AGA production was suppressed instead of increased for a CD associated risk genotype. These results are in contrast with our initial hypothesis that the level of food antigens would be increased in patients with *MYO9B, MAGI2* and *PARD3* variants predisposing for CD. This might be due to different immunological mechanisms in DS patients as a result of chromosomal aberrations, possibly involving altered induction and/or maintenance of tolerance.

The field of genetics in CD and other autoimmune diseases is moving very rapid. Multiple genes seem to contribute to CD and to each of the major autoimmune disorders, with significant genetic overlap between them. However we are only beginning to unveil the (patho) genetics of these diseases. Future studies will extend our knowledge both of the role of intestinal permeability and of associated genetic risk factors in CD and in other autoimmune diseases.

Chapter 9

Summary in Dutch
Nederlandse samenvatting

Coeliakie is een veel voorkomende ziekte die wordt veroorzaakt door een intolerantie voor gluten, een belangrijk bestanddeel van tarwe, rogge en gerst. Het is een multifactoriële aandoening waarbij naast gluten ook erfelijke aanleg een belangrijke rol speelt. Bij coeliakie patiënten lokt dit na inname van gluten een auto-immuun reactie uit, met als gevolg een chronische ontsteking van de dunne darm en vaak ook vlokatrofie. Hierdoor kunnen voedingsstoffen minder goed worden opgenomen en dit kan leiden tot symptomen als diarree, groeiachterstand, botontkalking en moeheid.
Coeliakie patiënten hebben een combinatie van genen geërfd waardoor zij een verhoogde kans hebben op een afwijkende afweerreactie tegen gluten. De belangrijkste component van deze erfelijke aanleg wordt gevormd door aanwezigheid van HLA-DQ2 en/of -DQ8 op de antigeen presenterende cellen. Vrijwel alle coeliakie patiënten zijn in het bezit van HLA-DQ2 en/of -DQ8 en afwezigheid hiervan sluit coeliakie zo goed als uit. Echter dit is niet de enige erfelijke factor die van belang is bij het ontstaan van coeliakie, aangezien bij 35% van de bevolking het HLA-DQ2 en/of -DQ8 aanwezig is, terwijl slechts bij enkele procenten van deze groep coeliakie ontstaat. De behandeling bestaat uit een levenslang glutenvrij dieet. Bij vrijwel alle coeliakie patiënten verdwijnen de symptomen en herstelt de darm zich na de start van het glutenvrije dieet.

Coeliakie kan goed behandeld worden maar het stellen van de diagnose is niet altijd even gemakkelijk. De ´typische´ coeliakie patiënt presenteert zich met klinische symptomen, positieve serologie en vlokatrofie (Marsh III) in het dunne darm biopt. De diagnose is echter pas definitief als door middel van een dunne darm biopt deze vlokatrofie ook daadwerkelijk is aangetoond. Daarnaast wordt in de internationale richtlijnen aanbevolen om bij alle patiënten die jonger dan 2 jaar waren bij diagnose een glutenbelasting met dunne darmbiopsiën uit te voeren. Een dunne darmbiopsie is een invasieve procedure waarvoor bij kinderen anesthesie noodzakelijk is. Idealiter zou men de diagnose daarom willen stellen zonder deze belastende dunne darmbiopsie. Om die reden wordt in het eerste deel van dit proefschrift de betrouwbaarheid van nieuwe serologische testen onderzocht en verder of het doen van een glutenbelasting bij jonge kinderen wel zinvol is.

Het tweede deel van het proefschrift is gericht op de rol van genetische factoren bij de pathogenese van coeliakie. De sterkst met coeliakie geassocieerde genetische factor is HLA-DQ2 en -DQ8 en deze factor wordt bij vrijwel alle coeliakie patiënten gevonden. Tweelingen familiestudies laten zien dat er ook niet-HLA gebonden genen betrokken moeten zijn bij het ontstaan van coeliakie. Verscheidene genetische studies hebben inmiddels ook op verschillende chromosomen dit soort gebieden geïdentificeerd. Meer specifiek zijn er recent drie genetische factoren gevonden die met coeliakie geassocieerd zijn (*MYO9B, MAGI2* en *PARD3*) en die allen mogelijk een rol spelen bij darmdoorlaatbaarheid. Zij lijken met name betrokken bij het 'bij elkaar houden' van epitheelcellen door middel van ´tight junctions´.

Deze drie 'darmdoorlaatbaarheid' genen blijken niet alleen met coeliakie geassocieerd maar ook met andere auto-immuun ziekten, wat een deels zelfde pathogenese zou suggeren. Dit is intrigerend en zou ons mogelijk meer inzicht kunnen geven in de complexe- en overlappende pathogenese van coeliakie en andere auto-immuunziekten. In dit proefschrift wordt de rol van deze drie 'darmdoorlaatbaarheid' genen onderzocht bij patiënten met coeliakie welke niet op een glutenvrij dieet reageren en wordt in een andere populatie (bij patiënten met Down syndroom) gekeken of deze factoren de darmdoorlaatbaarheid inderdaad beïnvloeden.

Dit proefschrift begint met een overzichtsartikel (**hoofdstuk 2**) waarin de geschiedenis van coeliakie, de pathogenese, de klinische kenmerken, de diagnose en de betrokken genetische factoren, voor zover bekend, worden beschreven. De ontdekking van het glutenvrije dieet door de Nederlandse kinderarts Willem Karel Dicke wordt uitgelicht. Hij toonde aan dat een dieet zonder tarwe en rogge (dus zonder gluten) een dramatische verbetering gaf van de conditie van kinderen met coeliakie. Dit glutenvrije dieet is nog steeds de enige beschikbare behandeling voor coeliakie. Op een glutenvrij dieet verbeteren de klachten en ook de histologische afwijkingen in de darmmucosa verdwijnen bij vrijwel alle patiënten.
Vervolgens beschrijven we dat de klassieke presentatie met gastro-intestinale symptomen ten gevolge van malabsorptie nog steeds veel voorkomt, maar dat ook steeds meer patiënten worden gediagnosticeerd met aspecifieke symptomen zoals anemie, klachten die zouden kunnen passen bij een prikkelbare darm en osteoporose. Antilichamen tegen IgA-anti-endomysium (IgA-EMA) en IgA-anti tissue transglutaminase (IgA-tTG) zijn de eerste stap bij de screening van coeliakie. Echter, een dunne darmbiopsie is nog steeds de gouden standaard bij de diagnose van coeliakie. De afgelopen jaren is er een enorme vooruitgang geboekt in het ontrafelen van de oorzaken die tot de darmontsteking bij coeliakie leiden. Gluten veroorzaken de ziekte bij genetisch gevoelige personen doordat de hoge proline concentratie in gluten dit eiwit relatief resistent maakt tegen de eiwitafbrekende enzymen in de dunne darm. De onverteerde gluten eiwitten worden vervolgens gedeamineerd door het enzym tissue transglutaminase (tTG) in de darmmucosa wat resulteert in een sterkere bindingscapaciteit aan HLA-DQ2 en -DQ8 op de antigeen presenterende cellen. De gluten eiwitten worden vervolgens gepresenteerd aan de CD4+ T cellen wat een immuun respons veroorzaakt met als gevolg darmontsteking en darmschade. In afwezigheid van HLA-DQ2 en -DQ8 treedt deze reactie niet op en wordt de diagnose coeliakie vrijwel nooit gesteld. Niet-HLA genen zijn ook betrokken bij het ontstaan van coeliakie, echter deze factoren lijken moeilijker op te sporen aangezien elk individueel gen maar voor een klein deel bijdraagt aan de ontwikkeling van coeliakie.

Deel 1 Diagnostische factoren

In **hoofdstuk 3** worden de sensitiviteit en specificiteit van serum antistoffen tegen tissue transglutaminase (IgA-tTG) vergeleken met serum antigliadine antistoffen (IgA-AGA en IgG-AGA) en anti-endomysium antistoffen (IgA-EMA) bij het stellen van de diagnose coeliakie. In totaal werden 52 patiënten met coeliakie en 49 patiënten zonder coeliakie onderzocht, waarbij allen een dunne darm biopt hadden ondergaan. De sensitiviteit van humaan IgA-tTG was 96% en de specificiteit 100%. De sensitiviteit van cavia IgA-tTG was 96% en de specificiteit 92%. De sensitiviteit van IgA-EMA was 92% en de specificiteit 90%. Zowel IgA-AGA als IgG-AGA hadden beiden een sensitiviteit van 83% met respectievelijk een specificiteit van 86% en 80%. Op basis van dit onderzoek konden wij concluderen dat de bepaling van antistoffen tegen humaan IgA-tTG de beste methode voor de screening van coeliakie is, althans in de afwezigheid van IgA deficiëntie.

Vervolgens wordt in **hoofdstuk 4** onderzocht of de serum concentratie van intestinaal vetzuur bindend eiwit (intestinal fatty acid binding protein (I-FABP)) van toegevoegde waarde kan zijn bij de diagnose en follow up van coeliakie. I-FABP is een klein eiwit in het cytosol van de darmcel en recent werd ontdekt dat dit een goede niet-invasieve marker is voor mucosa schade in de darm. In hoofdstuk 4 werd daarom de serum concentratie van I-FABP geanalyseerd bij 49 kinderen met middels biopsie bewezen coeliakie en bij 19 kinderen zonder histologische afwijkingen in de darmmucosa die op coeliakie wezen. Alle onderzochte kinderen hadden verhoogde concentraties van serum IgA-tTG en IgA-EMA. De I-FABP concentraties waren significant hoger ($p<0.001$) bij kinderen met een middels biopsie bevestigde coeliakie in vergelijking met controle patiënten. Verder was er een significant verband tussen de I-FABP concentraties en de ernst van de vlokatrofie ($p<0.003$). In de groep patiënten met vlokatrofie was de I-FABP concentratie verhoogd bij 40 van de 49 patienten terwijl in de controle groep de I-FABP concentratie slechts bij 2 van de 19 kinderen verhoogd was. Eén van deze twee kinderen bleek na een glutenbelasting vervolgens toch coeliakie te hebben. Dus in deze groep zou slechts bij één kind een verkeerde diagnose zijn gesteld wanneer de combinatie van een positieve IgA-tTG, IgA-EMA en I-FABP zou zijn gebruikt voor de diagnose van coeliakie.
Bij alle coeliakie patiënten daalde de I-FABP concentratie snel na de start van het glutenvrij dieet en normaliseerde de waarde binnen 12 weken bij 80% van de patienten, wat sneller was dan de normalisatie van het serum IgA-tTG. Dus, I-FABP lijkt een goede niet-invasieve marker voor mucosaschade in de dunne darm en van toegevoegde waarde bij zowel diagnostiek als follow up van coeliakie.

In **hoofdstuk 5** wordt het nut van het routinematig verrichten van glutenbelastingen onderzocht bij jonge kinderen. De ESPGHAN richtlijn adviseert om dit ter bevestiging van de diagnose te doen bij ieder kind gediagnosticeerd onder de 2 jaar. Dit is wellicht niet nodig en zou een onnodige belasting kunnen zijn. Daarom werden 100 kinderen onderzocht bij wie de diagnose coeliakie was gesteld onder de 2 jaar en bij wie een glutenbelasting was verricht. Bij 97 kinderen werd de diagnose bevestigd en was achteraf gezien de glutenbelasting niet nodig geweest. Drie kinderen hadden geen afwijkingen in het dunne darmbiopt na de

glutenbelasting. Retrospectief bleek dat bij twee van deze drie kinderen de initiële diagnose op oneigenlijke gronden was gesteld. Bij de derde patiënt kon een tijdelijke gluten intolerantie niet worden uitgesloten. Twee van de drie kinderen zonder afwijkend biopt na glutenbelasting hadden een negatieve serologie ten tijde van het initiële biopt en de derde patiënt had alleen IgA anti-gliadine antistoffen, maar negatieve IgA anti-endomysium antistoffen (IgA-EMA). Alle patiënten met een verhoogde IgA-EMA bij diagnose hadden na glutenbelasting inderdaad coeliakie. Derhalve kunnen we concluderen dat het routinematig verrichten van glutenbelastingen in de groep van patiënten gediagnosticeerd onder de 2 jaar niet geïndiceerd is wanneer er sprake is van vlokatrofie in het initiële dunne darm biopt in combinatie met verhoogde IgA-EMA. Glutenbelasting is wel zinvol bij kinderen met onduidelijke histologische veranderingen in het dunne darmbiopt, (deels) negatieve serologie of indien de kliniek en serologie onvoldoende verbeteren na start van het glutenvrije dieet.

Deel 2 Genetische factoren

Coeliakie is een multifactoriële ziekte waarbij erfelijke aanleg een belangrijke rol speelt en verschillende genen betrokken zijn. In **hoofdstuk 6** wordt een overzicht gegeven van de recente genetische ontwikkelingen en wat de implicaties hiervan zijn voor de dagelijkse praktijk. De belangrijkste genetische factor is het HLA-DQ2 en -DQ8. Afwezigheid hiervan sluit de diagnose coeliakie vrijwel uit. Aanwezigheid van het HLA-DQ2 of -DQ8 kan leiden tot de ontwikkeling van coeliakie maar doet dit bij het overgrote deel van de dragers niet. Dus HLA typering is zinvol om coeliakie uit te sluiten gezien de hoge negatieve voorspellende waarde (bijna 100%), maar niet om coeliakie aan te tonen. Daarnaast lijken er grote aantallen niet-HLA genen betrokken te zijn bij de pathogenese van coeliakie, waarbij de bijdrage van iedere aparte genvariant op het ontstaan van coeliakie waarschijnlijk klein is. In de toekomst zullen genetische risicoprofielen voor coeliakie, bestaand uit zowel HLA- als niet-HLA genen, kunnen helpen om uit te maken welke patiënten een verhoogde kans hebben op het ontwikkelen van coeliakie en het ontwikkelen van complicaties van de ziekte.

In **hoofdstuk 7** wordt onderzocht of genvarianten in het MYO9B gen een risicofactor kunnen zijn voor de ontwikkeling van refractaire coeliakie (RCD) type II en enteropathie-geassocieerd T-cel lymfoom (EATL). Genotypering van MYO9B en moleculaire HLA-DQ2 typing werd verricht bij 62 RCD II en EATL patiënten, 421 ongecompliceerde coeliakie patiënten en 1624 controles. Een bepaalde genvariant in *MYO9B* (rs7259292 T allel) was significant vaker aanwezig bij RCD II en EATL patiënten in vergelijking met controles (p=0.00002). Het rs7259292 T allel kwam significant vaker voor bij RCD II en EATL patiënten in vergelijking met coeliakie patiënten (p=0.0003, OR 3.61 (95% CI 1.78-7.31)). De frequentie van het haplotype met de T allel van deze SNP was significant hoger bij RCD II en EATL patiënten (11%) in vergelijking met controles (2%) en coeliakie patiënten (3%) (OR 6.76 (95% CI 3.40–13.46), p=2.27E-09 and OR 4.22 (95% CI 1.95–9.11) p=0.0001, respectievelijk). Zowel de *MYO9B* rs7259292 genvariant als homozygotie voor HLA-DQ2 verhoogden het risico op het ontstaan van RCD II en EATL in gelijke mate in vergelijking met ongecompliceerde coeliakie patiënten (OR 4.3 (95% CI 1.9–9.8) en 5.4 (95% CI 3.0–9.6), respectievelijk) maar er waren geen aanwij-

zingen voor interactie tussen deze twee risicofactoren. Zowel *MYO9B* als homozygotie voor HLA-DQ2 lijken derhalve van invloed op de prognose van coeliakie en de kans op het ontwikkelen van RCD II en EATL.

In hoofdstuk 8 werden drie met coeliakie geassocieerde genvarianten (*MYO9B*, *MAGI2* en *PARD3*) onderzocht. Deze genen coderen mogelijk voor ´tight junction´ eiwitten en zouden een rol kunnen spelen bij de regulering van darmdoorlaatbaarheid. In geselecteerde populaties met een mogelijk verhoogde darmdoorlaatbaarheid zou bovengenoemde hypothese getest kunnen worden, bijvoorbeeld bij patiënten met Down syndroom. Ongeveer 20% van hen hebben verhoogde antigliadine antistoffen (AGA) in het serum, zonder dat er sprake is van coeliakie en dit percentage is aanzienlijk hoger dan bij de algemene bevolking. Deze verhoogde AGA zou het gevolg kunnen zijn van een verhoogde darmdoorlaatbaarheid. De hypothese was dat de *MYO9B*, *MAGI2* en *PARD3* genvarianten die geassocieerd zijn met coeliakie ook gepaard gaan met een verhoogde darmdoorlaatbaarheid welke dan indirect gemeten zou kunnen worden met AGA, of met een andere antistof, ASCA.
In totaal werden 126 patienten gegenotypeerd voor zes SNPs: rs1457092 en rs2305764 in *MYO9B*; rs10763976 en rs6962966 in *MAGI2;* en rs9640699 en rs1496770 in *PARD3*; allen zijn geassocieerd met de ontwikkeling van coeliakie. Een allel dosis associatie van de genetische variaties van deze tight junction genen met de concentratie van AGA werd vervolgens verricht. Er werd een sterke correlatie gevonden tussen AGA en ASCA ($p<0.01$). De groep patienten met één of meer risico genotypes hadden gemiddeld lagere AGA concentraties (trend test P=0.007). In deze groep waren ook een significant groter aantal patiënten met normale AGA concentraties aanwezig ($P=9.3x10^{-5}$). Er werd consistent een lagere AGA concentratie gevonden in het geval van een coeliakie geassocieerd genotype. Deze resultaten zijn in tegenspraak met onze eerdere hypothese dat AGA productie juist verhoogd zou zijn bij patienten met de *MYO9B, MAGI2* en *PARD3* genvarianten welke geassocieerd zijn met coeliakie. De verklaring hiervoor zou kunnen zijn dat er bij Down patiënten andere immunologische mechanismen een rol spelen door de chromosomale verstoring, met mogelijk een veranderde tolerantie drempel als gevolg.

Het genetisch onderzoek naar coeliakie en andere auto-immuunziekten gaat snel vooruit. Verscheidene genen lijken bij te dragen aan de ontwikkeling van coeliakie en andere auto-immuunziekten, met een overlap tussen deze verschillende groepen. Het ontrafelen van de onderliggende genetische afwijkingen en het effect op de pathogenese is een van de uitdagingen voor de toekomst. Deze studies zullen verder duidelijkheid gaan verschaffen omtrent de rol van darmdoorlaatbaarheid en andere geassocieerde genetische risico factoren bij de ontwikkeling van coeliakie en overige auto-immuunziekten.

Chapter 9

List of publications

Donderwinkel PF, van der Vaart H, Wolters VM, Simons AH, Kroon G, Heineman MJ. Treatment of patients with long-standing unexplained subfertility with in vitro fertilization. *Fertil Steril* 2000; **73**(2): 334-337.

Wolters VM, Nikkels PGJ, Van Der Zee DC, Kramer PPG, De Schryver JEAR., Houwen RHJ. A gastric diverticulum containing pancreatic tissue and presenting as congenital double pylorus: case report and review of the literature. *J Pediatr Gastroenterol Nutr* 2001;**33**(1):89-91.

Wolters VM, Vooijs-Moulaert AFSR, Burger H, Brooimans R, De Schryver JEAR, Rijkers GT, Houwen RHJ. Human tissue transglutaminase enzyme linked immunosorbent assay outperforms both guinea pig based tissue transglutaminase assay and anti-endomysium antibodies when screening for coeliac disease. *Eur J Pediatr* 2002;**161**(5):284-287.

Posthouwer D, Wolters VM, Fischer K, Houwen RH, Berg HM, Mauser-Bunschoten EP. Hepatitis C infection in children with haemophilia, a pilot study. *Haemophilia* 2004;**10**(6):722-726.

Wolters VM, Verbeek WHM, Zhernakova A, Onland-Moret C, Schreurs MWJ, Monsuur AJ, Verduijn W, Wijmenga C, Mulder CJJ. The *MYO9B* gene is a strong risk factor for the development of refractory celiac disease. *Clin Gastroenterol Hepatol*; 2007;**5**(12):1399-405.

Wiggelinkhuizen M, Wolters VM, Hoekstra MO, Houwen RHJ, Meijer Y. Allergische reacties na tarwe-ingestie; diagnostiek en classificatie van tarwegerelateerde klachten. *Nederlands Tijdschrift voor Allergie*, jaargang 7, nr 6, dec 2007- jan 2008, pagina 218-223.

Wolters VM, Wijmenga C. The genetic background of celiac disease and its clinical implications. *Am J Gastroenterol*. 2008;**103**(1):190-195.

Wolters VM, Nadort C van de, Kneepkens CMF, Ten Kate FJW, Gijsbers CFM, Schweizer JJ, Nikkels PGJ, Benninga MA, Houwen RHJ. Is gluten challenge really necessary for the diagnosis of coeliac disease in children under the age of two years? *J Pediatr Gastroenterol Nutr* 2009;**48**(5):566-70.

Coenen MJH, Trynka G, Heskamp S, Franke B, van Diemen CC, Smolonska J, van Leeuwen M, Brouwer E, Boezen MH, Postma DS, Platteel M, Zanen P, Lammers JWJ, Groen H, Mail W, Mulder CJ, Tack GJ, Verbeek WHM, Wolters VM, Houwen RHJ, Mearin ML, van Heel DA, Radstake TRDJ, van Riel PLCM, Wijmenga C, Barrera P, Zhernakova A. Common and different genetic background for rheumatoid arthritis and celiac disease. *Hum Mol Genet* 2009 Jul 31. Epub ahead of print.

Wolters VM, Alizadeh BZ, Weijerman ME, Zhernakova A, Hoogstraten IMW, Mearin ML, Wapenaar MC, Wijmenga C, Schreurs MWJ. Tight junction gene variants may not explain the increased levels of antigliadin antibodies, suggesting other mechanisms than altered permeability. *Submitted*

Chapter 9

Curriculum Vitae

Victorien Wolters werd op 19 december 1973 geboren te Leidschendam. In 1992 deed ze eindexamen aan het Rijnlands Lyceum te Oegstgeest. Na uitloting voor de studie geneeskunde haalde zij haar propedeuse farmacie aan de Universiteit van Groningen en startte vervolgens met de studie geneeskunde te Groningen. Tijdens haar studie liep ze 2 maanden stage op de afdeling infectieziekten in Jeruzalem, Israël, en deed ze haar wetenschappelijke stage in Bloemfontein, Zuid Afrika. Na haar co-schappen in Enschede startte zij op 1 januari 2000 als AGNIO kinder-maag-darm-leverziekten (kinder-MDL, Dr. R.H.J. Houwen) in het Wilhelmina Kinderziekenhuis (WKZ/UMC) te Utrecht, waarna op 1 januari 2001 de opleiding tot kinderarts volgde (Prof. Dr. J.L.L. Kimpen). De perifere stage vond plaats in het Catharina Ziekenhuis te Eindhoven (Dr. J.J.J. Waelkens). Op 1 juli 2005 werd de opleiding tot kinderarts afgerond en was ze gedurende een half jaar kinderarts- chef de clinique op de afdeling kinderneurologie in het WKZ/UMC. Op 1 januari 2006 startte zij haar fellowship kinder-MDL in het WKZ/UMC (Dr. R.H.J. Houwen). Bij aanvang van dit fellowship startte zij eveneens met haar promotie onderzoek. Op 1 juni 2009 rondde zij haar fellowship af. Op 1 juli 2009 vertrok zij naar Canada. Ze doet onderzoek op de afdeling Paediatric Gastro-enterology and Hepatology (kinder-MDL) van het Hospital for Sick Children, Toronto, Canada (Prof. Dr. A.M. Griffiths en Dr. A.M. Muise) naar genetische risicofactoren bij chronische darmziekten. Na terugkomst in 2010 zal ze als kinderarts MDL in het WKZ/UMC Utrecht gaan werken. In 2004 is zij getrouwd met Mervyn Vergouwen en ze hebben twee zonen; Koen (2005) en Gijs (2007).

Chapter 9

Dankwoord

Dit boekje was er niet geweest zonder ieders hulp en enthousiasme!

Alle kinderen en hun ouders die deelgenomen hebben aan de studie, dank voor het vertrouwen!

Professor Dr. C. Wijmenga, beste Cisca, in 2006 stelde Roderick je aan me voor en daar ben ik nog steeds heel blij om. Dankzij jouw enorme gedrevenheid heb je een heel goedlopende onderzoeksgroep neergezet. Jij hebt gezorgd dat ik kwam waar ik nu ben. Als ik zaterdagavond een stuk naar je stuurde, had jij er op zondagochtend vroeg alweer naar gekeken. Ambities verwezenlijken zoals jij doet is een combinatie van creatief talent en heel hard werken, iets waar ik enorm respect voor heb!

Professor Dr. J.L.L. Kimpen, beste Jan, eerst mijn opleider kindergeneeskunde en nu promotor. Het allermeest wil ik je bedanken voor je vertrouwen, dat hielp enorm. En je overzicht en hulp door rake nuanceringen. Ik heb enorm veel waardering voor wat jij allemaal hebt bereikt in zowel het UMC als daarbuiten. Dank voor je hulp en steun!

Dr. R.H.J. Houwen, beste Roderick, jij gaf mij de kans om me als AGNIO bij de kindergastroenterologie te bewijzen. Eigenlijk is dit het resultaat van dat begin. Je vertelde per maand of ik nog een maand langer mocht blijven, dus continu vlammen! Die onzekerheid van wat er komen ging, maakte ook dat je het onderste uit de kan haalde. Dat doe je nog steeds en daardoor kon ik promoveren naast mijn fellowship terwijl dat in mijn ogen aanvankelijk niet haalbaar leek. Je hebt altijd het overzicht en tegelijk ook oog voor detail waar ik erg veel aan heb gehad en nog steeds heb. Ik vind je een hele goede onderzoeker. Daarnaast valt er met jou echt te lachen en dat maakt het werken erg gezellig en leuk en zorgt ervoor dat we ook bij verschillen van mening prima door een deur kunnen. De kansen die je mij hebt gegeven, je snelheid van werken, goede revisies en je hulp bij de eindsprint heb ik heel erg gewaardeerd! Dank!

De leden van de promotiecommissie; Prof. Dr. W. Kuis, Prof. Dr. E.A.M. Sanders, Prof. Dr. Ir. Y.T. van der Schouw, Prof. Dr. P.D. Siersema en Dr. M.L. Mearin. Dank voor jullie bereidheid om dit proefschrift te beoordelen. Dr. Luisa Mearin wil ik bovendien bedanken voor de prettige samenwerking en persoonlijke interesse de afgelopen jaren.

Prof. Dr. C.J. Mulder, beste Chris, bedankt voor je vele ideeën (die in no time werden gelanceerd) en hulp bij het RCD stuk en de review. Knap om kliniek en onderzoek zo met elkaar te verweven!

Drs. F.T.M. Kokke, beste Freddy, jij bent een echt 'mensen' mens, en altijd geïnteresseerd en dat waardeer ik heel erg. Bedankt voor het inspringen als ik voor onderzoek wat moest regelen. Heel veel succes met de afronding van jouw proefschrift!

Drs. J.E.A.R. De Schryver, beste Jan, of 'pensionada', dank voor de prettige samenwerking en altijd goede adviezen zowel ten aanzien van werk als op culinair gebied!

Collega's van de afdeling kindergastro-enterologie, Cathelijne van der Feen, wat een welkome verandering om een mede-fellow te hebben na twee jaar alleen als fellow te zijn geweest! Jij brengt echt gezelligheid met je mee! Janneke Stapelbroek, hoe vaak we wel niet even wat hebben bijgekletst fietsend naar de mooiste buurt van Utrecht! Heel erg bedankt voor al je gezelligheid en hulp als ´chef vouwen´! Hubert van der Doef, dank voor je praktische 'ICT helpdesk' hulp en dat je altijd bereid was even mee te kijken als mijn Excel vastliep en succes bij het afronden van jouw boekje! Amani Mubarak, succes met het voortzetten van dit onderzoek!

Winny van der Hoeven, Janet Feenstra, Ellen Sevenhoven en Marianne Krijger, bedankt voor de hulp bij inclusie van patiënten, jullie gezelligheid en attentheid! Dat waardeer ik enorm en maakt het werken leuk. Ellen en Debby, bedankt voor de gezellige lunches.

Drs. A. Zhernakova, beste Sasha, jij hebt mij enorm geholpen met de RCD studie en allerlei praktische zaken. Daarnaast hielp je me up to date te blijven. Dank!

Dr. B.Z. Alizadeh, dear Behrooz, you were the one to guide me in the land of the statistics. You've taught me a lot! It was really helpful to speak to a doctor who is now a scientist with a perfect overview. When it became too complicated your one-liners about women got me sharp! Thanks a lot!

Beste Wieke Verbeek, Caroline Hogen Esch en Anita Vreugdenhil, bedankt voor jullie hulp bij de verschillende artikelen en het soms halen van mijn deadlines!

Jackie Senior, thank you for editing the English texts of the papers! Harry van Someren, dank voor het verwerken van alle patiëntengegevens in een database. Alienke Monsuur en Martin Wapenaar wil ik bedanken voor hun hulp bij het wegwijs worden in 'coeliakieland' en bij het opstarten van de genetica studies.

Dr. C.M.F. Kneepkens en Dr. J.J. Schweizer, beste Frank en Joachim, dank voor jullie hulp en tijd tijdens het promotietraject. Jammer dat het uiteindelijk niet gelukt is om er een gezamenlijke multicenter studie van te maken, maar wie weet volgt dat in de toekomst!

Dr. P.G.J. Nikkels, beste Peter, dank voor de prettige samenwerking de afgelopen jaren en in het bijzonder natuurlijk je hulp bij de revisie van menig biopt voor twee artikelen van dit proefschrift. Je achtergrondmuziek zal ik even moeten missen!

Studenten Cissy van de Nadort en Susan Gerritsen, dank voor jullie hulp met statusonderzoeken en invoeren van de SPSS database en presentaties tijdens mijn verlof!
Marie-Jeanne van der Ploeg en Irene Wilson, dank voor jullie secretariële hulp!

Dr. E.E.S. Nieuwenhuis en Dr. J.C. Escher, beste Edward en Hankje, dank voor jullie hulp en vertrouwen in de eindfase van dit proefschrift en bij het contact leggen met Toronto. Jullie zijn een super voorbeeld!

Dr. J. Frenkel, beste Joost, als arts-assistent mijn mentor en nu nog altijd supportive, dank!

Beste Astrid Uyterwaal, bedankt voor je praktische hulp bij het verzamelen van alle bloedsamples!

Beste Barbara, Dank voor je ideeën, tempo en de prachtige vormgeving!

Het ´vouwteam´ voor het vouwen en verspreiden van alle boekjes! Dank Janneke, Cathelijne, Jenneke, Lianne, Marieke, Annemarie, Esther, Winny, Janet en Ellen!

Lieve collega´s, in het bijzonder Lianne, Marieke, Dorien, Annemarie en Esther, jullie zijn een ultieme vorm van 'uitlaatklep' en leuk om bij te kletsen over het wel en wee zowel binnen als buiten het UMC/ WKZ! Lianne en Marieke, bedankt voor jullie vriendschap en dat jullie nu als paranimfen aan mijn zijde staan!

Lieve jaarclub, farmacie- en geneeskunde vrienden, en ´thuis-thuis´ vrienden, dank voor jullie vriendschap, dat betekent heel veel!

Lieve schoonfamilie, dank voor de support en Driek voor al het oppassen en Brun zelfs tot in Canada op dit ´moment suprême´.

Lieve Thomas en Myrna, Heleen en George, een warm en gezellig nest is het belangrijkste wat er is en dat zijn jullie! Altijd relaxed, meelevend en lachen. Verder helpen jullie om het 'belangrijkste eerst' goed in het oog te houden, ook al is dat in de drukte van alle dag soms even wat op de achtergrond.

Lieve mam, wat fijn om zo'n lieve en stimulerende moeder te hebben! Je staat altijd klaar en het is altijd gezellig om je (al fietsend over de Uithof) even te spreken na het werk! Je hebt ons ook in praktische zin enorm geholpen om alles thuis goed georganiseerd te krijgen. Mam, ik vind het heel erg knap hoe je je leven na het overlijden van papa weer hebt opgepakt. Papa en jij hebben ons altijd enorm gesteund en jullie hebben ons geleerd dat je eruit moet halen wat erin zit op een goede manier. Dank voor alles wat jullie ons hebben meegegeven en bovenal een gezellige thuisbasis!

Lieve Mervyn, wat een geluk dat we elkaar in Enschede tegenkwamen en nu samen met Koen en Gijs in Toronto zitten! Je bent heel relaxed en dat geeft mij rust en dat is heel prettig! Jouw enthousiasme voor onderzoek is heel stimulerend en dat was redelijk essentieel voor dit boekje! Lieve Merv, ik ben super blij met jou en onze twee mannen!
Lieve Koen en Gijs, jullie zijn het 'where it's all about'! Op jullie fietsjes door High Park met big smiles! Ik ben super trots op jullie!