PhD Thesis:

Shifting management paradigms in gastrointestinal disorders associated with gluten

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Thesis Corrections

ERRATA

1. p19 para 3, line 6: “increased” for “increase”
2. p19 para 3, line 6: delete sentence commencing “At a more macro protein level…”
3. p28 point 1, line 4: “faint” for “feint”
4. p28 point 1, line 6: “to” for “do”
5. p32 para 2, line 4: “improve” for “improved”
6. p35 para 3, line 2: “significantly” for “significant”
7. p36 para 2, line 3: delete parenthesis between references 135 and 136
8. p40 para 3, line 1: “healing of the small bowel” for “the treatment goal”
9. p60 para 2, line 11: “of permeability” for “if permeability”
10. p86: “Aims 3.1 to 3.8” for “Aim 3.1 to 3.8”
11. p93 bullet point 2, line 2: “caused” for “cause”
12. p107 Figure 3.4 label: “Change in body composition indices grouped according to Body Mass Index (BMI)” for “Change in body composition indices”
13. p128 line 1: insert superscript reference “119” after “Karinen et al”
14. p148 para 4, line 2: delete “non-specific”
15. p158 para, line 5: “iron studies, B12 and folate” for “haematinics”
16. p168 para 2, line 12: “to” for “do”
17. p169 para 3 line 2: delete “at”
18. p207 para 2 line 2: insert close bracket after reference 486
19. p238 Declaration: “In the case of Chapter 5.3” for “In the case of Chapter 5.1”

ADDENDUM

1. p31, para 2 line 7: “CD may be four times more common in those with IBS compared to a control population.” for “up to a four-fold”
2. p50, para 3 line 1: “Although reversal of fulminant hepatic failure has been reported in a small group of patients with CD treated with a GFD[235], in general abnormalities of liver function tests are mild.” for “Generally abnormalities of liver function are mild, but more severe complications have been reported with the reversal of fulminant hepatic failure in four patients reported by one group[235].”
3. p54, para 3 line 4: “Claudin” for “Structurally, claudin”
4. p54, para 3 line 5: after “junction” insert “(as reviewed in detail in Arrieta et al [279]).”
5. p101, end of para 1: insert “Only one participant had an elevated EMA at 5 years (titre 1:160) and was the same participant seen as an outlier in Figure 3.3.”
6. p164 insert subheading “**Participants**” before para 3
7. Regarding the examiner’s query on p 201 para 3, “what does GEE indicate?” this has been defined on page 198 para 2 line 2 (generalized estimating equation)
8. p 104 section **“Bone mineral density” -** replace paragraph with:

   “Baseline bone mineral density characteristics are summarised in Table 3.4. In the lumbar spine, 7 (10%) patients (6 female, 5 post-menopausal) were osteoporotic, 17 (24%) osteopenic (14 female, 6 post-menopausal), and 48 (79%) (36 female, 11 post-menopausal, had normal BMD. At the femoral head, these figures were 3 (11%) (2 female, both post-menopausal), 20 (18%) (8 female), and 56 (78%) (22 female), respectively. Ten of 32 (31%) pre-menopausal women had reduced BMD, which was significantly fewer than 14 of 25 (56%) post-menopausal women (p=0.046; Fisher’s exact test).”
9. p123 para 2: “a similar trend was” for “similar findings were”

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This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution and affirms that to the best of my knowledge the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

A number of publications have arisen from this thesis work and the declarations at the beginning of the relevant Chapter outline these contributions.

Dr Evan Newnham MBBS FRACP

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Augurix®

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Publications arising from this thesis

1. **Newnham, E.D.**, Muir, J.G. & Gibson, P.R. (2014). Other dietary confounders: FODMAPS et al. *Digestive Diseases* (in press). doi: 10.1159/000371401

2. Ryan, D., **Newnham, E. D.**, Prenzler, P. D., & Gibson, P. R. (2014). Metabolomics as a tool for diagnosis and monitoring in coeliac disease. *Metabolomics* (in press). doi:10.1007/s11306-014-0752-9

3. Lichtwark, I. T., **Newnham, E. D.**, Robinson, S. R., Shepherd, S. J., Hosking, P., Gibson, P. R., & Yelland, G. W. (2014). Cognitive impairment in coeliac disease improves on a gluten-free diet and correlates with histological and serological indices of disease severity. *Alimentary Pharmacology & Therapeutics*, *40*(2), 160–170.

4. Lichtwark, I. T., **Newnham, E. D.**, Robinson, S. R., Gibson, P. R., & Yelland, G. W. (2014). "Brain Fog" and coeliac disease – evidence for its existence: authors' reply. *Alimentary Pharmacology & Therapeutics*, 1–1. doi:10.1111/apt.12867

5. Biesiekierski, J. R., **Newnham, E. D.**, Shepherd, S. J., Muir, J. G., & Gibson, P. R. (2014). Characterization of Adults With a Self-Diagnosis of Non-celiac Gluten Sensitivity. *Nutrition in Clinical Practice*, *29*(4), 504–509.

6. Biesiekierski, J. R., Peters, S. L., **Newnham, E. D.**, Rosella, O., Muir, J. G., & Gibson, P. R. (2013). No effects of gluten in patients with self-reported non-celiac gluten sensitivity after dietary reduction of fermentable, poorly absorbed, short-chain carbohydrates. *Gastroenterology*, *145*(2), 320–328.e3.

7. Ferrante, M., Karmiris, K., **Newnham, E. D.**, Siffledeen, J., Zelinkova, Z., van Assche, G., et al. (2012). Physician perspectives on unresolved issues in the use of conventional therapy in Crohn's disease: results from an international survey and discussion programme. *Journal of Crohn's & Colitis*, *6*(1), 116–131. doi:10.1016/j.crohns.2011.09.009

8. Biesiekierski, J. R., **Newnham, E. D.**, Irving, P. M., Barrett, J. S., Haines, M., Doecke, J. D., et al. (2011). Gluten causes gastrointestinal symptoms in subjects without celiac disease: a double-blind randomized placebo-controlled trial. *The American Journal of Gastroenterology*, *106*(3), 508–14; quiz 515. doi:10.1038/ajg.2010.487

9. **Newnham, E. D.** (2011). Does gluten cause gastrointestinal symptoms in subjects without coeliac disease? *Journal of Gastroenterology and Hepatology*, *26 Suppl 3*, 132–134. doi:10.1111/j.1440-1746.2011.06653.x

10. **Newnham, E. D.** (2011). Letter - Potential confounders in observed association between coeliac disease and tuberculosis. *Alimentary Pharmacology & Therapeutics*, *33*(10), 1175–1176. doi:10.1111/j.1365-2036.2011.04621.x

Abstract

Gluten has been linked to two human conditions – coeliac disease (CD) and non-coeliac gluten sensitivity (NCGS). The role of gluten in causing CD is unequivocally proven, and there is a pressing need for robust prospective data that clarify the natural history of a condition that is increasing in incidence, but reducing in symptom severity. In contrast, it is unclear whether gluten alone can cause gastrointestinal symptoms in patients without CD, and there has been a paucity of blinded randomised and placebo controlled trials dissecting the role of gluten in human disease. This thesis aims to address these important areas.

Two prospective studies in CD are presented in this work. In the first, a cohort of patients with newly diagnosed coeliac disease was reassessed at diagnosis and again at one and five years after commencing a gluten-free diet (GFD) with respect to their symptoms, intestinal healing, body composition and routine pathology testing. Response at the mucosal level was noted in the vast majority by 5 years, but coeliac antibodies correlated poorly with these outcomes. Weight gain was greatest in those with an average BMI compared to obese patients whilst skeletal muscle mass increased irrespective of baseline weight. Bone mass increased, but only in those with reduced BMD at diagnosis. An increased HLA-DQ2 and/or DQ8 dose was associated with a more severe clinical phenotype at diagnosis, but it held poor predictive value for long-term outcomes. Abnormal liver function tests occurred at any time during follow-up but overall tended to improve with treatment, whilst mild neutropenia is described as a new association. Point-of-care testing for coeliac antibodies appeared to have little place in the follow-up of CD.

In the second prospective study, the issue of 'brain fog' was explored in patients with newly diagnosed CD in a pilot study in which patients were cognitively assessed regularly over one year. Cognition was found to improve in concert with endoscopic and serological markers of CD.

NCGS was examined initially in a pilot parallel-group, double-blind, randomised, placebo-controlled trial of FODMAP-deplete wheat protein in a group of 37 patients, in which wheat protein was associated with greater gastrointestinal symptoms than placebo. However, a subsequent two trials utilizing gold-standard methodology for identifying a food intolerance – adequately-powered, double blind, placebo-controlled crossover rechallenge trials where all food was provided – found no evidence for wheat-protein-specific induction of symptoms in patients in a low FODMAP background. Lastly, an analysis of surveys received from applicants to the last two randomised trials identified short-comings in the diagnosis of NCGS that included inadequate exclusion of CD and ongoing moderate-to severe symptoms despite adherence to a GFD.

In conclusion, these studies have provided important data that inform management of CD and NCGS. The positive effects of a GFD for multiple outcomes in patients with newly-diagnosed CD underline the clinical value of dietary adherence even in the apparently asymptomatic. In contrast, the use of gluten-free diet in those with gastrointestinal symptoms but without CD was not supported and raises issues regarding the entity of NCGS.

PART A: General Declaration

Monash University

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 4 original papers published in peer reviewed journals. The core theme of the thesis is coeliac disease and non-coeliac gluten sensitivity. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within Monash University under the supervision of Professor Peter Gibson.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 4, 5.1, 5.2 and 5.3 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
4	Cognitive impairment in coeliac disease improves on a gluten-free diet and correlates with histological and serological indices of disease severity	Published	Study conception, study design, patient recruitment, conducting cognitive tests and study visits, data analysis and preparation of the manuscript
5.1	Gluten causes gastrointestinal symptoms in subjects without celiac disease: a double-blind randomized placebo-controlled trial	Published	Patient recruitment, study visits, collection and analysis of samples, data analysis and preparation of the manuscript
5.2	No effects of gluten in patients with self-reported non-celiac gluten sensitivity after dietary reduction of fermentable, poorly absorbed, short-chain carbohydrates	Published	Study design, patient recruitment, study visits, data analysis and preparation of the manuscript
5.3	Characterization of Adults With a Self-Diagnosis of Non-celiac Gluten Sensitivity	Published	Design of the questionnaire, patient recruitment, data analysis and preparation of the manuscript

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: [redacted] **Date:** 16/12/14

Chapter 1: Literature Review - Gluten in gastrointestinal disease

Cereals and grains have only been part of the human food supply for 12,000 years when our diet was driven to grasses upon reduction of the more traditional meat and fruit basis just prior to the ice-age. The predictability of crops and harvesting, and the unique characteristics of wheat, rye and barley when combined into dough have led to them becoming a core component of the Western diet, and wheat is the principal grain harvested in many Western Countries. Wheat is also the greatest source of gluten in the Western diet.

In Australia, wheat and wheat-based cereals provide up to 45% of the fibre consumed (National Nutrition Survey ANZ) and, therefore, provide important health benefits. Human gluten consumption now approximates 12-13 g/day[1], an amount that has subsequently been used as a basis for gluten challenge in clinical trials. It is conceivable that gluten consumption has trebled in recent times as a result of the food industry's increasing reliance on vital gluten[2]. Although of high nutritional value, it has been long recognised that gluten can cause human diseases.

Coeliac disease

The role of gluten in contributing to human disease has been recognized for centuries. First references to coeliac disease (CD) stem from ancient Greece in the second century where the physician Arateus used the term 'koiliakos', derived from 'koilia' (abdomen), to describe a syndrome of abdominal pain, weight loss and diarrhoea. Coeliac disease again rose to prominence when Samuel Gee in 1887 described the 'coeliac affection' defined as a condition affecting all age groups, manifesting as a 'kind of chronic indigestion' with stool reminiscent of steatorrhoea. Not until 1953 was the responsible agent in causing this condition identified. Willem Dicke described the wheat-responsive nature of a cohort of children with malabsorptive symptoms, an observation further refined by Charlotte Anderson where characteristic

histological changes and the responsible antigen were described. Central to many of these early observations in CD was the clinical syndrome of malabsorption and malnutrition in the majority. The discovery of the causative antigen in CD revolutionised the treatment and prognosis of these sick patients. In early studies, resolution of profound malnutrition and gastrointestinal symptoms was noted.

Gluten forms the main structural protein of wheat. In nature, the principal biological function of gluten is as a storage protein for elements and minerals vital to seed propagation and survival[3]. Gluten's component proteins are defined by their solubility within alcohol. Gliadins, which represent the main prolamin protein content in wheat protein, are soluble in 70-90% alcohol, and glutenins are alcohol insoluble[4]. Due to a shared genetic lineage, similar prolamin-rich peptides are found in rye (secalins), barley (hordeins), and oats (avenins).

A number of other proteins exist in wheat that include alpha-amylase/trypsin inhibitors, albumins, globulins and peroxidases that have been implicated in allergic conditions[4]. Alpha-amylase/trypsin inhibitors have also been implicated in non-coeliac gluten sensitivity and may contribute to the innate immune response in CD[5].

Fortuitously, when mixed with water and yeast, kneading releases gluten proteins that instill an elastic and 'doughy' property. Specifically, the ability of wheat (and its major protein component, gluten) to expand on the addition of water, maintain air upon proving with yeast, elasticity and 'stickiness' make it an ideal substrate for baking. The combination of its aerated doughy texture and taste from the carbohydrates contained in flour has led to the widespread adoption of bread as a base dietary carbohydrate in Western Society. Moreover, when wheat flour is washed of its water soluble components, the residual protein ('vital gluten') also forms

the basis for food additives when elasticity is desired (for example, in chocolate bars and sweets).

The changing face of coeliac disease

The epidemiology of CD is changing. No longer is CD typically a disease of profound protein-calorie malnutrition as identified by Drs Gee, Dicke and Anderson, but a condition affecting equally those underweight and overweight, symptomatic and asymptomatic, and nutritionally replete and deficient[6]. Atypical presentations of CD are now the rule rather than the exception[7]. In concert with these epidemiological shifts, evidence from several populations indicates an increasing incidence of CD independent of physician recognition and practice[8,9]. As the epidemiology changes, the concepts of disease management and outcomes are also being challenged.

Somewhat paradoxically, the early insights gained into the immunopathology of CD have held back clinical research into CD and only in recent years has this been substantially advanced. There remains only one treatment for CD, but the recognition of the substantial limitations of a gluten-free diet (GFD), including compliance, efficiency and cost, has spawned greater interest in CD therapeutics and alternatives to a GFD.

The worldwide incidence of CD has some minor variability. Sero-prevalence studies in Europe and the USA suggest an incidence of between 1 and 1.5%, but recent data from Australia imply a prevalence of up to 1.4%[10]. Although screening would probably be acceptable to the broader community, adherence to the World Health Organisation (WHO) criteria cannot justify it. In particular, the natural history of untreated CD is not well understood, the cost effectiveness of treating CD is not well established and controversy still exists regarding diagnostic criteria.

Pathophysiology of coeliac disease

Coeliac disease is a chronic small intestinal immune-mediated enteropathy caused by exposure to dietary gluten derived from wheat, rye, barley and oats in genetically susceptible individuals[11]. Coeliac disease is a serious medical condition affecting up to 1 in 70 Australians[10] and is an increasing public health problem with a myriad of extra-intestinal manifestations. Left untreated, it can lead to complications such as other autoimmune diseases, liver disease and cancer as well as early onset osteoporosis and infertility[9,12-14]. Seven in eight Australians with CD are unaware they have CD and up to 50% of people with CD are asymptomatic[15]. The only available treatment is a lifelong, strict GFD.

As a minimum requirement, the diagnosis of CD requires the confirmation of characteristic findings on biopsies (at least 6) taken at the time of gastroscopy (endoscopy). A tissue diagnosis is required because currently available antibody tests have established false-positive and false-negative rates as high as 10%, and there is acknowledged variability amongst laboratories in interpretation of antibody results[16].

Coeliac disease is one of the most common and also most understood autoimmune diseases. The inducing antigen has been identified, the genetics understood and the mucosal processes resulting in intestinal inflammation are well defined. In addition, there are sensitive and specific antibodies that aid in disease detection, whilst the local and systemic cytokine response has been documented.

At the ultrastructural level, the gliadin constituent of gluten contains prolamine- and glutamine-rich peptides that favour its specific interaction with immune cells in genetically susceptible

individuals. The toxicity of gluten to the human immune system is restricted to a specific 33-mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF). Due to the high proline content this peptide is highly resistant to digestive and synthetic proteases[17].

The translation and presentation of this 33-mer into the submucosa is poorly understood. This may relate to increased paracellular permeability, the evidence for which has been derived from multiple studies using urine intestinal permeability markers and has been more recently supported by the finding of MyD88-dependent expression of zonulin by chemokine receptor 3 (CXCR3). Further evidence for increased permeability has been derived from the finding of circulating gluten peptides and observed transepithelial transport of the 33-mer in rhesus macaque monkeys[18-20]. In addition, variants in Myosin IXb, which encodes epithelial tight junction proteins, have also been found in the epithelium of subjects with CD and are associated with the development of refractory disease[21,22]. Increased intestinal permeability has also been noted in first degree relatives of those with CD perhaps pointing towards inherent mucosal susceptibility to the development of overt CD[23]. Moreover, infections that are likely to result in abrogation of the intestinal barrier may lead to an increased incidence of CD[24].

In the lamina propria, deamidation of these peptides by tissue transglutaminase (tTG) results in glutamine residues being converted to glutamic acid (or glutamate) and permits presentation of the more negatively charge gluten-derived antigen with high affinity to T-cells in genetically susceptible individuals[25]. Whether tTG has any pathogenic role in CD is unclear. Deamidation of these peptides by tTG is crucial to permitting the interaction between gluten and the immune system. In addition, tTG activity is increase in the context of mucosal inflammation[26]. At a more macro-protein level, a 33-mer protein has been identified. More recent analysis has identified three peptide sequences that share distinct abilities to stimulate the immune system in

coeliac subjects[27].

Presentation of gliadin peptides to the subepithelial milieu results in enterocyte apoptosis and upregulation of dendritic cells. The concomitant release of interleukin-15 (IL-15) results in activation of intraepithelial lymphocytes and induction of immune responses of the innate type - enhanced dendritic and natural killer (NK) cell activity - and adaptive type - proinflammatory cytokines (particularly interferon gamma (IFN-γ)) and antibodies. Although poorly understood until recently, IL-15 activation of intraepithelial lymphocytes (IEL) likely contributes to the villous atrophy and crypt hyperplasia that define CD.

Diagnosis of coeliac disease

Several robust guidelines have been published for the diagnosis of CD[28-30]. Although there is some variability across these guidelines, consistent between all is the need for an accurate diagnosis at disease onset; the need for adequate gluten consumption to interpret coeliac antibodies and small bowel histology; and the need for treatment with a GFD under the guidance of an appropriately skilled dietitian. There continues to be a significant delay in reaching a diagnosis of CD of between 9 and 12 years, and this may have associated consequences on costs of healthcare and quality of life[31].

Coeliac antibodies

Assays assessing anti-reticulin antibodies and whole gliadin antibodies have now been superseded by more sensitive and specific tests. Whole anti-gliadin antibodies continue to be investigated in assessing the role of wheat and gluten intake in conditions such as autism, schizophrenia and non-coeliac gluten sensitivity, but their pathophysiological role in these

conditions and the role of gluten remains unclear and controversial. The more accurate antibodies now in routine use are outlined below.

Endomysial antibodies:

The technique for interpretation of endomysial antibodies (EMA) is inherently subjective requiring the visual detection of fluorescence on serial dilutions of patients' sera on monkey oesophagus. Assessment of EMA is thus labour and resource intensive and requires skilled technicians. These technical aspects, the reduced availability of a reliable substrate and the greater availability of higher throughput enzyme-linked immunosorbent assays (ELISA) have led to the reduction in the routine use of EMA in many laboratories. Nevertheless, the presence of circulating EMA remains the most sensitive and specific tests available for the diagnosis of CD and continues to be used in studies and therapeutic guidelines to increase the accuracy of diagnostic algorithms[28,32,33].

Tissue transglutaminase antibodies

The deamidation of gliadin peptides in the submucosa allows antigen presentation to the immune system with high affinity and is facilitated by tissue transglutaminase (tTG). Tissue transglutaminase is a ubiquitous enzyme in the human body and is present in a number of organs including the small intestine, heart and liver. Its main physiological function relates to regulation of the cellular cytoskeleton as well as cellular apoptosis.

Tissue transglutaminase is also the autoantigen in CD and the antigen recognised by EMA[34]. There are several isoforms of tTG. The target isoform in CD is tTG-2 whilst in dermatitis herpetiformis it is tTG-3[35]. The isoform tTG-6 may partly explain a role for tTG in

neurodegenerative conditions[36]. This isoform is principally located in the brain and elevated tTG-6 antibodies have been associated with gluten ataxia and schizophrenia[37].

Circulating tTG-2 antibodies are easily measured and older assays reliant on guinea pig tTG as a substrate have been replaced by high throughput and more accurate ELISA based kits that have proved to be a valuable asset for CD diagnosis and monitoring[16]. At CD diagnosis, published sensitivities and specificities range from 90-100% and 95-100% respectively in populations biased toward a high prevalence of CD and using a variety of commercially available kits[16,38,39].

Deamidated gliadin peptide antibodies

Antibodies to deamidated gliadin peptides (DGP) have more recently become available and are just as accurate as tTG antibodies[40]. Being measured by ELISA, it also allows for high throughput assessment and is accurate when combined with a tTG assay[41].

The recent European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) guidelines have suggested that paediatric patients with high titres of antibodies (greater than 10 times the upper limit of normal) and with symptoms consistent with CD can be diagnosed without the need for intestinal biopsy[28]. This remains a contentious recommendation for several reasons. First, there is inter-laboratory variation in the assessment of ELISA assays and many different ELISA kits are commercially available[16]. Adoption of an antibody threshold may therefore be impractical in this circumstance[42]. Secondly, there is an acknowledged false positive incidence of coeliac antibodies even at these high titres. Thirdly, antibody titres are known to fluctuate during childhood and early adolescence meaning that assessment of small bowel histology remains important[43]. At the time of writing, translation of these guidelines to

the adult population cannot be recommended on these bases and this view is supported by more recent guidelines[29].

Consistent with Baye's theorem, the positive and negative predictive values of coeliac serology vary with disease prevalence in the population being studied[44]. Most of the data derived above are from populations with a high prevalence (>45%) of CD and the study populations are relatively small in number[38,39]. Large screening studies have been limited by a lack of histological correlation with the largest noting a positive diagnosis of CD in 83% with elevated antibodies in children[45]. In a recent Australian study, 4.6% were found to have elevated tTG-IgA antibodies but a third did not have the appropriate genotype for CD and ultimately the disease prevalence was found to be 1.6%. Even in those with the susceptibility genotypes, less than half with positive antibodies were ultimately diagnosed with CD but a confirmatory EMA increased the accuracy of diagnosis[10]. These observations have also been encountered when examining patients attending for endoscopy where 7 of 33 seropositive individuals reached accepted diagnostic criteria[46] and where the sensitivity (81-90.9%), specificity (90-99.3%) varied[44,47,48]. The 'real-world' accuracy of antibody tests is difficult to calculate from many of the above studies due to the lack of histology in control populations in the vast majority of these studies as well as the reluctance of some of the seropositive groups to undergo confirmatory biopsy[15].

Regardless of which assay is utilised, all antibody assessments are contingent upon gluten consumption. Quantifying gluten consumption prior to assessment is a prerequisite and it is acknowledged that this step frequently does not occur[49]. Further, IgA deficiency can affect up to one in twenty with CD and thus IgA antibody assays may represent a false negative result in

these circumstances[50]. Therefore, the accurate interpretation of coeliac antibodies requires the assessment of total IgA or the measurement of an IgG coeliac antibody.

As a result of the technical and disease-related issues outlined above, the combination of an accurate assay assessing both an IgA and IgG antibody has an intuitive attraction[41]. This has been validated in a prospective study examining tTG-IgG and DGP-IgG and found to have excellent sensitivity and specificity, albeit in a population with a very high incidence of CD[51]. But in populations with a lower prevalence of CD, this combined assay has reduced accuracy and cases of CD would be misdiagnosed if relied upon alone[52].

A further, more practical limitation of serological testing is the need to collect peripheral blood from a vein, transport it to the laboratory and wait for a result. This then requires two points of contact with the person having the test. Furthermore, a patient's gluten consumption can reduce after blood has been collected and this can affect interpretation of confirmatory histological analysis. Such characteristics are not favourable to community screening programs. Point-of-care testing (PoCT) has clear theoretical advantages in such an application.

Point-of-care testing in the diagnosis and monitoring of coeliac disease

Qualitative PoCT has developed considerably over recent years, particularly as a population-based screening tool for patients with undiagnosed CD and has been reported to reduce the time to diagnosis of CD[53,54]. Early cumbersome immunochromatographic assays have now been superseded by commercially available kits[55-57]. PoCT provides a means of coeliac antibody testing in real time during a patient consultation. Results are available promptly (within 10 minutes) and the test is well tolerated, requiring only a finger prick blood sample or microliter

serum sample. The available kits rely on serum or blood being combined with a haemolysing buffer that releases the relevant antibody. Capillary action of the haemolysed sample through a conjugate pad results in the appearance of a coloured line if coeliac antibodies are present (lateral flow immunochromatography). A positive test is indicated by the presence of a coloured line that in some kits can be compared to a control line. The currently available kits are summarized in Table 1.1.

Table 1.1 Currently available Point of Care Tests for coeliac disease

Test Kit	Antibody	Substrate	Control Line	References (1st author, yr)
Biocard[a]	tTG IgA	Whole blood	Yes	Raivio, 2007; Korpanay-Szabo, 2005; Korpanay Szabo, 2007[58-60]
Stick-CD1[b]	tTG IgA and IgG	Serum	No	Ferre-Lopez, 2004[61]
Stick CD-2[b]	tTG IgA and AGA	Serum	No	-
Simple CD1WB[b]	tTG IgA, IgG and IgM	Whole Blood	Yes	-
Simple CD2WB[b]	tTG IgA and AGA	Whole blood	Yes	-
Coeliac Quick Test[c]	tTG IgA, IgG and IgM	Whole blood	Yes	-
CoeliacScreen Pro (or Xeliac test)[d]	tTG IgA and IgG	Whole blood	Yes	-
Simtomax[e]	DGP IgA and IgG, Total IgA	Whole blood, serum, heparinised and EDTA plasma	Yes	Benkebil, 2013[53] Bienvenu, 2012[54]

a Ani Biotech Oy, Vantaa, Finland

b Operon Immuno and Molecular Diagnostics, Zaragoza, Spain

c Biohit Healthcare, Cheshire, United Kingdom

d Personal diagnostics, United Kingdom

e Augurix SA, Monthey, Switzerland

The evidence base for point-of-care testing in coeliac disease

Qualitative PoCT is relatively new to the field of CD diagnosis and the evidence to date is summarised in Table 1.2. When examined in serological studies in subjects with known CD and controls (i.e. where the study population prevalence of CD is high), the test performs well with inherent advantages of kits that examine both IgA and IgG antibodies[62]. In the context of population screening, PoCT does not perform as well as serum antibodies but still performed adequately with sensitivities and specificities ranging from 79-93% and 95-96% respectively, and a positive predictive value of 71% when compared to serum antibodies[53,54]. In those attending for endoscopy (and thus with histological results), PoCT was inferior to standard serological tests in diagnosing CD[63]. Nevertheless, PoCT has been demonstrated to reduce the time to biopsy, which has implications for a condition where the diagnosis is often delayed[31].

There may also be a role for PoCT in monitoring of CD, but this has not been prospectively evaluated[59,60]. The use of PoCT in CD follow-up will be limited by test accuracy as the antibody titres approach the reference range due to the colorimetric nature of the test[54], although such an application has not been reported.

Although this technology would be appropriate for applications such as targeted or population screening, at the time of writing there were no data on the cost-effectiveness of PoCT in this context. In acknowledgement of this issue, the British NICE guidelines suggest PoCT awaits further evaluation and should not be used in the place of laboratory testing [64]. Recommendations from this guideline predated some of the more recent publications but further studies are needed.

Table 1.2 Summary of evidence for point-of-care testing in coeliac disease

Reference	Comparator	PoCT	Population	Clinical context	Sensitivity / Specificity
Ravio, 2006[65]	Serology/Histology	Biocard	Positive serology	Retrospective serum samples (n=334: new CD, controls and CD follow-up)	96.7 / 93.5
Korponay-Szabo, 2007[58]	Histology	Biocard	Children	Population Screening (n=2690)	78% / 100%
Bienvenu, 2012[53]	Serology	Simtomax	Children	High risk population (n=250)	93.1 / 95
Zanchi, 2013[66]	Serology	Eu-tTG Quick, Eurospital	Children/adults	CD follow-up (n=350)	84 / 98.5
Benkebeil, 2013[54]	Serology/Histology	Simtomax	Children/adults	Known CD (n=112)	78.9 / 95.7
Popp, 2013[67]	Serology/Histology	Biocard	Children/adults	1st degree relatives (n=148)	Unable to calculate
Mooney, 2014[63]	Histology	Biocard	Adults	Endoscopy (n=576)	70.1 / 96.6

Who should administer point-of-care tests for coeliac disease?

To date all studies have evaluated trained clinicians (nursing and medical) interpreting the PoCT results. The ESPGHAN guidelines note that adequate training of those interpreting the PoCT is a core component of PoCT administration and governance[28].

Advantages of point-of-care testing

Point-of-care testing is comparatively cheap (approximately A$30-60 per test), well tolerated and easy to administer. In addition, results are easy to interpret after appropriate education and interobserver agreement has been found to be very good to excellent[54,66]. In the paediatric population, PoCT has a particular advantage in avoiding venepuncture.

Problems with point-of-care-testing

Several problems or issues have been identified with PoCT.

1. Interpretation: Although a visual cue for interpretation PoCT is an attractive technology, there is an inherently subjective nature to the reporting of a positive or negative result. The degree of antibody binding to the conjugate pad will affect the intensity of colour on the test lines. Thus, whether a feint positive result represents a positive, negative or equivocal antibody result has not been clarified. To overcome this issue investigators have suggested a colorimetric scale do define positivity of the PoCT. Although a Rann scale cut-off of 2 accurately separated diseases from controls the technique may prove a cumbersome barrier to the implementation of an otherwise easy to use technology[54].
2. Qualitative testing: PoCT provides a qualitative result. To enable accurate basis for further investigation and to provide a baseline comparator to guide follow-up, quantitative laboratory based ELISA assays are crucial.
3. Accuracy: False positive results have been reported in up to 10% of kits and similarly false negatives in 15-20%[63]. For reasons that are unclear false positives are more common in Type I diabetes[53]. PoCT is, therefore, inferior to standard serological tests and it is critical that a positive test is followed up with formal serological ELISA based assays. Equally, if the clinical suspicion is high, laboratory testing should follow a negative test, and consideration should be given to seeking specialist advice.
4. Reporting: There is no accepted nomenclature for reporting of PoCT results and this is an inherent issue of this subjective technology. As alluded to above, reporting 'Positive' and 'Negative' may be overly simplistic but it is unclear how a 'Faint Positive' result should be communicated. A key component of interpretation and accurate reporting will be familiarity with the relevant kit in order that true positives are not missed.

Conclusions regarding point-of-care testing in coeliac disease

Point-of-care testing is an attractive technology that is well tolerated and easy to interpret. Nevertheless, interpretation of test results requires training and sound clinical governance is required in test administration given the implications of both a positive and negative test. Further studies are awaited before PoCT can be adopted into routine clinical practice.

Small bowel histology

Older methods of obtaining small bowel tissue such as the Crosby capsule have been superseded by gastroscopy which is better tolerated, does not require radiological positioning, has lower risks and is more accurate for tissue sampling[68]. Diagnostic features of CD during standard white light endoscopy have a limited sensitivity (between 50 and 75%) but high specificity (up to 99%) in detecting CD[69-72]. Accuracy has been improved by implementing technologies such as chromoendoscopy, narrow band imaging (NBI) and confocal endomicroscopy. In uncontrolled studies, chromoendoscopy with either indigo carmine or methylene blue have yielded very high sensitivities and specificities[73-75] but was disappointing in the only prospective study to date in average risk individuals[76]. Similarly NBI has good sensitivity for detecting CD and accuracy is improved when combined with magnification endoscopy[77]. Confocal endomicroscopy has also been shown to be accurate but is a time-consuming procedure that has practical limitations in routine endoscopy[78]. Although these newer endoscopic techniques and technologies have improved accuracy, a histological diagnosis remains important both due to its high accuracy and providing a validated baseline upon which healing can be monitored.

Therefore, a histological diagnosis is required for the accurate diagnosis of CD although this has been challenged in recent guidelines for symptomatic paediatric CD with high antibody titres as discussed above[28]. Guidelines suggest at least two biopsies be taken from the first part of the

duodenum (D1) and at least four from the second part (D2) [28]. As a generalisation, CD follows a proximal-distal gradient of disease severity[79], but recommendations regarding biopsies from D1 have only recently arisen from several studies in both adults and children suggesting that CD can either be its most severe in this area or, in some cases confined to the first part only[80-82]. First part duodenal biopsies had traditionally been avoided due to the perception of false positive biopsies, particularly in mucosa overlying Brunner's glands. These concerns have been addressed where a 0% false positive rate was noted when routine biopsies are taken from D1 [79]. Up to 13% of CD in adults can be isolated to D1 [83] and between 7-10% of children with positive coeliac serology may have disease confined to D1 [84,85 86,87].

Correct orientation of biopsy specimens is important to avoid tangential slicing of intact villi (and resultant false-positive interpretation) and an experienced pathologist familiar with CD diagnosis is also helpful. The most accepted means of histological grading CD is via the Marsh-Oberhuber classification[88,89] but the villous-height to crypt-depth ratio (Vh:Cd) is a more dynamic and continuous measure that lends itself well to clinical trial settings[90]. It is important that histology is interpreted in concert with the clinical picture as there are alternative causes of small bowel villous atrophy that include immunoglobulin deficiency, autoimmune enteropathy, tuberculosis and HIV.

Although sensitive and specific, there are some pitfalls in the histological assessment. Gastroscopy is well tolerated by patients, but it is an invasive test with small procedure related risks. The procedure is resource-intensive and requires patients to miss work on the day of their procedure. There are also limitations in biopsy attainment and interpretation. Coeliac disease can be patchy reinforcing the need for multiple biopsies to be taken[82,91]. A biopsy strategy to allow for this patchiness has been proposed with a D1 biopsy at the 9 o'clock or 12 o'clock

position and four D2 biopsies being the most accurate[92]. Rarely, CD can also be confined to the jejunum leading to a false negative endoscopic assessment as demonstrated in a study examining capsule endoscopy[93] and there may even be variation in lesion severity within a biopsy specimen[82].

Clinical presentation

As the epidemiology of CD changes, so too does the mode of clinical presentation. True malabsorption is now encountered uncommonly in adults. Perhaps explaining the under-diagnosis of CD, most cases come to the attention of treating practitioners due to the development of gastrointestinal symptoms or disease associations. It remains controversial as to whether gastrointestinal symptoms are more common in CD[94] as best evidenced by population screening studies where participants with CD could not be identified by symptoms[15,45]. Meta-analyses have suggested up to a four-fold incidence of CD in irritable bowel syndrome[95,96], but this has recently been challenged by a large prospective study in the USA[97].

The aetiology of symptoms in CD is not well understood and highlighted by the overlap of most gastrointestinal symptoms with other inflammatory bowel diseases, irritable bowel syndrome and non-gastrointestinal diseases. Upon superficial consideration, the development of diarrhoea, malabsorption and anorexia in CD is easy to explain. Coeliac disease results in a significant reduction in the small bowel surface area and thus could be expected to result in an increase in stool water, malabsorption of nutrients and relative deficiency of brush border enzymes. But the degree of villous atrophy does not appear to correlate with clinical presentation[72,98] and symptoms do not accurately identify CD in those attending for endoscopy[46]. In an older population with a prevalence of CD of 0.2%, there was no differences in the symptom profile

between those with CD and controls, with even a tendency toward more diarrhoea in controls[99]. Up to 50% of patients are asymptomatic at presentation and only a third present with the 'classical' symptoms of diarrhoea and weight loss[15,100]. Further, in a study of 180 patients who had not met the established criteria for the diagnosis of CD, re-challenge with gluten found that 51/180 had CD and 55% of the non-coeliac patients experienced a worsening of their symptoms[101]. As demonstrated in screening studies, malnutrition is also uncommon in children[102].

Discrepant clinical presentations such as constipation, reflux disease and obesity may in part be explained by disease burden where longer segments of small intestine might be involved[93] but the high incidence of asymptomatic CD is difficult to rationalize on these grounds[45]. To improved the accuracy of diagnosis and follow-up, a validated score assessing symptoms has now been developed and is a useful adjunct to clinical trials but remains a subjective score open to reporter bias[103]. Coeliac disease is also identified in at-risk groups such as those with disease associations or a family history of CD. As detailed below, the role of genetics is crucial in CD pathophysiology and 1 in 25 with a family history of CD will develop overt disease[104].

Genetics

Genetics clearly underpin the susceptibility for the development of CD. In monozygotic twins there is up to 86% concordance for CD[105] and up to 20% of first degree relatives can be affected[104]. Although these rates are high in comparison to other immune diseases, penetrance falls well short of 100% and, therefore, environmental factors such as diet, breast-feeding and infection may also play a role. In those with CD more than 99% express either the DQ2 or DQ8 haplotype or a combination thereof[10]. The DQ Class II molecule is derived from chromosome 6

and is responsible for the presentations and recognition of peptides from outside of the cell. The nomenclature for genotypes is derived from WHO classifications and has been more recently rationalised (G1-G5) to allow for more accurate and relevant grouping (Table 1.3, adapted from[106]). These genotypes can either be inherited in cis (on the same chromosome) or in trans (on different chromosomes). In CD, the dominant HLA DQ molecule is DQ2.2. At the cellular level, the αβ subunit of antigen presenting cells can be encoded by a number of coeliac susceptibility genotypes. Up to 50% of the Australian population carry the susceptibility genotype[10,107] but there is variability in this incidence worldwide particularly in South East Asia, China and Japan[108,109].

With up to 50% of the population carrying genetic susceptibility genes, and a worldwide incidence of between 1-1.5%, there are clearly other factors leading to the development of CD. To this end, genome wide association studies (GWAS) have endeavoured to localize non-HLA genes of which up to 40 have so far been elucidated. Strengthening the relationship of these non-HLA loci with CD is the observed overlap with other immune diseases such as rheumatoid arthritis and other inflammatory bowel diseases. Despite significant advances in this field, there is still a significant knowledge gap with only 50% of disease development explained by genetics alone[110]. As proposed recently, a 'multiple-hit' hypothesis with an interplay between genetics and the environment may underpin the pathophysiology[111].

Genotype dose has been linked to increased risk of developing CD and its associated complications. The first indication that a "double dose" of these genes might predict phenotype was that people who are homozygous for DQB1*02 (i.e., DQ2) have a much higher likelihood of developing CD than those who are heterozygous[112-114]. At the level of antigen presentation,

DQ2 homozygosity is associated with greater affinity of presentation of t-cell epitopes and a 5 fold increased risk of developing CD[115]. It has been proposed that this may be due to the lower availability of DQ2 molecules in heterozygotes for presentation to antigen presenting cells[116].

Several studies have addressed the relationship of DQ2 dosage to clinical presentation, histological severity of the duodenal lesion at diagnosis, immunopathology, and the likelihood of complicating of T-cell lymphoma. The findings have been heterogeneous, ranging from DQ2 homozygosity being associated with more severe presentation and duodenal histology[117-120] to no association at all[113,121,122]. That homozygosity for DQ2 is common amongst those with refractory coeliac disease (RCD) and predisposes to t-cell lymphoma is worthy of noting[123].

Table 1.3 Genetic classification of coeliac disease*

Genotype Subclass				***HLA Genotype***
G1	H1/H1 or H1/H2	**H1**	DQA1*0501 DQB1*0201	DQ2.5
G2	H2/H3	**H2**	DQA1*0201 DQB1*0202	DQ2.2
G3	H1/H3 or H1/H4 or H1/H5	**H3**	DQA1*0505 DQB1*0301	DQ7.5
G4	H2/H2 or H2/H4	**H4**	DQA1*0301 DQB1*0302	DQ8
G5	H4/H5 or H5/H5	**H5**	OTHERS	

*Adapted from [106]

Treatment of coeliac disease

Despite CD being perhaps the best understood autoimmune disease, there remains only one available treatment – a lifelong strict gluten free diet (GFD). Discovery of the source of the antigen driving CD revolutionised treatment 60 years ago and dramatically improved the quality

of life in those diagnosed with CD. But the epidemiology of CD has evolved and, with that evolution, challenges in treatment are being increasingly uncovered. Important questions are being asked regarding the efficacy, palatability, cost and need for lifelong treatment. The definition of 'gluten free' also continues to evolve as more sensitive tests for detection become available. Only in relatively recent years has the realisation of these shortcomings generated research into therapeutics in CD.

Undoubtedly, treatment of CD with a GFD has well-recorded benefits. Coeliac antibodies tend to fall, histology tends to improve and symptoms when present. Patients compliant to a GFD are more sexually active, have less autoimmune disease, improved bone mineral density, better dentition and an improved health related quality of life[124-126]. Some authors have questioned the requirement of a lifelong GFD with successful reintroduction of gluten in a small subset of patients with CD[127-129]. Of note, some patients can achieve therapeutic success even with poor compliance as seen in a small group of patients in one retrospective study[100]. But even with the apparent achievement of tolerance, complications of CD may still ensue[128,130]. Despite these findings and as observed in gluten re-challenge studies in CD, intolerance to gluten appears to be a lifelong phenomenon[90,131]. Although it is the only available treatment, a GFD has several problems including cost, nutritional adequacy, compliance, and adverse effects.

The GFD is costly. Several studies have addressed its cost in North America, Canada and the UK with a significant increased cost noted when compared to gluten containing alternatives[132,133]. Although government rebates are available in several countries to aid in meeting this cost, it remains an issue in several developed countries including the USA and Australia and may impact upon dietary compliance in lower socioeconomic groups.

The GFD may also be low in some nutrients. Studies conducted in the USA, UK and Australia suggest that patients with CD following a GFD may be receiving inadequate amounts of non-starch polysaccharides, calcium, vitamin D, folate and zinc[134,135]}[136]. These nutritional deficits may be offset by the benefits noted in body composition and bone mineral density upon treatment of CD but are a concern in those who do not require these benefits and in those following a GFD for non-medical indications.

There may be other adverse effects of treatment with a GFD. In one study, depression, smoking and greater alcohol consumption was found in those strictly compliant[124]. The GFD may also have adverse consequences on the gut microbiome. A GFD results in reduction in *Bifodobacteria* and *Lactobacilli* and increase in *E. coli* and *Enterobacteriaceae*. In association with this observation lower immunostimulatory (tumor necrosis factor-alpha (TNFα) and IFN-γ) as well as lower anti-inflammatory (IL-10) cytokines have been noted in the faecal stream after introduction of a GFD[137].

In summary, although mostly successful in achieving the desired therapeutic goals from the patient's perspective, there are several shortcomings of the GFD that has driven recent developments in CD therapeutics and alternatives to a strict lifelong GFD. The management of an increasing population with no obvious symptoms remains a significant challenge in modern gastroenterology. We continue to rely on population studies outlining the benefits of good compliance and the risks of poor compliance to guide treatment.

The natural history of coeliac disease

As detailed above, the only available treatment for CD is a lifelong strict GFD and intuitively the aims of treatment are to relieve symptoms and to resolve the defining histology. However, there is controversy as to how CD should be monitored and there is a discrepancy between published guidelines and clinical practice[138]. Further confusing the issue, available measures of intestinal inflammation such as coeliac antibodies are inaccurate markers of intestinal healing during disease follow-up[139]. Repeated endoscopic evaluation is the only accurate means of assessing small bowel healing but endoscopy is invasive, has small procedure-related risks and regular endoscopic evaluation has resource implications for already stretched health systems.

Current practice is to monitor patients by assessing compliance, coeliac antibodies and gastrointestinal symptoms in conjunction with small bowel histology. As detailed below, all of these tools have shortcomings and there is an unmet need for reliable non-invasive markers of intestinal inflammation in both the diagnosis and monitoring of CD. Novel technologies need to be considered.

Available methods to monitor coeliac disease

Monitoring compliance

Clearly, compliance with the prescribed treatment improves outcomes in any chronic disease, and this is also true of CD[140]. As little as 1mg of gluten can be enough to re-initiate mucosal inflammation demonstrating the need for compliance and the need for adequate education and support of patients at diagnosis[141]. Poor compliance with a GFD results in increased mortality, increased complications (such as cancer) and persistent mucosal inflammation[142,143]. Conversely,

good adherence is associated with improvements in quality of life, body mass index and bone mineral density[144-146], and reduces the risk of infertility, depression and autoimmune disease[125,147].

In a meta-analysis compliance to the GFD in CD was found to vary from 46-92% depending on the population[148]. Methods of assessing compliance vary markedly in the literature from mailed questionnaires to direct interviews by either physicians or dietitians. Furthermore, the terminology used to grade adherence also makes comparisons between studies challenging. These difficulties culminated in the development of a validated means of assessing compliance with a short questionnaire the Celiac Dietary Adherence Test (CDAT), developed after consultation between members of an expert multidisciplinary panel[149]. The application of the CDAT in subsequent studies has revealed compliance rates to vary between 47 and 56% [150,151].

A number of strategies have been devised to improve compliance. In one randomised study from Australia, a weekly educational online tool has shown promise[152] whilst regular follow-up in a coeliac clinic with the support of a dietitian also improves adherence[153]. In another study, psychological support to a group with anxiety improved compliance as well as rates of depression[154].

Whether it be specialist assessment by personal interview, completion of a validated questionnaire or review of a food diary, all available assessments of compliance remain subjective. Until reliable objective measures of compliance are available, the true effect of GFD adherence on disease outcomes will be reliant on imperfect assessments and population study.

One such objective measure is the quantification of intact gluten peptides in faeces[155] but other technologies such as metabolomic analysis of serum or urine show promise in this area.

Symptoms

As most patients ultimately diagnosed with CD have presented with gastrointestinal symptoms, their resolution is highly desirable. Symptoms can predate the diagnosis of CD by up to 12 years[156]. Gastrointestinal symptoms have also been used to assess clinical response to the GFD in CD, but up to 50% of screen-detected individuals have no symptoms and symptoms are poor discriminators for CD when relied upon as a screening tool[15,45]. Complete clinical response may only be seen in 56% of patients even after 5 years[100]. In this same population, persistent villous atrophy was found in 62% of those with a clinical response and there was no association between clinical response and mucosal recovery. Similar results have been noted in other populations; symptoms appear to be a poor guide to mucosal architecture in the setting of long term follow-up of CD and also in the context of gluten challenge[157,158].

Coeliac Antibodies

Complicating adherence to follow-up guidelines, reliable tools to predict mucosal healing have been lacking and prospective data on currently available tools are limited[159]. When coeliac antibodies are elevated at the diagnosis of CD they provide a useful monitoring tool. CD antibodies fall in the majority and may provide an initial guide as to disease response. The titre of EMA is poorly predictive of mucosal recovery in CD[160] but the concentration of IgG antibodies to DGP may be a more accurate marker if a lower cut-off is used[161]. Coeliac antibodies can remain elevated in up to 46% after a year and of these up to 48% will be associated with normal mucosa (Marsh 0)[139]. In another retrospective study, antibodies were

negative in 77% of patients with persistent villous atrophy[162] a finding confirmed by two more recent publications[100,157]. Thus over-reliance on coeliac antibodies as an assessment for both dietary compliance and histological response can result in misinformation and stigmatisation of patients that can be unconstructive in achieving treatment goals.

Available evidence suggests that all coeliac antibodies fall with treatment of CD. This observation has been extrapolated in clinical practice to use antibodies as a measure of disease response and dietary compliance. Persistently elevated antibodies can suggest dietary transgression and this should first be excluded as a cause. However, there are a group of patients whose antibodies remain elevated despite adequate compliance. EMA have been previously demonstrated to be poor at monitoring response to treatment[160,163] and are a poor marker for dietary compliance[164]. Similarly, available data for tTG and DGP antibodies have been disappointing when compared to healing and measures of compliance[139,163,165-167]. Furthermore, in a one year long prospective study, coeliac antibodies did not distinguish between those with partial and complete histological response[166]. In an effort to optimise coeliac antibodies as a non-invasive measure of both compliance and mucosal healing some have proposed using a lower cut-off in reference range[161]. This awaits validation in other populations. In children however, antibodies may be a more reliable measure of histology[168].

Small bowel histology

It is intuitive that the aim of treatment in CD be the treatment goal in a condition that is defined by its mucosal morphology. In this way histology should be the only means of accurately assessing disease response. Although guidelines lack some specifics, it is generally recommended that patients undergo repeat histological evaluation to assess response. It is likely

that similar numbers of biopsies are obtained in follow-up as at diagnosis (i.e., at least four from the second part of the duodenum and two from the first part) although this has not been addressed directly. However there is some debate as the timing of repeat biopsy[169]. Such timing has also been muddied by only relatively recent data linking poor mucosal recovery to disease outcomes. In order to determine the appropriate timing of re-evaluation, the velocity of mucosal recovery needs to be completely understood, but this has not been prospectively evaluated. From the available retrospective studies, in some mucosal recovery may be very rapid in some, whilst years of treatment may be required in others.

In other inflammatory bowel diseases, the importance of mucosal healing is acknowledged. In Crohn's disease it has been established that the attainment of mucosal healing reduces hospitalization and the need for abdominal surgery as well as being predictive of long term remission[170]. In addition, absence of mucosal healing after surgical resection is predictive of clinical relapse[171]. While treatment of CD is largely restricted to diet, some correlates to intestinal healing are seen in other inflammatory bowel diseases and their response to therapy. For example in Crohn's disease, immunosuppressive agents such as azathioprine and methotrexate may take between 3 to 6 months to achieve full clinical efficacy and mucosal healing[172,173]. In the setting of biological agents, clinical responses can be seen much earlier and assessment between 4 to 6 weeks has been suggested[174]. Corticosteroids are effective in relieving symptoms in Crohn's disease[175], but high-dose corticosteroids induce complete mucosal healing in only 13%[176].

The importance of mucosal healing in CD has been highlighted by the association of ongoing mucosal inflammation with osteoporosis, and risks of autoimmune disease and cancer[100,177-179].

Although mucosal healing in children appears to occur in the vast majority[159,180], the converse may be true in adults. Depending on the criteria used to define healing, retrospective studies have shown healing rates between 8 and 66% at differing time points with a tendency for these healing rates to improve over time from diagnosis[100,157,162]. Population studies have also alluded to an improvement in the rates of healing in the past 10 years compared to 30 years ago[181]. Risk factors for poor mucosal healing have included severe baseline histology, male gender, increased age, lower education level, severe clinical presentation and HLA DQ gene dosage[100,166,181]. A summary of the available histological follow-up studies in CD is presented in Table 1.4.

The reasons for lack of mucosal recovery in CD may include poor dietary compliance and secondary pathologies (such as immune deficiency, human immunodeficiency virus infection and tuberculosis). Mucosal recovery in some may simply represent the natural history of their disease and this may be more the truth in adults rather than children. Micro-contamination of the diet with trace amounts of gluten may also explain a degree of ongoing mucosal damage with up to 50% apparently compliant with a GFD showing histological response to a more restrictive diet, the so-called "super sensitive diet"[139].

Table 1.4 Summary of published case series where histological follow-up of coeliac disease was reported

Reference	Number of patients	Length of follow up	Methods	Definition of healing	Healing	Antibodies	Compliance
Kumar, 1988[182]	44 teenagers	Unknown	Retrospective	Villous height	27/44 (61%)	Not reported	Self-reported and dietitian assessment (57/102 compliant)
Grefte, 1988[183]	22 adults	14 months	Retrospective	Surface: volume ratio compared to controls	Surface to volume ratio 22% to 48% after 9 to 19 months 24 to 48 months on GFD increased to 68%	Not reported	Assessed retrospectively if histology abnormal
Ciacci, 2002[143]	390 adults	6.9 yrs	Retrospective: GFD (110), transient GFD (85), Not gluten free (165)	Marsh 0	44%	EMA positive in 25% at follow-up (70% with severe histology at follow-up)	Physician assessment
Wahab, 2002[180]	158 adults	2 to 5 years	Retrospective	Marsh 2 or better	65% at 2 years; 85% at 5 years; 89% after 5 years	Not reported	Not reported
Lee, 2003[162]	39 adults	Mean 8.5 yrs	Retrospective	Marsh 0	21% None had normal Vh:Cd ratio despite clinical response in all	EMA negative in 77%	Physician assessment

Reference	Number of patients	Length of follow up	Methods	Definition of healing	Healing	Antibodies	Compliance
Tursi, 2006[184]	42 (aged 15 to 72 years)	2 years	Prospective	Marsh 0	59%	Not reported	Nor reported
Kaukinen, 2007[177]	591 adults	3 to 30yrs	Retrospective Push enteroscopy	Vh:Crypt depth ratio >2.0	574/591 (97.1%)	Positive in 5/13 non-responders	Dietitian and food diary
Bardella, 2007[185]	135 children 114 adults	2 yrs 2 yrs	Retrospective	Marsh 0	Children 74%, Adults 17.5%	Not reported	Questionnaire (100% compliance)
Carroccio, 2008[186]	42 adults with and 27 without symptoms	6.5 yrs	Retrospective	Normal Vh:Cd ratio	35% (villous atrophy more common in those with symptoms)	EMA negative in 100%	Interview by trained dietitian
Lanzini, 2009[157]	465 adults	16 months	Retrospective	'normalisation' Marsh 0; 'remission' Marsh 0 or 1	8% normalisation; 65% remission	Negative (tTG, AGA or EMA) in 87%	GFD program run by dietitians (85% compliance)
Rubio-Tapia, 2010[100]	241 adults	5 months to 35 years	Retrospective	Marsh score <40% IEL's Vh:Cd >3:1 Vh:Cd improvement by >2 points	37% total Improvement in 45% 34% at 2 years and 66% >5 years	tTG seroconversion in 64% and 51% with and without mucosal recovery; these figures were 89% and 63% for EMA)	By dietitian Good compliance in 66%. Poor compliance associated with poor recovery

Reference	Number of patients	Length of follow up	Methods	Definition of healing	Healing	Antibodies	Compliance
Lebwohl, 2013[178]	7648 adults	11.5 years	Population based	Resolution of villous atrophy	43%	Negative (tTG, AGA or EMA) in 59% (higher rates in those with healing)	Not assessed
Sharkey, 2013[139]	391 adults	12 to 24 months	Retrospective	Normal: Marsh 0 Minor changes: Marsh 0 or 1	26% normal 57% minor changes	15% seronegative poor correlation between serology and histology	Dietitian supervised
Galli, 2014[166]	65 adults	12 months	Prospective	Marsh 0	66% (0% in those non-adherent)	Negative (tTG or EMA) in 70% (irrespective of compliance)	4 question interview (81.5% compliance)

Bone Mineral Density

Coeliac disease has been clearly linked to osteoporosis and osteopenia. Evidence from population studies suggests an incidence in CD of between 20 and 76% [187-189] with similar incidences found in those with treated CD despite apparent dietary adherence[190]. In addition, reduced bone mineral density (BMD) may be the earliest manifestation in those who are asymptomatic[191]. Serological studies also suggest those with positive coeliac antibodies have a lower BMD than healthy controls and have an elevated risk of fragility fractures[192,193]. Prospective data are lacking, but, in osteoporotic coeliacs, BMD improved with treatment GFD[194-196] while, in children and adolescents, recovery of BMD can be rapid and complete[197]. The only long term prospective study in the effect of CD treatment on BMD evaluated patients at 1 year and 5 years after diagnosis[198]. In addition markers of bone turnover were analysed. Most of the improvement in BMD occurred during the first year and levels of alkaline phosphatase inversely correlated with increase in BMD.

The cause of reduced BMD has been proposed to relate to lowered serum vitamin D and consequent secondary hyperparathyroidism[188,199]. Reduced calcium absorption may also play a role, and consequent improved absorption with treatment may contribute to improved BMD[200]. With treatment, bone mineral gain appears to be more pronounced in the axial than peripheral skeleton[199]. Secondary hyperparathyroidism at diagnosis has also been linked to poor bone remineralisation and impaired intestinal recovery[201]. As demonstrated in other inflammatory bowel diseases and by the systemic cytokine response seen in CD[202], intestinal and systemic inflammation are also likely to play a role.

Prospective studies on the effect of GFD on bone mass have been biased towards populations with a high incidence of osteopenia and have been in small cohorts[203] with only one prospective

study examining patients to 12 months on a GFD[189]. The longest follow-up was to a median of 37 months[203]. Only one study of 28 individuals compared these BMD changes to histology, in which bone mineral deposition was noted to occur particularly in the first year[198]. Somewhat supporting the role of systemic inflammatory cytokines and perhaps malabsorption, BMD has been related to the severity of mucosal inflammation[204]. Few studies have addressed the relationship between these bone indices on the one hand, and mucosal healing status and serological markers of disease activity on the other.

Body Composition

In both children and adults, patients with CD have a lower body mass index (BMI), weight and height compared to gender- and age-matched controls[145,146,205,206]. Clinical presentation has also been shown to relate to body composition[207]. Those who were more symptomatic had more severe deficits in fat and lean body mass. Perhaps consistent with a changing epidemiology, these concepts have been challenged by a large population study where only modest reductions of weight and BMI were noted[102].

Long term prospective data on the effect of a GFD on BMD and body composition are lacking but many retrospective studies and some short-term prospective studies have been performed. Evaluation of body composition in CD has noted normalisation of both low fat and lean body mass in adolescents after one year[146], but studies in adults have either lacked measures beyond fat indices, used superseded antibody assays and/or have not correlated changes with histology[205,206,208,209]. Data have suggested an increase in BMI with treatment and some have suggested that this increase may be more so in those who are underweight with reduction in those who are overweight[210].

Previous prospective studies have analysed patients using inferential measures of fat-free mass in a group of patients with high (100%) rates of mucosal and serological response and limited to 12 months of treatment with a GFD[145,205,209]. The observed increase in body fat has particular relevance to the use of ghrelin as a non-invasive marker of disease activity[211]. However, the utility of ghrelin may be offset by the body composition and thus metabolic changes observed in this group.

The effect of gene dosage

The effect of genotype on long term phenotypic and disease outcomes in the longer term is unclear[118,212]. The majority of the studies have examined retrospective cohorts and these were mostly from tertiary referral hospital populations[213]. Both of these factors open the studies to considerable selection bias. Many studies have been performed in paediatric populations and there is no certainty that findings in children are directly applicable to patients diagnosed as adults. Furthermore, varying methodologies and definitions, particularly with regards to symptom assessment, have been used rendering comparison of findings difficult. To date, all studies are relatively small case-control or population data, and no study has prospectively evaluated the effect of disease treatment with outcomes such as BMD, histology and body composition.

Other coeliac disease associations

There are a myriad of associations with CD and these have been previously outlined in detail[214]. Since particular targets in this thesis will be the effects of treatment on cognition, abnormal liver

function tests and neutropenia, discussion will focus on these (a possible new association described within this work).

Cognition

There is significant body of information to suggest that chronic illness has an adverse effect on cognition. Authors have noted deficits in verbal and non-verbal IQ in illnesses such as systemic lupus erythematosis, multiple sclerosis and other autoimmune diseases[215,216]. These findings have also been noted in inflammatory bowel disease[217,218], but there is a paucity of literature available in CD.

Neurological manifestations reported in patients with CD include amnesia, ataxia, acalculia, epilepsy, chronic neuropathies, confusion and personality changes[219-222]. Some of these severe neurological symptoms can improve upon treatment with a GFD[219,223]. For gluten ataxia, antibodies to tTG-6 have been proposed as a marker of disease and subsequent response [224]. General clinical experience is that patients with CD commonly report more subtle cognitive deficits that have been colloquially termed 'brain fog'. This might include difficulty concentrating, problems with attentiveness, lapses in short-term memory, word-finding difficulties, forgetfulness, temporary loss in mental acuity and creativity, and confusion or disorientation[225]. In clinical experience, patients often report that brain fog dissipates after treatment on a GFD or returns after inadvertent gluten exposure. However, few studies have investigated such subtle cognitive deficits. In a group of 8 'cognitively normal' participants with CD and idiopathic cerebellar ataxia significant impairments were found in immediate recall from episodic and semantic memory, and there was a trend towards deficits in phonemic verbal fluency and executive function[226]. Further, a study of 15 elderly patients with cognitive decline

that began within two years of the onset of CD symptoms suggested a link might exist between cognitive impairment and CD[219]. The possibility of such a link has been strengthened by the findings of a retrospective study of elderly patients in which CD was diagnosed after the age of 60 years[227]. Of the 7 patients identified, two presented with cognitive decline that had been attributed to Alzheimer's disease, but was ameliorated after the initiation of a GFD, while a third patient had peripheral neuropathy that completely resolved after the initiation of a GFD.

Abnormal liver function tests

Abnormalities in liver function have been extensively described in association with CD and may be one of the most common extra-intestinal manifestations, but thorough prospective longitudinal data are lacking in the adult population. Older studies suggest an incidence of abnormalities in liver function tests (LFT) of up to 42%[228,229] but more recent prospectively-collected data have placed this figure closer to 10% [230]. One case control study from Finland has even questioned whether LFT abnormalities are more prevalent in CD[231]. Conversely, the incidence of CD within those with LFT abnormalities varies from 1%-10% [232-234].

Generally abnormalities of liver function are mild, but more severe complications have been reported with the reversal of fulminant hepatic failure in four patients reported by one group[235]. Histologically, CD-associated abnormalities in liver function have been attributed to a chronic periportal infiltrate of lymphocytes or a 'non-specific' histological picture[228,236]. Due to shared genetics and clustering of autoimmune conditions, there is also an overlap with the biochemical, histological and autoantibody stigmata of primary biliary cirrhosis (PBC), autoimmune hepatitis and primary sclerosing cholangitis (PSC)[237-239].

There are many possible explanations for liver function test abnormalities in CD. As with routine evaluation, the isolated elevation of alkaline phosphatase should prompt a search for associated osteomalacia and vitamin D deficiency, whilst predominance of cholestasis or elevated transaminases should prompt evaluation for primary biliary cirrhosis and autoimmune hepatitis respectively[229]. Outside of these conditions, other contributors have been proposed to be steatohepatitis secondary to undernourishment, but, as outlined earlier, malnutrition is now an uncommon presentation. Due to the increasing worldwide problem of obesity and the metabolic syndrome, steatohepatitis may explain an increasing proportion of LFT abnormalities in CD due to factors independent of CD but this has not been well studied. Only one prospective study has examined BMI in concert with liver function tests and, although the serum concentration of alanine aminotransferase (ALT) increased in a proportion of patients, BMI did not significantly change[231].

Data on the effect of a GFD on these abnormalities are somewhat conflicting, but the overwhelming majority report numerical improvement in liver function[229-231,240]. In a prospective study of 350 children with CD, 40% had an elevated aspartate aminotransferase (AST) (n=133 'cryptogenic', n=7 autoimmune hepatitis) and 98% achieved normalisation with a GFD[241]. In this same population, of those who failed to normalise, all had autoimmune hepatitis suggesting the GFD is ineffective in this context – a finding supported by one study in adults[232]. Somewhat paradoxically, adherence to a GFD may prevent the development of autoimmune diseases that include autoimmune hepatitis[125]. One of the earliest studies in adults noted improvement in LFT as early as three months after the initiation of a GFD, but the improvement was not universal with 10% failing to normalise[229]. In a more recent prospective study of 130 adults, all with elevated transaminases at baseline (10%) returned to normal values, but there was a sub-group whose ALT increased from normal values with treatment[231].

In exploring the above concepts further, only one study has evaluated the effect of gluten challenge on liver function tests in a group of 25 patients with CD. After a median 51-day unblinded gluten challenge an increase in ALT in 11/25(44%) was observed, with all returning to normal values upon reinstitution of a GFD[231].

Haematological associations

A number of haematological manifestations have been described in CD. The population incidence of neutropenia is less than 1% with higher rates observed in the black population of North America[242]. In CD, a higher incidence of leucopenia has been observed in a selected paediatric population[243] and lymphopenia has been reported in adults[244]. There may also be a higher incidence of coeliac antibodies and clinical CD in patients with idiopathic thrombocytopenic purpura (ITP)[243,245,246]. Prolongation of the prothrombin time due to vitamin K deficiency has been reported, and, paradoxically, a number of case reports and a single population study have associated hypercoagulability with CD[247-249].

Of potential relevance to the above observations regarding leucopenia, CD is associated with a higher incidence of bacterial and mycobacterial infections[250-252]. This may in part be explained by the high incidence of functional hyposplenism in CD due to the reduced production of IgM memory cells crucial to defence from encapsulated organisms, and has prompted guidelines to recommend pneumococcal vaccination[29,253]. The increased rates of infections with non-encapsulated organisms have not been adequately explained. The incidence of functional hyposplenism in CD has been reported to be as high as 77% [254]. Hyposplenism may be seen in

up to 20% of patients with uncomplicated CD[255] and splenic hypofunction may be a harbinger for other complications of CD[256].

More recently, links between CD and eosinophilic disorders have been described. In both children and adults, eosinophilic oesophagitis appears to be over-represented in patients with CD, but the pathophysiological basis for this is unclear. There is no shared genetic predisposition from GWAS and the histological lesion is quite distinct. But a pathological case series identified a proportion of CD patients with eosinophilic infiltration of the lamina propria in the absence of a peripheral eosinophilia[257]. The incidence of peripheral eosinophilia has not been prospectively assessed in CD but the population incidence of peripheral eosinophilia may be as low as 0.1% [258].

Novel non-invasive markers to monitor coeliac disease

As discussed above, there is a lack of good non-invasive markers of disease activity in patients with CD. This has parallels in patients with Crohn's disease where there is also a discrepancy between clinical response and mucosal disease activity. In Crohn's disease, the routine clinical assessment of patients both in clinical practice and trials, utilises the Crohn's disease activity index (CDAI). This tool is almost entirely subjective and has few organic components. Several studies have demonstrated the limitations of the CDAI in assessing mucosal disease activity when Crohn's disease is treated with either corticosteroids or biologic agents[176 173,259]. As a result, more attention is being placed on mucosal disease assessment and clinical disease endpoints are being questioned[260,261]. Several non-invasive markers have been examined in other inflammatory bowel diseases. Serum C-reactive protein (CRP) correlates well with disease activity and mucosal healing in Crohn's disease[262263] as well as being predictive of disease

response[264] and relapse[265], but CRP correlates poorly with the activity of small bowel Crohn's disease[263]. Faecal bio-markers of inflammation, such as calprotectin and lactoferrin, are superior to serum CRP in diagnostic accuracy of intestinal inflammation[266]. Levels of faecal calprotectin correlate well with mucosal healing in adults and children[267,268] and an elevated faecal calprotectin is predictive of subsequent clinical relapse in those with Crohn's disease in clinical remission[269,270].

Unfortunately, the non-invasive markers of inflammation that have found place in clinical practice in patients with Crohn's disease are not useful in CD. For example, faecal calprotectin may be modestly elevated in children with newly diagnosed CD[271] but studies in adults have failed to confirm this finding[271,272]. However, several other markers have received attention in CD.

Assessment of intestinal permeability

The human gastrointestinal tract is lined by a continuous epithelium that has a profoundly complex role in regulating digestion, immunity and protection from disease. Structurally, each epithelial cell is joined by a tight junction and adherens junction that in themselves are complex in structure and function. Structurally, claudin and occludin proteins form the basis of the tight junction. Disruption of the tight junction, such as during infection with cholera that targets zonula occludens (via zonula occludens toxin) or *Clostridium perfringens* that affects some of the claudin family, can result in profound electrolyte losses, demonstrating the important physiological role these proteins have. Of particular relevance to CD therapeutics, zonulin has been found to be endogenously secreted in humans and have a role in increasing tight junction permeability[273]. Pro-inflammatory cytokines such as TNF and IFN also regulate tight junction

structure and may in part explain the therapeutic efficacy of anti-TNF agents in inflammatory bowel disease[274]. Deeper to the above protein network are actin filaments that also have a role in preserving barrier integrity and variants of genes involved in controlling actin have been linked to an increased risk of CD[21]. Thus, measures of intestinal permeability (IP), that have included both direct measures to assess the tight junction, such as electron microscopy, and methods such as transepithelial resistance *in vitro* and urine intestinal permeability studies *in vivo*, particularly dual sugar permeability tests, have been studied extensively.

To begin to understand IP studies using dual sugars, an understanding of intestinal carbohydrate absorption is needed. A number of theories have been proposed for the absorption of the sugars used as probes in IP studies. Carbohydrates may cross the epithelium either through paracellular or transcellular routes. It is presumed but not unequivocally proven that larger molecules travel via paracellular pathway due to their nearly 100% recovery after intravenous injection[275]. Fihn and others, have proposed two systems for absorption by examining rat ileum[276]. First, larger molecules are proposed to be transported by pores that are small in number at the base of the crypts in the epithelium. Smaller pores in large frequency may be located at the villous tips explaining the relative high absorption of smaller sugars such as mannitol and rhamnose. This theory explains the noted increase in lactulose absorption in villous atrophy where the crypt bases are exposed and the crypt tips are reduced in number creating an increase in the lactulose:mannitol ratio. One tangible anatomical correlate to this theory is that claudin expression varies greatly between the villous crypts and tips[277]. Paracellular absorption of sugars has also been demonstrated for glucose. Where the saturable capacity of the Na-glucose transporter SGLT-1 is exceeded, there are observed changes in tight junction structure[278].

There are many determinants of sugar absorption that should be considered when evaluating IP studies. These include: bacterial fermentation (particularly in bacterial overgrowth); the concentration gradient between the lumen and subepithelium; the surface area of the epithelium; intestinal transit time (itself being reliant upon gastric empting and small bowel motility) and the intrinsic properties of the intestinal barrier (e.g., health versus disease)[279]. After absorption, the measurement of sugars in the urine is also impacted upon by hepatic metabolism and renal function. Further, administration of hyperosmolar compounds increases epithelial stress and increases the permeability of some sugars[280]. In order to account for these many variables, it has been proposed that a ratio between sugars that intrinsically account for some of these issues are used. The earliest validation study showed this to be the case in CD treated with a GFD and upon gluten challenge[281].

Although measurement of the administered carbohydrates in the serum may be just as accurate, concentrations are 100 times lower than in the urine and serum levels not been thoroughly investigated[282]. Therefore IP studies now routinely assess the ratio of two sugar carbohydrates measured in the urine collected over several hours after consumption by fasting subjects. Although the probe carbohydrates vary, common to all methods is the consumption of a higher molecular weight (MW) sugar (such as lactulose) together with a lower MW carbohydrate (such as rhamnose or mannitol). Substrates above a size of 0.5nm have restricted perfusion and thus a size lower than this is the most appropriate for assessing IP and various probes have been used[283]. As outlined in Table 1.5, a variety of probes have been used. Lactulose in combination with either rhamnose or mannitol are ideal markers of small intestinal barrier function as under normal physiological conditions. Lactulose resists hydrolysis by small intestinal disaccharidases and urinary excretion is rapid. Its absorption and measurement is also unaffected by renal and hepatic function[280]. Also, lactulose, rhamnose and mannitol all have high urinary recovery rates

after intravenous injection suggesting low systemic utilisation[275,284]. The ideal dose of lactulose is between 5 and 7.5 g as higher doses have been shown to affect the absorption of rhamnose[285]. The use of polyethylene glycol (PEG) as a substrate has been limited by its variable lipid solubility and variable recovery after intravenous injection suggesting peripheral metabolism. PEG may also traverse the epithelium by a combination of transcellular and paracellular routes[285]. These physiological tenets need to be borne in mind, particularly in the quantification of single carbohydrates used to attach significance to observations in more recent studies[286]. The length of time over which the urine collection takes place is also important. Whilst up to 11 hours may be required to retrieve all of the ingested substrate, most is retrieved between 3 and 7 hours and thus 5 hours has been accepted by most as adequate[287].

Alterations of permeability have been proposed to increase susceptibility to many human diseases[279]. These studies are summarised in Table 1.6. Increased IP in diarrhoea-predominant IBS may be regulated by micro-RNA and glutamine[288] but the mechanism for the observed increased zonulin levels in Type I diabetes is unclear. In one case report, it has been suggested that increased IP may be a harbinger of the development of Crohn's disease in later life whilst it might predict relapse in established Crohn's disease[289-291]. An observed increase in IP in unrelated cohabitants of those with Crohn's may point toward an environmental trigger for disease development[292]. Links to elevated IP in these and other non-gastrointestinal illnesses have been embraced by alternative health practitioners. In this context, the use of these IP studies is anecdotally widespread and has been used as evidence of a 'leaky gut'.

Table 1.5 Available substrates for assessment of intestinal permeability

SUBSTRATE	MW	Radius	Effect of Hyperosmolar stress
Inulin	5000 (approx)	15-20A	-
Cellobiose	342	0.5nm	-
Cr-EDTA		5.25A	-
Glucose	180	?0.4nm	-
Lactulose	342	0.54nm	Increased
Lactose/sucrose/melibiose	342	0.5nm	-
Raffinose	504	0.59nm	Increased
Stachyose	666	0.62nm	Increased
Dextran	3000	1.25nm	Increased
L-rhamnose	164	0.4nm	-
D-Mannitol	182	0.4nm (3.35A)	Not affected (Cobden, Hamilton et al. 1985)

Table 1.6 Summary of conditions found to be associated with increased intestinal permeability

Condition	Context	References
Diarrhoea predominant IBS	Patients	Dunlop, 2006[293] Mujagic, 2014[294]
Crohn's disease	Prediction of relapse Cohabitants	Hollander, 1986[295]
Primary Biliary Cirrhosis	Patients	Feld, 2006[296]
Autism	Patients Relatives	de Magistris, 2010[297]
Mental illness	Schizophrenia Depression	Wood, 1987[298] Maes, 2008[299]
Type I Diabetes	Patients	Sapone, 2006[300]

Coeliac disease provides an attractive model for studying IP as it has a well defined histology along with easily obtained peripheral markers of disease activity (coeliac antibodies) that have allowed comparisons with disease progress. An anatomical basis for impaired barrier integrity has been demonstrated by freeze-fracture electron microscopy while expression of tight junction proteins is affected by gluten challenge potentially allowing the passage of toxic gluten-derived peptides[301,302]. Some anatomical deficits persist despite treatment, a finding supported by one

study examining the use of Cr-EDTA as a urinary marker for intestinal permeability[303]. With rare exceptions, CD affects the proximal small bowel where the absorptive capacity of many of the sugar substrates used in IP studies is greatest and where, under normal conditions, the surface area is greatest[304].

Fundamental to the pathophysiology of CD is the exposure of the mucosal immune system to gluten peptides that have been deamidated by tTG and presented with high affinity to T-cells in those with an appropriate genotype. The mechanism for passage of these gluten peptides to the submucosa is yet to be fully understood but intact gluten peptides been found in the plasma of both animals and humans after gluten challenge[18,305]. Transcytosis of the 33-mer has been observed in cell lines and in human epithelial cells, perhaps mediated by IFN-gamma[306]. Also, genetic linkage studies have attributed a 2 to 3 fold increased risk of developing CD to a variant of myosin IXB, a gene involved in regulation of actin filaments and thus tight junction permeability[21]. This interaction has been proposed to be facilitated by an epithelium that is more permeable even before CD develops. Urinary IP studies have been used for several decades to measure the degree if permeability in CD and has been proposed as a non-invasive marker of response to therapy. Some have found urine IP studies to have higher sensitivity and specificity than coeliac antibodies[307].

Arising from the above observations, a potential therapy for CD disease has been developed. Lorazetide is an orally active zonulin receptor antagonist that has been proven to prevent tight junction opening both anatomically and by using urine IP studies. In CD this has potential benefit in preventing transepithelial passage of the causative antigen. Although early studies

have not demonstrated any effect on urine IP, lorazetide may attenuate the antibody response in gluten challenge[308]. Results from later phase clinical trials are awaited.

Intestinal permeability studies have been investigated for decades in many intestinal and non-intestinal diseases. On superficial analysis, IP studies are an attractive technology. The substrates used are cheap and readily available, they appear to be accurate and reproducible in many of the studies published and analysis is also relatively simple[280,309,310]. However the long collection periods (at least 5 hours), patient compliance with complex instructions and variability of methodology in the available studies have limited broad acceptance (Table 1.7). In an attempt to rationalise the many published methods, a compromise in methodology has recently been proposed and awaits validation in clinical trials[311].

In the absence of proven therapies that alter IP and as long as assessment is hampered by the practical issues outlined above, urine IP studies largely remain a research tool and results should be interpreted with knowledge of their limitations. Recent advances in the assessment of the mucosal barrier such as tight junction gene expression measured by real time PCR may be a more sensitive and specific means of assessing barrier function[286].

Table 1.7 Summary of intestinal permeability studies in coeliac disease (C=cellobiose; Ma=mannitol; LL=lactulose; Rh=rhamnose)

First author, year	Population	Sugar probes	Sugar ratio	Collection period	Osmolality (mOsmol/L)	Main results
Cobden, 1980[310]	Controls n=55 Untreated CD n=24	cellobiose 5g, mannitol 2g, lactose 20g, sucrose 20g, water 100ml	C:Ma	5 hours	1500	Cut-off ratio of 0.1 identified 24/25 CD
Hamilton, 1982[281]	Controls (n=55) compared to CD: 50g gluten challenge n=6; newly diagnosed n=18; treated n=10, refractory n=3	cellobiose 5g, mannitol 2g, lactose 20g, sucrose 20g, water 100ml	C:Ma	5 hours	1500	Ratio 0.02 in controls, 0.29 in newly diagnosed CD (0.02 after treatment). Cellobiose reduced early with treatment of CD Gluten challenge resulted in increased ratio
Strobel, 1984[312]	Controls n=15 Untreated CD n=135	cellobiose 5g, Mannitol 2g, lactose 20g, sucrose 20g, water 450 mls	C:Ma	5 hours	1500	Ratio above 0.04 considered abnormal. No patient with villous atrophy had normal ratio
Cobden, 1985[287]	Controls n=55 Untreated CD n=37	cellobiose 5g, mannitol 2g, lactose 20g, sucrose 20g, water 100ml	C:Ma	5 hours	1500	Ratio unaffected by gastric emptying, intestinal transit or renal/liver failure. Sensitivity 89%, specificity 100% for villous atrophy
Juby, 1989 (Gut)[313]	1010 with GI symptoms	cellobiose 5g, mannitol 2g, lactose 20g, sucrose 20g, water 100 ml	C:Ma	5 hours	1500	Normal range 0.004 to 0.03 For diagnosis of CD: Sensitivity 96%, specificity 70%

First author, year	Population	Sugar probes	Sugar ratio	Collection period	Osmolality (mOsmol/L)	Main results
Juby, 1989 (Gastroenterology)[314]	Comparison of ratios Possible CD n=82 CD n=17	lactulose 5g, mannitol 2g, glucose 22.3g, water 100ml VERSUS cellobiose 5g, mannitol 2g, sucrose 20g, lactose 20g, water 100ml	LL:Ma vs. C:Ma	5 hours	1500	Cutoff ratio of 0.028 yields 94% sensitivity and 100% specificity for CD Both ratios equivalent accuracy
Greco, 1991[315]	Gluten challenge in: Controls n=19 CD on GFD for 2 years n=27	lactulose 5g, rhamnose 1g, water 100ml	LL:Rh	5 hours	220	Ratio for controls 0.05 Ratio increased form 0.1 to 0.3 in CD after gluten challenge More palatable solution for patients
Fernandez-Calle, 1993[316]	CD n=29 (20 compliant, 9 non-compliant)	lactulose 5g, rhamnose 1g, water 50ml	LL:Rh	5 hours	440	Ratio for those with healing 0.05 IP not useful in assessing compliance
Catassi, 1993[141]	Gluten challenge in CD n=20	cellobiose 5g, mannitol 2g	C:Ma	5 hours	iso-osmolar	No correlation between histological findings and IP results
Vogelsang, 1995[317]	Controls n=30 Untreated CD n=33 1st Degree relatives n=111	lactulose 10g, mannitol 5g, glucose 10g, water 100ml	-	5 hours	1300	Used 'permeability index' (ratio of percentages of each sugar recovered) Increased ratio in CD versus controls IP may be a useful adjunct to screening

First author, year	Population	Sugar probes	Sugar ratio	Collection period	Osmolality (mOsmol/L)	Main results
Smecuol, 1997[318]	Controls n=30 Untreated CD n=27 (n=15 with follow-up IP at 8 weeks) Diarrhoea with normal biopsy n=2	lactulose 5g, mannitol 2g, sucrose 100g, water 450ml	LL:Ma	Not stated	1800	Ratio 0.36 at diagnosis and reduced to 0.13 with treatment. Increased ratio in diarrhoea from other causes IP in very few returned to normal values
Smecuol, 1999[319]	1st degree relatives of CD n=66	lactulose 5g, mannitol 3g, sucrose 100g, water 450 mls	LL:Ma	Not stated	1800	Ratio 0.02 in relatives, 0.10 in those found to have CD Elevated L:M had 100% sensitivity and 83% specificity for CD
Cummins, 2001[320]	Controls n=75 CD n=36	lactulose 5g, rhamnose 1g, glucose 22.6g, water 100ml	L:Rh	5 hours	1500	Ratio 0.09 for controls IP improved from 0.47 to 0.16 after 2 years IP improved more rapidly than histology
Abazia, 2003[321]	Controls n=25 CD n=22	lactulose 7.5g, rhamnose 1g, sucrose 40g, water 220 ml	LL:Rh	5 hours	690	Ratio in controls of 0.04 Ratio in CD 0.4 Validated use of gas chromatography to measure sugar probes
Duerksen, 2005[322]	Controls n=19 CD: GFD for 1 (n=3), 1 to 12 (n=9) and >12 (n=46) months	lactulose 5g, mannitol 1g, sucrose 100g, water 300ml	LL:Ma	5 hours	Not stated	IP testing superior in detecting dietary transgression compared to coeliac antibodies

First author, year	Population	Sugar probes	Sugar ratio	Collection period	Osmolality (mOsmol/L)	Main results
Vilela, 2007[323]	Controls n=11 CD n=22 at baseline and 12 months of treatment	lactulose 6g, mannitol 3g, water 120mls	LL:Ma	6 hours	iso-osmolar	Ratio controls 0.08 Ratio CD 1.02 – higher in those who has persistently positive antibodies

Cytokines

Coeliac disease is being increasingly recognised as a systemic disease with the observation of increasing extraintestinal manifestations and symptoms. Circulating cytokines may have a role in initiating or perpetuating these symptoms and investigators have begun to explore this interaction. At the tissue level, analysis of cytokine profiles has provided insights into disease mechanisms and potential therapeutic targets. For example, increased tissue levels of IL-15 have been noted in refractory CD and may be a crucial link in the generation of intraepithelial lymphocytes and subsequent villous atrophy[324].

Peripheral markers of disease activity offer a non-invasive alternative to endoscopically-obtained tissue and may be a tool for disease monitoring. On a practical level, methodologies to assess cytokines can be complex and time-consuming. Samples need to be centrifuged, mononuclear cells need to be isolated and then cultured and incubated for 72 hours in a humidified atmosphere[325]. Alternatively, reverse transcriptase polymerase chain reaction (PCR) can be used on isolated leucocytes but is an indirect measure of protein production[326]. Whole blood analysis has some efficiency gains with a shorter incubation time[327,328]. Serum measurement of cytokines by ELISA based assays is an attractive alternative but may not be as accurate or sufficiently sensitive[202,326].

In IBS, mucosal and peripheral cytokine profiles have suggested an inflammatory basis that is not present on routine H&E stains of intestinal tissue[329]. Differences were also noted in subsets of patients with IBS with increases in stimulated release of TNF-α, IL-6 and IL1β in diarrhoea predominant IBS (D-IBS).

Several studies have examined peripheral and mucosal cytokine expression in CD and these are summarized in Table 1.8. Using a serum-base ELISA, Fornari and others observed an increase in IL-6 that paralleled improvements in BMD in participants with CD after treatment with a GFD[202] and IL-6 has a recognised role in bone resorption. Similar observations were reported in a later study[330]. Others have been able to demonstrate that the cytokine expression seen in CD is unsurprisingly tissue-specific[326] and that some cytokines (for example IL-18) can correlate with intestinal damage and increase after prolonged gluten challenge[331]. Some studies have lacked histological correlation which hampers assessment of results as a correlate to disease response[332]. In an effort to suggest a change in inflammatory profile of cytokines, others have proposed using a ratio (IFNγ:IL-10) suggesting that more of a Th-1 profile occurs in CD[333]. Recently, a number of peripheral cytokines were assessed in patients at different stages of CD including refractory CD[334]. Consistent with prior studies, IL-8 was found to be upregulated in active CD but serum levels of IL-6 were only elevated in EATL. Levels of IL-17 were also elevated consistent with some other autoimmune diseases[335]. Elevated levels of IL-8, IL-17, IL-22 and sCD25 were found in RCD but the types of RCD were not able to be differentiated.

In summary, measuring circulating cytokines as an indicator of disease activity and response is challenging both methodologically and in their interpretation. Accurate measurement requires complex and time-consuming methodologies although ELISA based serum measurement may provide a more practical alternative. Cytokines have a relatively short half-life in the serum and measuring messenger RNA (mRNA) in the tissue is an indirect measure of activity.

To counteract these variables, the measurement of cytokines in peripheral blood mononuclear cells (PBMC) after subjects have undergone gluten challenge has been proposed as a more

accurate means of cytokine assessment[336]. This technology (ELISPOT) has not only been utilised in gluten challenge studies to measure response to therapies but has also enabled mapping t-cell specific epitopes in CD[337].

Table 1.8 Peripheral and mucosal cytokine response in coeliac disease

	Cytokine	Mucosa	Reference (first author, year)	Serum	Reference	PBMC	Reference
Th1	IL-2	↑ (high at baseline) ↑ (after exposure of Bx to Gluten)	Lahat, 1999[326] Nilsen, 1998	↔	Lahat, 1999[326]	↑	Lahat, 1999[326]
	IFNγ	~↑ (difference in titre significant) ↑	Lahat, 1999[326] Nilsen 1998[338]	↔	Lahat, 1999[326]	↔ (same in inactive vs. active but more than control)	Lahat, 1999[326]
	TNFβ	↑	Lahat, 1999[326]			↔ (as above but none in control)	Lahat, 1999[326]
	IFN:IL-10 ratio					↑	Mizrachi, 2002[333]
Th2	IL-4	↑ (vs. inactive coeliac), ↑ (but same as untreated)	Lahat, 1999[326] Nilsen 1998[338]	↔	Lahat, 1999[326]	↑	Lahat, 1999[326]
	IL-10	↑, ↔	Lahat, 1999[326] Nilsen, 1998[338]			~↑	Lahat, 1999[326] Mizrachi, 2002

	Cytokine	Mucosa	Reference (first author, year)	Serum	Reference	PBMC	Reference
	IL-5	↔, ↔	Lahat, 1999[326] Nilsen, 1998[338]			↔	Lahat, 1999[326]
Pro-inflammatory	IL 1β	↑	Lahat, 1999[326]	↓	Fornari, 1998[202]	↑	Lahat, 1999[326]
	IL 6	↑, ↑ (after Bx given gluten)	Lahat, 1999[326] Nilsen 1998[338]	↓ ↑	Fornari, 1998[202] Romaldini, 2002	↑ (n=2)	Lahat, 1999[326]
	IL-1 RA			↑	Fornari, 1998[202]		
	TNF α	↑, ↑ (“)	Lahat, 1999[326] Nilsen, 1998[338]	↔	Romaldini, 2002		
	IL-18			↑	Fornari, 1998[202]		
Anti-inflammatory	TGFβ	↑, ↔	Lahat, 1999[326] Nilsen, 1998[338]			~↑	Lahat, 1999[326]
Other	sIL-2R			↑ ↑	Lahat, 1999[326] Romaldini, 2002[332]		

	Cytokine	Mucosa	Reference (first author, year)	Serum	Reference	PBMC	Reference
	IL-21	↑	Fina, 2008[339]				
	IL-12	↔	Nilsen, 1998[338]				

Assessment of small bowel surface area

Up to 50% of the proximal small bowel surface area is contained in the first 25% of the small bowel[304]. Being largely confined to the proximal small bowel, CD lends itself well to non-invasive assessments of enterocyte mass.

Breath tests

Sucrose is a disaccharide metabolised by sucrase produced from the brush border of the proximal small intestine[340]. When ^{13}C-sucrose is administered orally, metabolised sucrose can be measured indirectly via expired carbon dioxide ($^{13}CO_2$) and quantification might reflect small bowel surface area. The ^{13}C-sucrose breath test was first evaluated in rats and subsequently in children with chemotherapy-induced mucositis[341,342]. Although accurate in these situations, the test has not been subjected to analysis in adults or in patients with CD. Thus, its implementation has been very limited.

Citrulline

Citrulline is an amino acid that can be measured in the serum and has been proposed as a non-invasive measure of small bowel mass. Citrulline is produced almost exclusively from the small bowel epithelium from glutamine and does not undergo any appreciable first-pass metabolism[343,344]. In patients with short gut syndrome, fasting plasma citrulline as assessed by ion exchange chromatography accurately separated patients with short gut from controls and also correlated with small bowel length[344]. The same authors found that a fasting citrulline of less than 20 μmol/L was accurate in predicting villous atrophy in a variety of small bowel disorders, including HIV enteropathy and that it might be useful in monitoring response to therapy in

CD[345,346]. Indeed, in children with CD, citrulline levels increased from low baseline levels after commencement of a GFD although histological correlation has not been performed[347]. These results above have been contradicted by other investigators who compared bomb calorimetry to serum citrulline in a groups of patients with CD to and refractory CD, and in healthy controls. Although citrulline concentration was lower in refractory CD than in healthy controls, it was within the quoted reference range and those with uncomplicated CD had levels similar to controls[348]. A citrulline generation test has subsequently been proposed whereby an oral dose of amino acids is administered to patients. The aim of this methodology was to refine the accuracy of serum citrulline measurements but this has not been validated elsewhere and is complex to administer as a routine monitoring test[349]. Thus, after initial promise, little has been published in recent years regarding the use of citrulline as a marker of enterocyte mass in CD.

Intestinal fatty acid binding protein

Intestinal fatty acid binding protein (i-FABP) is a small protein located exclusively in the small bowel and released into the circulation in circumstances of intestinal enterocyte damage, such as that seen in small bowel ischaemia or infarction[350,351]. Levels of i-FABP have also been assessed as an adjunctive tool in the diagnosis and follow-up of both adults and children with CD[352-354]. In children, circulating levels of i-FABP decline rapidly to normal levels upon commencement of a GFD in children with CD[354]. In a retrospective study of adults, levels of i-FABP correlated with the degree of villous atrophy at diagnosis of CD. However, during follow-up, levels did not return to normal in some despite histological response[352]. The discrepancy between children and adults may in part be explained by the shorter duration of disease and by the higher histological response rates that are seen in children[168]. Thus far, all publications evaluating i-FABP have originated from the same group. Studies in other populations with prospectively collected data are awaited.

Other non-invasive tests

Metabolomics

Metabolomics (or metabonomics) is a relatively new field in disease diagnostics. It involves interrogating metabolites in a biological sample (usually urine or serum) and observing responses to disease and treatment. It may provide a tangible link between patient genotype and phenotype[355]. Traditional metabolite profiling has been applied to study CD, but the results are limited. Significantly elevated levels of nitric oxide (NO) in urine of children with CD consuming a gluten-containing diet has been found, and NO concentrations generally double between 2-4 weeks of consuming approximately 10 g of gluten per day during a gluten challenge[356]. A positive correlation between gluten intake and increases in both intraepithelial lymphocytes (IELs; from gut biopsies) and mean urinary NO concentrations were found, but not between IEL count and NO concentrations. The authors, therefore, suggested that NO products and IELs have different metabolic origins and are not just different markers of mucosal inflammation. Similar findings have been noted with other gastrointestinal diseases including IBD[357].

One of the earliest metabolomics studies of CD used NMR spectroscopy to analyse serum and urine samples from patients with CD and from control subjects. Sera of coeliac patients showed decreased levels of amino acids, lipids, pyruvate and choline, and higher levels of glucose and 3-hydroxybutyric acid, while urines showed higher levels of indoxyl sulfate (IS), meta-[hydroxyphenyl]propionic acid (mHPPA) and phenylacetylglycine (PAG)[358]. Lower levels of pyruvate could suggest impaired glycolysis whilst elevated levels of mHPPA, IS and PAG have all been linked to changes in the gut microbiome and may have important implications for the understanding of the pathophysiology and response to treatment in patients with CD[358,359].

Whether metabolomics alterations are associated with mucosal inflammation in CD has also been studied by comparing overt and potential CD[360]. Comparison of urine and serum samples from control, overt coeliac and potential coeliac cohorts via ^{1}H NMR spectroscopy showed that the metabolic profile of patients with potential CD is close to patients with overt CD. With respect to urine samples, many more metabolites could differentiate potential CD from overt CD than from controls, and key differences were related to metabolites originating from gut microbiota (m-HPPA, IS, PAG; as previously demonstrated[361]). This suggested a relationship exists between overt CD, villous atrophy and the bacterial consortia of the host. Based on the similarity in serum profiles and dissimilarity in urine profiles between overt and potential coeliac patients, the authors surmise that whilst alterations in urine profiles may follow intestinal damage, an abnormal immune response to gluten exists before intestinal damage can be detected. In this way, metabolomics may be used to detect CD when its clinical manifestation is not fully evident and identify patients who should have more vigilant follow-up to identify those who may benefit from the introduction of a GFD before complications ensue[360].

The results support the hypothesis that CD is associated with intestinal and faecal dysbiosis. The metabolomic approach provides a means of monitoring whether such a dysbiosis can be corrected by, for example, prebiotics and probiotics[362].

To date, metabolomic analysis of mucosal biopsies has not been reported for CD. Application of HR-MAS NMR and/or NIMS to these samples may facilitate significant new information for improved understanding of the mechanisms underlying the onset and manifestations of CD and what metabolites/biomarkers are critical in determining the extent of villous atrophy and disease progression. While the ultimate aim for CD is to develop non-invasive approaches for diagnosis

and prognosis, this may require the intermediate step of concurrent metabolomic analysis of tissue blood, urine and possibly faeces to fully understand the metabolome associated with and altered biochemical pathways inherent to CD.

Prolactin

Elevated prolactin levels have been noted to be elevated in a number of autoimmune conditions including SLE and rheumatoid arthritis[363,364]. In children with newly-diagnosed CD, serum prolactin is higher than in control patients and decreases rapidly upon commencement of a GFD. This pattern is possibly driven by circulating cytokines[365].

Application of prolactin levels to adults with CD is likely to be hampered by variation caused by disease, mood disorders and concomitant medications. Its application has, however, not been studied.

Ghrelin

Ghrelin is a peptide hormone produced from entero-endocrine cells in the gastrointestinal tract and is involved in the regulation of appetite. Secretion of ghrelin is increased by emptiness of the stomach or when there is a positive energy balance. In a group of women with CD, associated nutritional deficiencies and a low BMI, serum ghrelin was higher at diagnosis than in disease controls, and fell significantly with treatment with a GFD[366]. In another group of patients with CD and nutritional depletion, ghrelin overall decreased with treatment of CD, but levels increased in a third of the patients[367]. Whether serum ghrelin may be associated with histological disease activity is controversial. In a study of underweight (mean BMI 19kg/m^2) patients ghrelin was

associated with histological severity at diagnosis and was greater than those with CD compared to controls. Whilst ghrelin reduced with treatment, it also inversely correlated with increasing BMI in all groups[211]. Tissue levels of ghrelin may also correlate with histological inflammation[211]. Only one prospective study assessing paired ghrelin levels in patients with CD has been undertaken. There was no correlation of baseline ghrelin with histology but levels did reduce with improving histology. Body mass index also increased with treatment and this may have confounded the observed changes[367]. Thus, despite correlation with disease response, the above findings would suggest ghrelin is more reflective of the changes in energy balance and nutrition with treatment rather than being accurately reflective of small bowel inflammation.

Antibodies to glycoprotein-2

Glycoprotein-2 antibodies have recently been found to be elevated in small bowel but not colonic inflammatory bowel diseases[368]. Glycoprotein 2A (GP2A) is derived from the pancreas. Antibodies to GP2 (GP2A-IgA) are proposed to represent abrogation of the intestinal epithelium caused by tissue inflammation. A recent study found elevated levels of GP2A-IgA in both CD and refractory CD[369]. Levels decreased with treatment with a GFD in CD but levels did not correlate with the degree of intestinal atrophy. Further studies are needed to define the role of GP2aA-IgA in the follow-up of CD.

Non-coeliac gluten sensitivity

Background: Wheat – the largest source of dietary gluten

The structure of wheat is complex. The wheat kernel is encased by a fibrous husk (peri-carp) which is easily removed during processing and used as high fibre bran. Beneath the peri-carp is a layer of starch (endosperm) rich in fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) but also rich in proteins that form the basis for many human diseases. The protein content of wheat varies and can vary from harvest to harvest[2], but is approximately 15-20%. These proteins consist largely of albumin, globulin and gluten, and vary in proportion according to regional and seasonal factors. Within the endosperm lies the germ. The germ contains lipids, more proteins (including lectins, such as wheat germ agglutinin), vitamins (particularly vitamin E) and minerals.

The role of wheat in non-gastrointestinal disease

Although not a key focus of this thesis, wheat does pay a role in some non-gastrointestinal diseases. The better-defined conditions include the following.

Baker's asthma

Sensitivity to wheat antigens is identified on skin prick testing in up to 15% of bakers. In a proportion, this can be manifest as bronchoconstriction and so-called Baker's asthma. Diagnosis can be confirmed by either high titres of specific IgE antibodies or by inhalational challenge with either wheat or rye derived antigens[370,371]. The main group of causative antigens in Baker's asthma is alpha-amylase trypsin inhibitors (ATI's) but also includes peroxidases, thioredoxin, non-specific lipid transfer protein and prolamines in gluten and gliadin[372,373]. Treatment of

Baker's asthma requires avoiding exposure to the causative antigen, but desensitisation, immunomodulatory and anti-asthmatic treatment have also been used[372].

Wheat allergy

Wheat allergy is though to be one of the more common food allergies and can result in typical IgE-mediated phenomena such as urticaria, angioedema, bronchoconstriction and occasional anaphylaxis[374]. Gastrointestinal manifestations can include nausea, abdominal pain and diarrhea and thus confusion exists with regards to an overlap with IBS. The use of skin prick testing is established but there is a wide background incidence of wheat allergen incidence (i.e. positive skin prick testing). In these circumstances, the incidence of wheat allergy may be as high as 9% in older children but fall to a prevalence as low as 0.5% in some populations[375,376]. Further complicating the picture is the discrepancy between the incidence of serological evidence of wheat allergy and reported symptoms of wheat allergy[376]. Although widely reported, wheat-dependent exercise induced allergy (WDEIA) has been poorly studied and may merely be representative of physical exertion unmasking or potentiating other allergic phenomena. Interestingly WDEIA has been linked to ω-gliadins and other high molecular weight glutenin subunits but alpha amylase trypsin inhibitors (ATI's) (which are water/salt soluble) have also been implicated[374]. In addition, lipid transfer proteins have also been identified in a sub group of patients with wheat allergy[377].

Atopic dermatitis

Wheat proteins also have a role in atopic dermatitis. After initial identification of gluten derived peptides causing atopic dermatitis[378], specific gluten epitopes have now been identified as allergens and share sequence homology with identified allergens in WDEIA[379]. Also consistent

with the observations in WDEIA, amylase trypsin inhibitors have been implicated in a study analyzing patients from Denmark, Italy and Switzerland[380].

The use of a gluten free diet to manage gastrointestinal symptoms outside of coeliac disease

In clinical practice, some patients have symptoms of irritable bowel syndrome (IBS) that respond well to a GFD but have no markers of CD. Until relatively recently, the published scientific literature had been devoid of the so-called "non-coeliac gluten intolerance" and "wheat intolerance", yet they were widely believed to be very common[381-383]. In the evaluation of exclusion diets, wheat has been found to be one of the most common factors inducing gastrointestinal symptoms[384], but it is not known whether gluten is the responsible agent, since wheat, the major cereal removed from the GFD, contains other proteins and carbohydrates, such as fructans, that are capable of inducing symptoms themselves[385]. There is now a solid evidence base indicating that fructans and some other poorly absorbed, short-chain carbohydrates (Fermentable Oligo-, Di- and Mono-saccharides And Polyols or FODMAPs) are responsible for the symptoms of IBS in the majority of people[386,387].

The role of gluten in CD is clear. The toxic peptide sequences have been defined, the genetic susceptibility loci identified and the pathological processes comparatively well known[25,27]. Deamidation of these gliadin epitopes by tissue transglutaminase (tTG) enables them to be presented with high affinity to MHC Class II T-cells in genetically susceptible individuals (HLA-DQ2 or -DQ8 being expressed in 99.4 % of patients with CD[388]). This process initiates a cascade of events resulting in mucosal inflammation, small intestinal villous atrophy, increased intestinal permeability, malabsorption of macro and micronutrients and resultant complications of CD[310,389].

Non-coeliac gluten sensitivity was first described in the late 1970's[390,391]. Prior to recent work, the literature regarding the effect of gluten outside of CD has been limited to experiments in cancer cell lines and to uncontrolled clinical studies[381,383,392-395]. Whether gluten itself can contribute to gastrointestinal symptoms and/or induce injury to the proximal small intestine in non-coeliac patients had not been directly assessed until the first randomised controlled trial presented in this thesis[396].

Non-coeliac gluten sensitivity – the evidence

Prior to the study presented in Chapter 5, few clinical studies had adequately evaluated the role of gluten in gastrointestinal disease. In animal models and cultured cell lines, gluten has been shown to disrupt tight junction structure[397], impair RNA synthesis[393] and alter gut contractility[398]. In one of the few studies performed in humans[381], gluten withdrawal was found to improve gut symptoms in those without CD. However this study was not blinded and a number of patients had Marsh I lesions on duodenal biopsy raising the possibility that they indeed had CD. Interestingly, this paper did suggest a higher incidence of 'non-coeliac gluten intolerance' in those expressing genetic susceptibility for CD. Older literature examining breath hydrogen responses to carbohydrate ingestion suggested that carbohydrates administered with gluten are more poorly absorbed than relatively gluten deprived carbohydrate[399]. Therefore, gluten may contribute to malabsorption and subsequent fermentation of wheat starch.

The avoidance of gluten is reported to be very common. Worldwide, the prevalence of this largely self-prescribed dietary treatment seems to vary. In New Zealand children, the prevalence is as high as 5%[400] whilst in the USA a recent study suggested as few as 0.6% follow a GFD[401]. In

perhaps the most robust evidence to date, avoidance of wheat in Australia maybe as high as 10% in adults and is more common in females and in those who avoid dairy products[402]. The results were derived from a subsection of a larger questionnaire distributed to the general population. This study may even have underestimated the prevalence due to the exclusion of patients who may not have CD.

The lack of a precise definition of these observations has also hampered progress in this field. Recently, an expert consensus was reached and, importantly, rationalised the nomenclature to non-coeliac gluten sensitivity (NCGS) which was formally defined in this publication as "immunological, morphological or symptomatic manifestations that are precipitated by the ingestion of gluten in people in whom CD has been excluded"[11]. It has also been proposed that NCGS be diagnosed dependent on symptomatic response to a blinded gluten challenge but this may have practical limitations[222]. Clinically, the most common symptoms of NCGS are bloating, abdominal pain and fatigue[49,402]. In a USA epidemiological study, patients with NCGS had similar demographics and BMI to those with CD and had a lower rate of hypertension than the control population[403]. In the clinical assessment of patients reporting NCGS, the adequacy of CD exclusion is also important. In this regard, conclusions have been difficult to make in several clinical studies in NCGS owing to the presence of patients that may fall on the spectrum of CD (either by having mucosal inflammation or elevated celiac specific antibodies)[381,404].

A recent randomised study evaluated 45 gluten consuming individuals with diarrhoea predominant IBS[286]. Participants were randomised to a GFD or gluten-containing diet (GCD) for 4 weeks with all food supplied from the investigating centre. Participants underwent assessments of gastrointestinal symptoms, intestinal permeability (via urine IP studies and mucosal tight junction

gene expression from small bowel and rectal biopsies) and intestinal motility. In addition, an in vitro assessment of cytokine response to in vitro stimulation of PBMC's with gluten was also performed. Stool frequency reduced in those randomised to a GFD whilst intestinal permeability was increased in the GCD compared to the GFD. Both stool frequency and IP were more reduced in those with an HLADQ2/DQ8 haplotype. Some alterations in mucosal tight junction gene expression were also noted in those possessing the coeliac genotype. No overall differences were observed in colonic permeability, stimulated cytokine response or GI motility. But increased TNFα was observed (but not IFNγ) pointing towards a possible innate immune response in NCGS, a finding supported by other groups[405].

An explanation for the disparate conclusions may lie in the dietary intervention being administered. Although the diet in the study by Vazquez-Roque was gluten free, whether there were differences between the dietary arms in wheat-derived carbohydrates or other dietary triggers for symptoms was not directly reported. Adoption of a GFD not only eliminates gluten, but also substantially reduces the consumption of carbohydrates due to the exclusion of high-FODMAP gluten containing grains[406,407]. Reduced FODMAP intake on a GFD might also explain observations in another retrospective study[404]. Whether FODMAP's have any effect on intestinal permeability has not directly been assessed, but high osmolar loads affect permeability of the intestine and undigested carbohydrates may exert similar effects[280].

In summary, there has been a great deal published over recent years in non-coeliac gluten sensitivity that has provided insights into proposed disease mechanisms and epidemiology, but the very existence of NCGS has indeed been challenged by the second of two randomised trials presented in this thesis[396,408].

Chapter 2: Hypothesis and aims

The overall aim of this thesis is to expand the evidence-base of the role of gluten and gluten-free diet in human disease. Particular focus is directed toward CD and NCGS.

Studies in coeliac disease

In defining evidence-based strategies for population screening and to better define treatment paradigms for CD, it is of paramount importance to understand the impact that adherence to a gluten-free diet has on multiple indices pertaining to the longer term outcomes and quality of life of patients. This has become especially important now that CD is increasingly diagnosed when there is a paucity of symptoms.

Hypothesis 1

In CD, a gluten-free diet is associated with correction of abnormalities that span from symptoms, to the intestinal lesion, to body composition and to cognitive function.

Aims 1.1 to 1.6

To perform a 5-year prospective follow-up study of a well-characterised cohort of patients with newly diagnosed CD examining pre-specified end-points and at defined time points (diagnosis, one year and five years). At each time point, assessment aimed to address the following issues by examining routine blood tests, coeliac antibodies, gastrointestinal symptoms, small bowel histology, body composition analysis and dietary compliance:

1.2 To characterise the patient cohort at diagnosis (Chapter 3.1)

1.3 To define the ability of adherence to the GFD to normalise coeliac antibodies and achieve mucosal response and remission (Chapter 3.1)

1.4 To identify patient characteristics that increase the risk of ongoing mucosal inflammation (Chapter 3.1)

1.5 To examine non-invasive markers of intestinal healing including gastrointestinal symptoms, coeliac antibodies, intestinal permeability and cognition (Chapter 3.1 and Chapter 4)

1.6 To determine the impact of HLA genotype on patient characteristics at diagnosis, one year and five years (Chapter 3.2)

1.7 To perform detailed analysis of biochemical and haematological abnormalities during the first 5 years of treatment with particular attention to:

a. The pattern of liver function test abnormalities in relationship to body composition (Chapter 3.3)
b. The incidence of neutropenia (Chapter 3.4)

Hypothesis 2

Qualitative point-of-care testing (PoCT) of coeliac antibodies offers an alternative to quantitative serum based antibody assays in the long-term follow-up of CD.

Aim 2.1

To evaluate the accuracy of PoCT in the follow-up of CD by comparing serum ELISA results obtained after 5 years of treatment to a PoCT kit utilising a combined DGP IgA/IgG calorimetric read-out (Chapter 3.5)

Studies in non-coeliac gluten sensitivity

The role of wheat protein/gluten in inducing gastrointestinal symptoms in patients without CD is controversial, particularly in view of the recent demonstration that short-chain indigestible carbohydrates (FODMAPs) in wheat can induce similar symptoms.

Hypothesis 3

In patients who do not have CD but believe they are sensitive to gluten, wheat protein devoid of FODMAPs can specifically induce gastrointestinal symptoms and does so by specific mechanisms.

Aim 3.1 to 3.8

To address these hypotheses by performing three randomised, double-blind, placebo-controlled dietary rechallenge trials and by reporting the results of a survey distributed to applicants for these studies.

The first trial randomised patients with NCGS following a gluten-free diet for at least 6 weeks to either gluten (16g) or placebo (Chapter 5.1). This trial aims to:

3.1 Assess differences in gastrointestinal and systemic symptoms between groups

3.2 To screen for potential mechanisms by which wheat protein causes symptoms by assessing coeliac antibodies, intestinal permeability and highly sensitive c-reactive protein

In the second trial, designed according to the gold standard of establishing causation of food antigens, participants with NCGS are randomised to 3 treatment arms in a crossover fashion (Chapter 5.2). This trial aims to:

3.3 Assess differences in fatigue, physical activity and gastrointestinal symptoms between intervention arms

3.4 Eliminate potential confounders to any differences between treatment arms by

a. Providing all main meals to study participants and ensuring these meals are low in FODMAPs

b. Commencing participants on a low FODMAP diet for at least one week prior to randomisation

3.5 To determine whether there is a dose response to gluten in patients with NCGS by randomising participants to high dose (16g), low dose (2g) and placebo (0g)

3.6 To screen for a potential mechanism by which gluten causes symptoms by examining coeliac antibodies, IFN-γ expression of gliadin specific T-cells (ELISpot), human eosinohil cationic protein, IgE antibodies to wheat, faecal calprotectin, faecal pH and faecal ammonia

The third is a randomised, double blind, placebo controlled, 3-day dietary rechallenge trial. (Chapter 5.2). The aims of this study are to:

3.7 Measure the response in gastrointestinal symptoms and fatigue to each of the three treatment arms (16 g/day whole-wheat gluten; 16 g/day whey protein isolate or no additional protein)

3.8 Exclude any further dietary confounders by ensuring the background diet of participants was not only gluten free and low FODMAP, but also dairy-free and low in food chemicals that have been shown to cause symptoms in other populations

Lastly surveys received from applicants to the above randomised trials are analysed (Chapter 5.3). The aims of this study are to describe the characteristics of patients who believe they have NCGS by reporting the diagnostic tests they have undertaken and the adequacy of exclusion of CD

Chapter 3: A five year longitudinal study of coeliac disease from diagnosis

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3.1 Adherence to the gluten-free diet can achieve the therapeutic goals in almost all patients with coeliac disease: a five-year longitudinal study from diagnosis

INTRODUCTION

The epidemiology of coeliac disease (CD) is changing. No longer is CD typically a disease of profound protein-calorie malnutrition but a condition affecting equally those underweight and overweight, symptomatic and asymptomatic, and nutritionally replete and deficient[6]. In concert with these epidemiological shifts, evidence from several populations indicates an increasing incidence of CD independent of physician recognition and practice[8,9]. As the epidemiology changes, the concepts of disease management and outcomes are also being challenged.

Guidelines for follow-up of CD vary and clinician adherence to these guidelines is patchy[409,410]. Intuitively, the aim of treatment in CD should be to heal the mucosal pathology that defines the condition. The importance of mucosal healing has been highlighted by the association of ongoing mucosal inflammation in CD with osteoporosis, autoimmune disease and cancer[177,179]. Although mucosal healing in children appears to occur in the vast majority[159,180], the converse may be true in adults. Even in those with adequate dietary adherence, mucosal remission rates can be as low as 8%[157]. Although the natural history of mucosal healing poorly understood, retrospective studies suggest that healing rates improve over time[100]. Risk factors for poor mucosal healing have included the degree of villous atrophy, severe clinical presentation and HLA DQ gene dosage[100].

Complicating adherence to follow-up guidelines, reliable tools to predict mucosal healing have been lacking and prospective data on currently available tools are limited[159]. The titre of endomysial antibodies (EMA) is poorly predictive of mucosal recovery in CD[160] but IgG antibodies to deamidated gliadin peptides (DGP) may be a more accurate marker if a lower cut-off is used[161]. Coeliac antibodies can remain elevated in up to 46% after a year and of these up to 48% will have normal mucosa (Marsh 0)[139]. Gastrointestinal symptoms have also been used to assess clinical response to the gluten free diet (GFD) in CD but up to 50% of screen-detected individuals have no symptoms and symptoms are a poor discriminator for CD when relied upon as a screening tool[15,45].

In concert with the aims of mucosal healing, treatment of CD should also aim to reduce long-term complications and nutritional consequences. Reduced bone mineral density is a well-recognised complication of untreated CD and is found in 20 to 76% at diagnosis[187-189] with similar incidences found in those with treated CD despite dietary adherence[190]. Reduced BMD may be the earliest manifestation in those who are asymptomatic[191] and those with positive coeliac antibodies have a lower BMD than healthy controls[192]. BMD seems to improve with treatment on a GFD[194-196] while in children and adolescents recovery of bone mineral density can be rapid and complete[197].

Evaluation of body composition in CD has noted normalisation of both low fat and lean body mass in adolescents after one year[146], but studies in adults have either lacked measures beyond fat indices, used outdated antibody assays and/or have not correlated changes with histology[205,206,209]. Long term prospective data on the effect of a GFD on bone mineral density and body composition are lacking.

In the present study, we aimed to evaluate the effect and time-course of treatment of patients with newly-diagnosed CD with a GFD on mucosal healing, body macro- and micronutrient composition, and coeliac serology in a population of patients with newly diagnosed CD followed prospectively for 5 years.

METHODS

Patients

Consecutive patients with a new diagnosis of CD, aged 18 years or older, referred to a single dietetic service that sees patients referred widely from gastroenterologists and general practitioners in both public and private settings, were invited to participate in this five-year prospective study. Inclusion criteria comprised the diagnosis of CD based on duodenal histology with Marsh 2 or greater lesions or Marsh 1 lesions with positive coeliac-specific serology, and the presence of an HLA susceptibility genotype (HLA DQ2 or DQ8). Those who were pregnant or had significant comorbidities, including severe psychiatric disorders, were excluded. It was planned to enrol approximately 100 patients for full assessment at baseline (i.e., untreated). The first 60 patients recruited were re-studied in detail at 1 year. After 5 years, all participants were again contacted with an invitation to undergo full repeat evaluation.

Protocol

All subjects underwent dietary education in the GFD by an expert dietitian (SJS) including the provision of written materials, food listings and referral to the Coeliac Society of Victoria. This education was refreshed after 6 weeks and again at 12 months by the same dietitian. At the one- and five-year assessments, adherence to the GFD was assessed (see below). At each time point, the subjects were assessed clinically and the concomitant medications including the use of vitamin D and nutritional supplements were recorded. They also had peripheral blood taken, and

body composition assessed by anthropometry and total body dual energy x-ray absorptiometry (DEXA). Endoscopic duodenal biopsies were also taken at one and 5 year time points. The protocol was approved by Eastern Health Research and Ethics Committee, Southern Health Ethics Committee and Monash University Standing Committee on Ethics in Research Involving Humans. All participants gave written, informed consent prior to entering study.

Gastrointestinal symptoms

The presence and severity of gastrointestinal symptoms were evaluated at each time point, but different measures were used.

- At the first interview prior to review of the histology and coeliac serology reports, the impact that gastrointestinal symptoms had on daily life was assessed. The patients were classified into three groups according to the following definitions: 'severely symptomatic' if experiencing debilitating symptoms that impaired ability to carry out daily activities (work or sleep), 'moderately symptomatic' if symptoms were sufficiently severe and frequent to be inconvenienced by them, but not to impair ability to carry out daily activities (work or sleep), or 'minimally symptomatic' when symptoms were absent or so trivial and infrequent that they were not considered by the patients as troublesome.
- At the one-year assessment, a previously validated, self-administered symptom questionnaire addressing the frequency and distress cause by those symptoms was used at entry to the study and at each follow-up visit[411]. The gastrointestinal symptoms addressed were abdominal bloating, abdominal pain, excess wind, loose watery bowel motions, and changes in stool habits over the preceding period since the previous review. Frequency was judged according to a scale of zero to three, where 0 = never, 1 = from time to time, 2 = frequently (more than 25% of the time) 3 = nearly always (more than 75% of the time). Distress caused was judged

according to a scale of zero to four where 0 = no distress, 1 = mild distress, 2 = intermediate distress, 3 = severe distress, 4 = very severe distress.

- At 5 years, gastrointestinal symptoms were assessed via a 100 mm visual analogue scale (VAS) assessing the presence and severity of overall abdominal symptoms, abdominal pain, bloating, wind, satisfaction with stool consistency and tiredness. Symptoms were then graded mild (≤20 mm), moderate (21-50 mm) or severe (≥51 mm)

Coeliac serology and vitamin D levels

Coeliac-specific antibodies were measured: endomysial antibodies were assessed via immunofluorescence using monkey oesophagus as substrate; tissue transglutaminase IgA (tTG-IgA) antibodies and DGP-IgA and IgG antibodies were assessed by ELISA (QUANTAlite™, INOVA Diagnostics Inc, San Diego, USA). DGP antibodies were not assessed at baseline or at 12 months due to lack of assay availability at the time. Coeliac genotype was assessed at baseline only by PCR amplification of DNA (Luminex-SSO®). Vitamin D levels were also examined at every time point via a polyclonal vitamin D3 assay (Elecsys, Roche Diagnostics).

Histopathological assessment

At approximately 1 and 5 years, subjects underwent upper gastrointestinal endoscopy performed under deep sedation at which time at least 4 biopsies were obtained from the second part of the duodenum and, for the 5-year test, at least two biopsies were obtained from the first part of the duodenum as per current best practice[80]. The most severe lesion in the biopsies was then graded according to Marsh scores by an experienced pathologist (PH) blinded to the baseline histology. *Mucosal remission* was defined as normal histology (i.e. Marsh 0) and *mucosal response* as either Marsh 1 or Marsh 0[139,157]. Routine gastric biopsies were obtained from the antrum and body to

ensure infection with *Helicobacter pylori* was not contributing to duodenal pathology[412]. Stained slides of the diagnostic biopsies were also obtained and reviewed blinded to patient information and scored as above.

Assessment of dietary compliance

At each follow-up assessment, dietary adherence was evaluated by a dietitian (SJS) via direct questions about any gluten-containing foods consumed, either accidentally or intentionally since last review, by specific questioning and via 7-day food diary entries. Dietary compliance was categorised as 'excellent' if gluten was never deliberately consumed, 'good' if minor gluten intake of no more than 300 mg gluten per episode, no more than 6 episodes per year occurred, equating to no more than 5 mg gluten per day, 'fair' if they had minor gluten intake of no more than 300 g gluten per episode, no more than one episode per week, equating to no more than 50 g/day, or 'non-compliance' if intake was greater than this.

Body composition analysis

Body composition analyses were performed at the Body Composition Laboratory of Southern Health, Monash Medical Centre, Clayton, Victoria. The following indices were measured:

- *Anthropometry:* Height, hip and waist circumferences were measured to the nearest 0.1 cm in triplicate by a qualified anthropometrist, and weight was measured to the nearest 0.1 kg using a calibrated digital scale. Body mass index (BMI) was calculated as kg/m^2 and classified by standard definitions.
- *Total body dual energy x-ray absorptiometry (DEXA):* This was performed using Lunar DPX, (Lunar Corporation software version 4.6, Madison, WI, USA). Bone mineral density (BMD) at L2-L4 region of lumbar spine and femoral neck, and whole body bone mineral content as a

measure of total bone mass were obtained. Bone density was described in terms of scores of SD from a reference population (T scores and Z scores for patients <20 years). Osteoporosis was defined as a T score <-2.5, whilst osteopenia represented a t-score between -2.49 and -1.0. Fat-free mass (sum of total body bone mineral and lean tissue mass), skeletal muscle mass calculated from appendicular lean tissue mass[413], trunk fat and limb fat were also measured and percentage trunk fat and limb fat were calculated by DEXA. Percentage body fat was calculated from fat mass relative to total body weight.

Statistics

Statistical analyses were performed using GraphPad Prism (version 6.0 for Mac, GraphPad Software, San Diego California USA) and IBM SPSS version 20. Comparison of continuous data between groups was made using paired or unpaired t-tests with Welch's correction or Wilcoxon rank sign test according to distribution. Proportions were compared using Fisher's exact or chi-squared tests where appropriate. Pearson's or Spearman's correlation was used for parametric or non-parametric data respectively. A repeated measures ANOVA was used to test comparison of measures over time. A p value ≤ 0.05 was considered statistically significant.

RESULTS

Patients

Of 119 invited into the study, 106 gave consent to participate, but three were excluded as they did not meet diagnostic criteria (two sero-negative with Marsh I lesions, one with normal histology) and four were excluded as they were HLA-DQ2 or DQ8 negative. This resulted in 99 patients being assessed at diagnosis and 59 who were enrolled in the one-year follow-up cohort. At 5 years, all participants (n=99) were contacted to undergo repeat evaluation and 46 agreed to

participate. Of the initial 99 participants consenting to involvement in the study, no follow-up data was available for 27 participants and were thus excluded (Figure 3.1). The baseline characteristics of the remaining 72 participants are presented in Table 3.1. Two participants had negative coeliac antibodies at baseline. Both had at Marsh 3A or greater on histology and either HLADQ2 or DQ8. Twenty-nine patients had a ferritin below the reference range and 4 of these were anaemic. All 29 received oral iron supplementation. Fifty-one had a reduced serum vitamin D but despite prescription to all, only 14 (27%) of these complied with vitamin D supplementation during the study. Three participants were smokers at baseline but all had ceased at 5 years.

Of the first 59 included participants invited to be part of the one-year follow-up study, complete data were available for 52. At 5 years, complete data were available for 46 participants, 25 of whom were also assessed at one year (Figure 3.1). At the 5-year time point, all participants underwent routine blood tests, 45/46 (98%) underwent endoscopy and 44/46 (96%) underwent repeat body composition analysis. Where data were missing, these patients were excluded from the relevant analysis only. The longitudinal groups were well-matched compared to the baseline group (Table 3.1).

Figure 3.1 Study Design

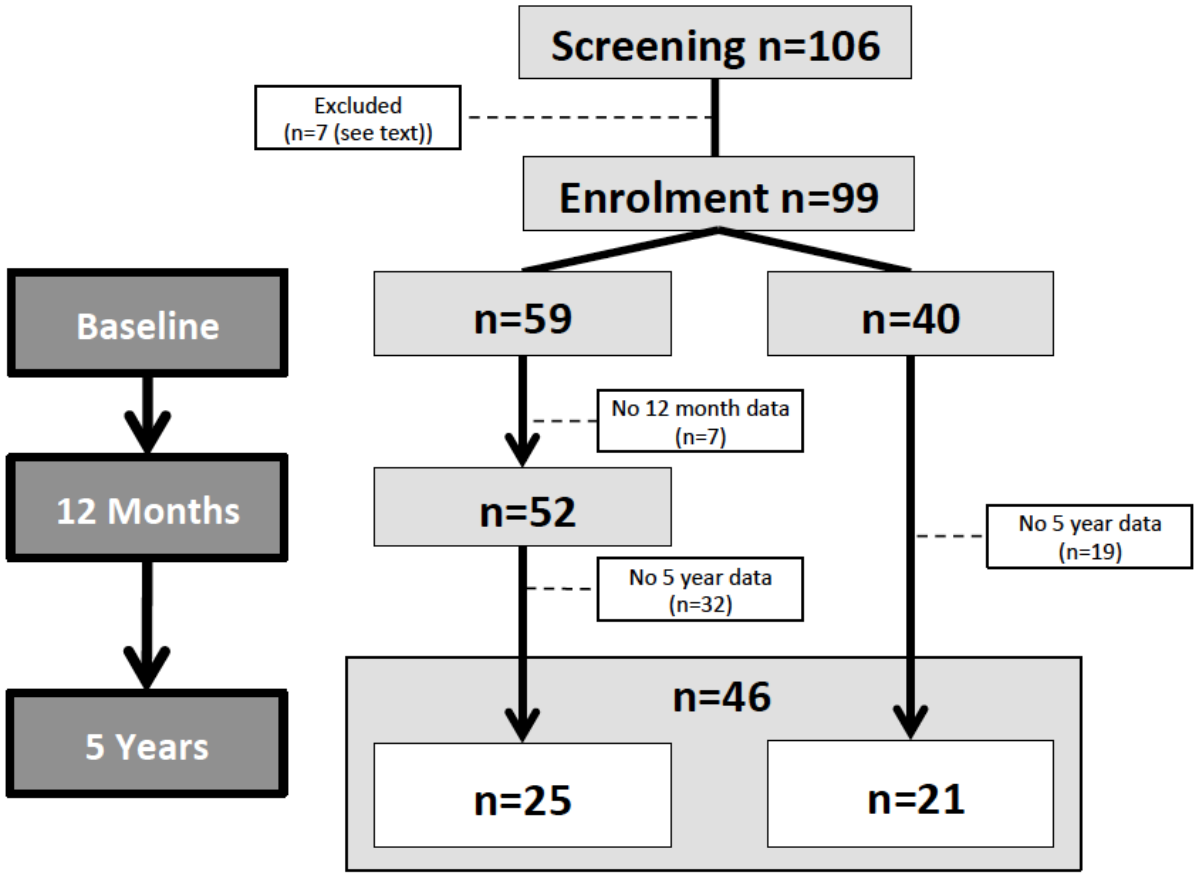


Table 3.1 Comparison of the three overlapping cohorts in terms of physiological characteristics at entry to the study.

		All patients (n=72)	Studied at 1 year (n=52)	Studied at 5 years (n=46)	Studied at 1 and 5 years (n=25)	p value
Male gender (%)		24 (33)	11 (21)	12 (26)	7 (27)	NS*
Mean age (range) years		40 (18-71)	42 (20-71)	42 (18-67)	45 (20-67)	0.999^
Mean BMI (range) kg/m^2		24.3 (15.7-40.4)	24.1 (18.1-40.2)	23.9 (15.7-40.2)	23.8 (18.3-40.2)	0.935^
Baseline histology (%)	Marsh 1	3 (4)	2 (4)	2 (4)	1 (4)	NS*
	Marsh 3A	15 (21)	9 (17)	10 (22)	3 (12)	NS*
	Marsh 3B	33 (46)	26 (50)	24 (52)	16 (65)	NS*
	Marsh 3C	21 (29)	15 (29)	10 (22)	5 (19)	NS*
Baseline symptoms (%)	Mild	30 (30)	15 (29)	13 (28)	9 (35)	NS*
	Moderate	44 (44)	23 (44)	19 (41)	8 (35)	NS*
	Severe	25 (25)	19 (38)	14 (30)	8 (31)	NS*
Bone mineral density (%)	Osteoporosis	7 (10)	5 (10)	4 (9)	2 (8)	NS*
	Osteopenia	17 (24)	15 (29)	15 (33)	9 (35)	NS*
	Normal	48 (66)	32 (61.5)	27 (59)	15 (62)	NS*

* z-test compared to all patients
^ one-way ANOVA

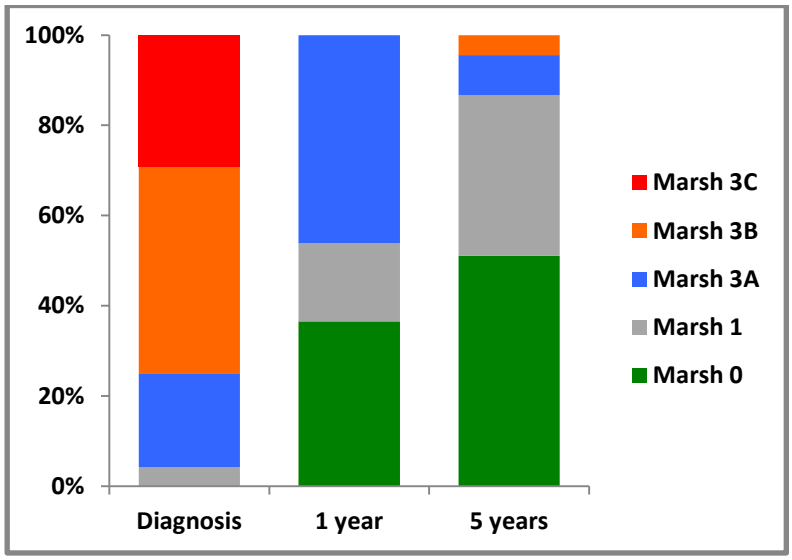
Compliance with gluten-free diet

At 1 year, compliance to the GFD was judged excellent in 40, good in 9, and fair in four patients. In other words, 25% of patients had some gluten exposure, albeit minimal. At 5 years, all had excellent compliance except one participant. Despite suboptimal compliance (classified as 'non-compliant'), this participant had mucosal remission but elevated coeliac antibodies.

Histology

The proportion of participants achieving each Marsh score after 1 and 5 years is illustrated in Figure 3.2. At 1 year, all participants had improved their Marsh score by at least one grade except two (1 male) with Marsh 3A lesions. Mucosal remission and response was noted in 37% and 54%, respectively. At 5 years, these had improved to 50% and 85%. Mucosal remission was associated with younger age at one year (39 [95% CI: 37-41] versus 46 [44-48] years; p=0.026), but not at 5 years (44 [42-46] vs. 41 [38-44]; p=0.445). Men were more likely to achieve mucosal remission at 5 (but not 1) years (OR 4.6 [1.049 to 20.31]; p=0.047). Neither severity of the lesion or coeliac-specific antibody levels was predictive of histological outcomes. The 25 patients examined at all time-points showed remission rates at 1 and 5 years were 35% and 50%, respectively, and response rates were 46% and 92%. No patient had *Helicobacter pylori* on gastric biopsies at baseline, 1 or 5 years, and none had received prior eradicative therapy.

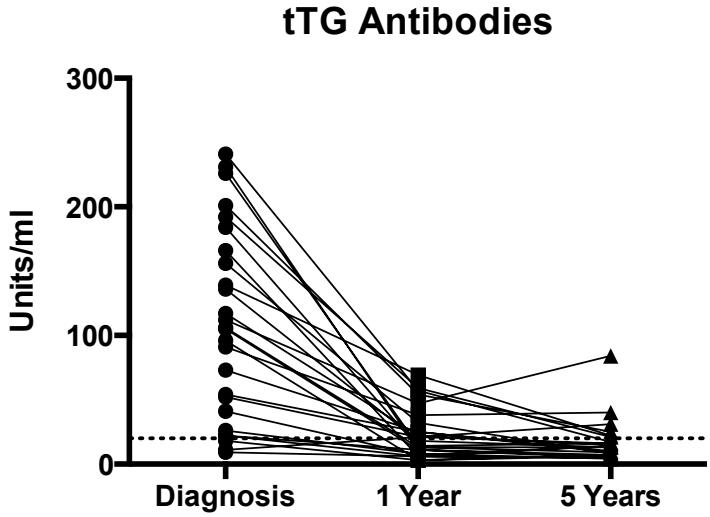
Figure 3.2 Histological outcomes over five years



Coeliac serology

All patients had serum IgA within the normal limits. Titres of EMA and tTG antibody concentrations fell significantly across the three time-points ($p<0.0001$ for both; one-way ANOVA), as shown for tTG in Figure 3.3.

Figure 3.3 Tissue transglutaminase antibody concentrations across the three time points. There was a significant change ($p<0.001$; ANOVA). The outlier at 5 years had excellent compliance and the biopsy was graded as Marsh 0.



All but 2 (4%) participants had at least one elevated coeliac antibody at baseline (Table 3.1). There was a poor relationship between serology and histology. At the one-year time-point, nine of the 44 patients (20%) whose EMA serology was positive at diagnosis were still positive at 12 months, with 2 (5%) of these having normal duodenal histology (both always compliant). Of the 49 whose tTG serology was positive at diagnosis, 23 (47%) were still positive at 12 months, with 7 (14%) of these having normal duodenal histology (4 strictly, 1 mostly, 2 poorly compliant). As seen in Table 3.2, antibody titres were poorly predictive of mucosal pathology. Conversely,

normalised serology at 1 year did not indicate a healed duodenal lesion, as ongoing villous atrophy (all Marsh IIIA) was present in 29 (66%) of the 44 patients with abnormal EMA, and in 25 (57%) of the 49 patients with abnormal tTG. A greater proportion of patients with initially mild lesions normalised their tTG and EMA serology (5 of 6 patients and 3 of 3 patients, respectively) compared with those with marked lesions (47% of 43 patients and 20% of 40 patients, respectively), but this was only statistically significant for EMA (p=0.013; Fisher's exact).

Table 3.2 Antibody performance at 1 year

	tTG IgA (Remission)	EMA (Remission)	Any Positive Antibody	tTG IgA (Response)	EMA (Response)
Sensitivity	44.1 (27.2 to 62.1)	18.2 (7.0 to 35.5)	44.1 (27.2 to 62.1)	30.3 (15.6 to 48.7)	15.2 (5.1 to 31.9)
Specificity	52.6 (28.9 to 75.6)	89.5 (66.9 to 98.7)	52.6 (28.9 to 75.6)	36.8 (16.3 to 61.6)	84.2 (60.0 to 96.6)
PPV	62.5 (40.6 to 81.2)	75.0 (34.9 to 96.8)	62.5 (40.6 to 81.2)	45.5 (24.4 to 67.8)	62.5 (24.5 to 91.2)
NPV	34.5 (17.9 to 54.3)	38.6 (34.9 to 96.8)	34.5 (17.9 to 54.3)	23.3 (9.9 to 42.3)	36.4 (22.4 to 52.23)
Likelihood ratio	0.93	1.72	0.93	0.48	0.96

At 5 years, 14 (30%) participants had at least one elevated coeliac antibody. Three subjects had elevated DGP IgA *and* IgG (DGP IgA/IgG values of 44/22, 31/34 and 21/30 U/ml respectively). A further 6 subjects had an elevated tTG alone (median 28, range 22 to 57 U/mL). Five subjects with negative DGP IgA and IgG were found to have an elevated tTG (median 42, range 20-84 U/ml). The positive and negative predictive values for each antibody assay are presented in Table 3.3. DGP IgG performed as the best predictor and EMA poorest of these assays. Lowering the cut-off for normative values did not improve the sensitivity, specificity or positive and negative predictive values for any of the antibody assays (data not shown).

Table 3.3 Antibody performance at 5 years (Remission)

	tTG (IgA)	DGP (IgA)	DGP (IgG)	EMA	Any Antibody
Sensitivity	54.5 (23.5-83.1)	66.7(11.5-94.5)	77.8 (40.1-96.5)	4.4(0.7-22.0)	81.8 (48.2-97.2)
Specificity	51.4 (34.0-68.6)	51.2(35.5-66.7)	56.8 (39.5-72.9)	100(85-100.0)	51.43 (34-68.6)
PPV	26.1 (10.3-48.4)	8.7(1.32-28.1)	30.43 (13.3-52.9)	100(16.6-100.0)	34.62 (17.3-55.7)
NPV	78.3 (56.3-92.5)	95.7(78.0-99.3)	91.3 (71.9-98.7)	51.1(35.8-66.3)	90.0 (68.3-98.5)
Likelihood Ratio	1.12 (0.59-2.13)	1.4 (0.6-3.2)	1.8 (1.1-3.0)	NA	1.68 (1.1-2.6)

Gastrointestinal symptoms

On average participants had been symptomatic for 1.5 (range 0.1-32) months at diagnosis. Fatigue was most commonly reported individual symptom (77%). Baseline gastrointestinal symptoms are shown in Table 3.1. Approximately two thirds had moderate to severe symptoms. All patients were virtually asymptomatic after 12 months of GFD. Hence, no relationship was evident between symptoms and healing. However, ten participants (22%) had severe ongoing symptoms at five years despite apparent adherence to a GFD. Further, 8 of these had achieved mucosal remission. The single non-compliant participant was asymptomatic. There was borderline correlation between severe ongoing overall symptoms and mucosal non-response (r^2=0.285; p=0.052), but no individual symptoms were associated with mucosal disease activity.

Body composition

Only 4% of patients were underweight (BMI<20) at diagnosis, but one in three patients were overweight or obese. A comparison of the indices measured between 0-1 and 0-5 years are shown in Table 3.4. While increases in lean body indices became evident at the five-year but not one-year assessment, indices measuring fat stores seemed to increase over the first year but not subsequently (see Table 3.5). As illustrated in Figure 3.4, the BMI at diagnosis was associated with change in body composition. Subsequent changes in BMI, weight and fat mass were significantly greater in those with a BMI at diagnosis <25 kg/m^2, in contrast to lean body mass indices (such as skeletal muscle mass shown in Figure 3.4), which increased independently of BMI at diagnosis. In the 25 patients with data at three time points, the same trends were observed. At both the one-year and five-year assessments, healing status, gender and antibody responses did not predict body composition or its changes (data not shown).

Bone mineral density

Baseline bone mineral density characteristics are summarised in Table 3.4. In the lumbar spine, 7 patients (10%), 6 female, 5 post-menopausal, were osteoporotic, 17 osteopenic (24%), 14 female, 6 post-menopausal, and 48, 36 female, 11 post-menopausal, (79%) had normal BMD. At the femoral head, these figures were 3 (11%), 2 female, both post-menopausal, 20 (18%), 8 female, and 56 (78%), 22 female, respectively. Ten of 32 (31%) pre-menopausal women had reduced BMD, which was significantly fewer than 14 of 25 (56%) post-menopausal women (p=0.046; Fisher's exact test).

Table 3.4 Body composition

Body composition index		Diagnosis	1 Year	5 Years	p-value
Anthropometry	Weight (kg)	68.1(64.8-71.5)	71.1(67.2-75)		**<0.0001**
		67.7(63.5-71.2)		72.0 (68.1-74.9)	**<0.0001**
	Body mass index (kg/m^2)	24.1(23.1-25.0)	25.0 (23.9-26.2)		**<0.0001**
		23.9(22.7-25.1)		25.4 (24.4-26.4)	**<0.0001**
	Waist-hip ratio	0.82 (0.80-0.84)	0.82 (0.79-0.84)		0.31
		0.82(0.79-0.85)		0.87 (0.85-0.90)	**<0.0001**
Lean body mass	Skeletal muscle mass (kg)	21.6 (20.3-22.9)	21.8 (20.3-23.3)		0.020
		21.6(19.9-23.2)		22.5 (20.5-24.4)	**0.0003**
	Fat free mass (kg)	46.5 (44.2-48.8)	46.4(43.9-49)		0.180
		45.9(42.1-49.8)		49.4 (46.3-52.5)	**<0.0001**
Fat mass	% Body fat	31.4(29.1-33.6)	34.0(31.6-36.4)		**<0.001**
		30.3(27.3-33.3)		31.1(28.8-33.5)	0.32
	Fat mass (kg)	20.4 (19.3-22.5)	24.68(21.9-27.5)		**<0.001**
		20.9(18.0-23.8)		22.8(20.2-25.5)	**0.005**
Bone indices	Bone mass (g)	2610 (2482-2738)	2640 (2513-2768)		0.089
		2642 (2449-2835)		2699 (2506-2892)	**0.035**
	Osteoporosis)	5 (9.6%)	2 (9.1%)		0.93
	Osteopenia	15 (17.3%)	9 (17.3%)		1.0
	Normal t score	32 (61.5%)	41 (78.8%)		0.73
	Osteoporosis	4 (2.2%)		4 (2.2%)	1.0
	Osteopenia	15 (32.6%)		14 (30.4%)	0.76
	Normal t score	27 (58.7%)		28 (60.9%)	0.85

Table 3.5 Body composition indices for the 25 who had measurements at each time-point

Body composition index		Diagnosis	1 Year	5 Years	p-value*
Anthropometry	Weight (kg)	69.1 (63.2-75.1)	71.4(65.7-77.1)[§]	74.4(68.8-80.1)[§]	**<0.0001**
	Body mass index (kg/m^2)	23.4(22.0-25.6)	24.7(23.0-26.4)[§]	25.6(23.8-27.5)[§]	**<0.0001**
	Waist-hip ratio	0.82(0.78-0.86)	0.82(0.78-0.86)	0.87(0.83-0.90)[§]	**<0.0001**
Lean body mass	Skeletal muscle mass (kg)	21.6(19.6-23.6)	21.8(19.7-23.8)	22.3(20.3-24.4)[§]	**0.012**
	Fat free mass (kg)	47.7(43.9-51.5)	47.7(43.7-51.6)	49.9(46.5-53.3)[§]	**0.002**
Fat mass	% Body fat	30.1(25.6-28.6)	32.7(28.6-36.7)[§]	32.2(28.7-35.7)[§]	**0.015**
	Fat mass (kg)	21.2(16.5-25.9)	23.7(19.2-28.2)[§]	25.7(21.2-30.2)[§]	**<0.0001**
Bone indices	Bone mass (g)	2661(2475-2848)	2705(2504-2906)	2739(2539-2939)	**<0.0001**

* RM one-way ANOVA

[§] $p<0.05$ compared to baseline (paired t-test)

Changes in bone mass over the first year depended upon bone mass at diagnosis. As shown in Figure 3.5, it increased between baseline and 1 year in those with osteopenia and osteoporosis, but not in those with normal BMD ($p<0.0001$). This improvement in BMD translated into an improved classification in 7 (14%) participants (3 from osteoporosis to osteopenia and 4 from osteopenia to normal). Total bone mass was greater overall at the 5-year assessment compared with that at baseline ($p=0.035$, Table 3.4), although it did not show the same relationship to bone mass at diagnosis as observed at one year (Figure 3.5). Improvement in BMD resulted in changed classification in 5 participants - 2 from osteoporosis to osteopenia (1 male aged 72 years and 1 female aged 44 years), and 3 from osteopenia to osteoporosis (both female aged 55 and 34). Three participants were reclassified into a worse category (2 from osteopenia to osteoporosis (both female, aged 57 and 38 y) and 1 from normal to osteopenia (female, aged 57 y).

Figure 3.4 Change in body composition indices (● 1 year, ■ 5 years)

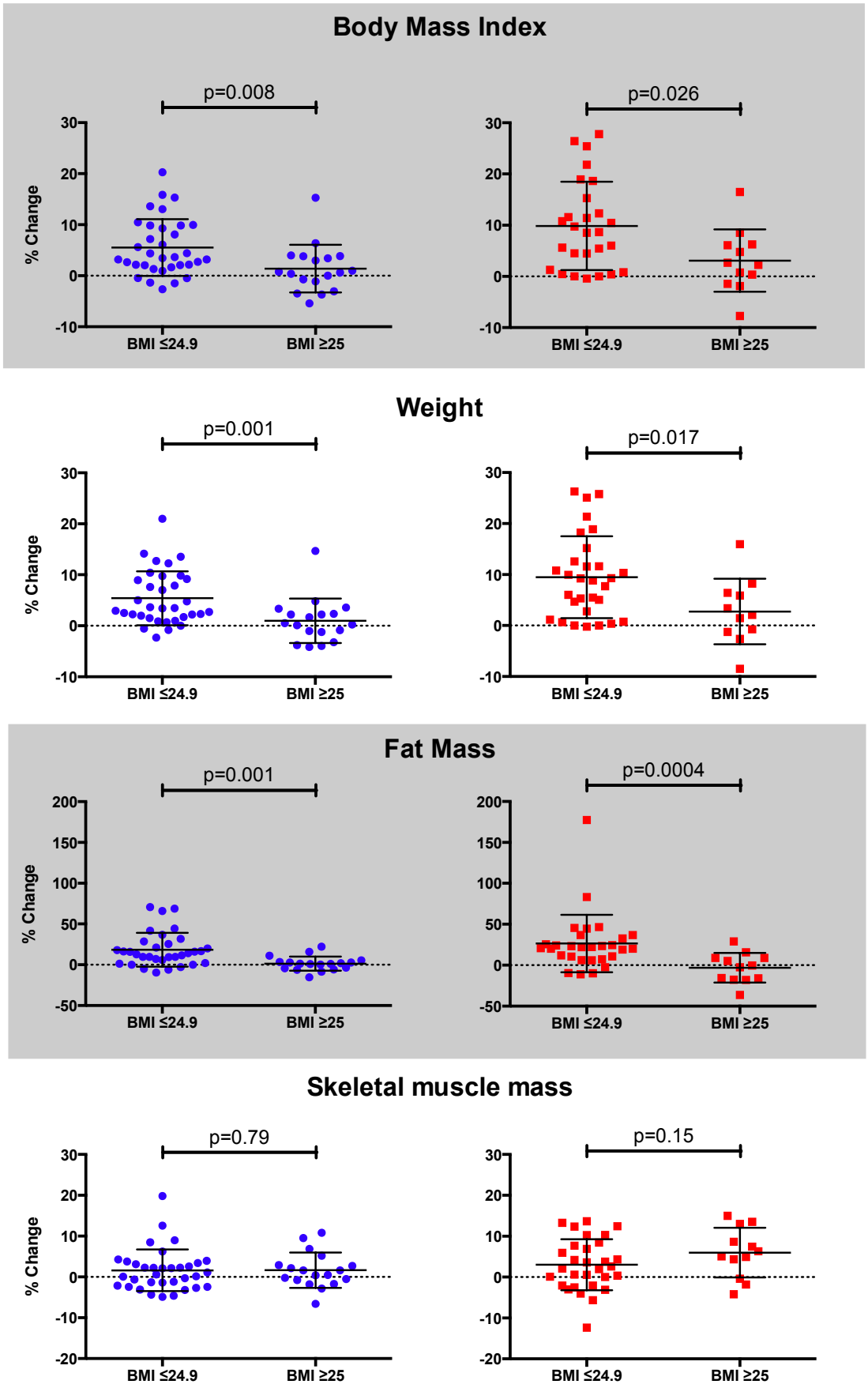


Figure 3.6 illustrates the changes over time in the 25 patients with data at the three time points. Those with normal BMD at diagnosis did not change bone mass over the first or subsequent four years, in contrast the continuing improvement throughout in those with osteopenia or osteoporosis at diagnosis. Over the 1-5-year period, two patients with reduced BMD at diagnosis had a considerable fall in their bone mass. Both participants were perimenopausal females who had reduced vitamin D levels at follow-up and who had persistently elevated tTG levels despite dietary compliance (age 54 and 51, tTG 31 and 40 Units/ml respectively). One had achieved mucosal remission and one had persistent villous atrophy (Marsh 3A) at 5 years. Vitamin D levels in this cohort increased irrespective of baseline BMD (from 69[55-84] to 85[74-95], p<0.0001, one-way ANOVA) but numbers were too small to draw conclusions regarding the relationship of vitamin D levels or supplementation to change in BMD.

Figure 3.5 Change in bone mass (● 1 year, ■ 5 years)

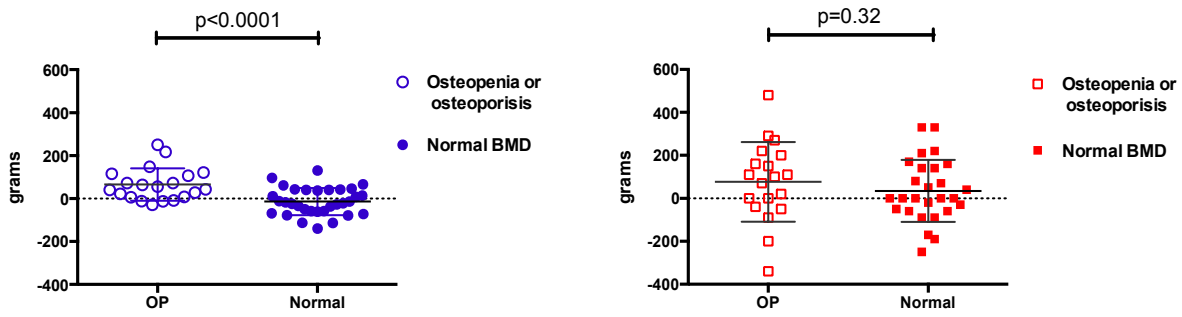
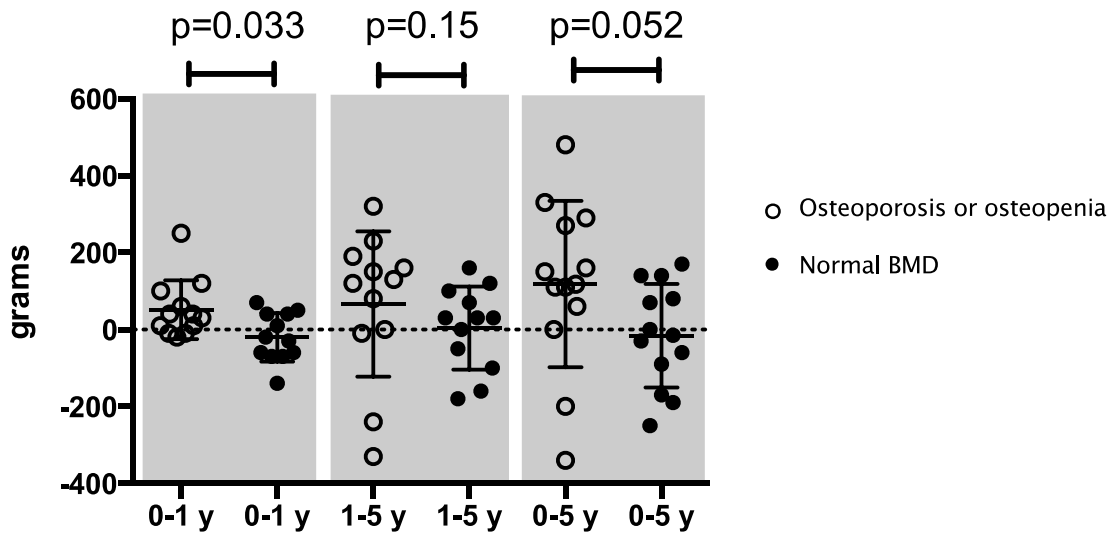


Figure 3.6 Change in bone mass in the patients who were studied at all time points



DISCUSSION

The primary goal of instituting a gluten-free diet as therapy for CD is healing of the intestinal lesion, with important secondary goals being the correction of the immunological abnormalities, minimisation of symptoms, and reversal of body compositional abnormalities that include protein-energy deficiency and abnormal bone health. Adherence to the diet would, on first principles, appear to be central to achieving such aims since the major driver is the immunological reaction between gluten peptides and the immune system. Previous studies have questioned whether such goals, particularly intestinal healing, are more aspirational that realistic in the majority of patients. The time course of the body's recovery has been poorly documented. The current study prospectively examined a relatively large group of newly-diagnosed patients with CD to address these key issues. A high degree of adherence to the GFD was achieved and, in the vast majority, a number of those therapeutic goals were obtained, although years of adherence were needed to succeed for some of them.

Response at the mucosal level in CD is both intuitive and desirable for a condition defined by the histological lesion. In other inflammatory bowel diseases, mucosal remission has been associated with improved long term outcomes and is now a recognised aim of therapy[414]. In CD the evidence base is still evolving. Poor mucosal recovery in CD has been associated with an increased risk of mortality, lymphoproliferative disorders, osteoporosis and autoimmune conditions[100,177-179]. Depending on the criteria used to define healing, retrospective studies have shown healing rates between 8 and 66% at differing time points with a tendency for these healing rates to improve over time from diagnosis[100,157,162]. We have shown here that, although mucosal remission only occurred in half, mucosal response was seen in the vast majority (89%) at 5 years. Although encouraging that mucosal response occurred in the vast majority, even mild ongoing inflammation can be associated with complications such as osteoporosis and autoimmune disease[14,415]. Prior studies have suggested that severe baseline histology, older age and high baseline antibody titres are associated with poorer long term response[100,185]. We also found that mucosal remission was associated with younger age at 1 year. In addition, male gender at 5 years was associated with remission, but no relationship to baseline histology or antibody titres was noted.

Most treatment algorithms for CD follow up have focussed on symptoms, particularly those related to the gut. In the current cohort, about one quarter of the patients had few symptoms that could be targeted for assessing response. Indeed, the symptoms were unrelated to the severity of the intestinal lesion as previously observed[177]. The discrepancy between symptoms and pathology is also supported by observations in the context of gluten challenge[158,416] and screening studies in children[45]. Persistence of symptoms is a well-recognised issue in CD and it is also recognised that complications can occur despite symptom improvement[100,177,417]. While patients seemed to improve markedly in their symptoms over the first year's observation, one in five had at

least moderate ongoing gastrointestinal symptoms when evaluated five years after diagnosis. This occurred despite excellent dietary adherence in and a complete or near complete mucosal response. By definition, these symptoms are unexplained by intestinal or immunopathology. Defining their nature was not a part of the aims of the current study.

Consistent with prior observations, coeliac antibodies were a poor guide to mucosal disease activity with positive and negative predictive values for any positive antibody of 35% and 90% respectively at 5 years[100,157,162]. An important insight provided by our study is the finding that antibodies can remain elevated despite adequate dietary compliance – a finding that challenges the conclusions drawn from other populations[100,143,418]. We did not find that antibodies disappear as a measure of dietary compliance - on the contrary, despite complete mucosal healing (and thus presumed successful compliance) 13% had elevated coeliac antibodies. In addition, we were unable to demonstrate better performance for a lower cut-off value for any antibody in predicting mucosal disease activity, as has recently been proposed[161].

A condition characterised by systemic inflammation and malabsorption is likely to be associated with macronutrient malnutrition particularly with reduced muscle mass as demonstrated for instance in Crohn's disease[419,420]. Few in this cohort were protein or energy malnourished comparing with healthy population norms, and more were overweight or obese, which is reflective of previous retrospective studies[208,210]. This might reflect the availability of food in the community and the eating habits and food choices made by the individuals, whereby malabsorption and increased consumption of energy might be easily offset by excessive consumption. While the energy consumption of patients did not apparently increase overall over the first year of the GFD in this patient cohort[136], this was assessed prior to and after one year by different methods and did

not stratify for body composition at diagnosis. Nevertheless, the energy status of patients, as judged by fat mass indices, increased over the first year and then plateaued, but only in those whose BMI was less than 25 kg/m^2. Although lacking detailed histological and biological correlation, similar increases in fat mass have been observed previously[206,209]. This relatively rapid correction toward population norms (i.e., overweight) may have related to increased energy intake in a subset of undernourished patients rather than to improved inflammatory or absorptive state, which took considerably longer to occur. The observed increase in body fat has particular relevance to the use of ghrelin as a non-invasive marker of disease activity[211]. However, the utility of ghrelin may be offset by the body composition and thus metabolic changes observed in this group.

In terms of lean body mass, comparing to population norms is a blunt tool to assess whether an individual is reaching his/her potential. A measure of protein deficiency in the individual might be regarded as the response of indices of lean body mass to correcting the inflammation and intestinal lesion. In this population, there were no extraneous confounders such as purposeful muscle-building, ingestion of anabolic steroids or high protein supplements, or excessive weight-bearing exercise. It is reasonable to postulate that changes in skeletal muscle mass then might reflect effects of treating the disease itself with the gluten-free diet. The observations that (a) lack of improvement in lean body mass indices did not improve over the first year despite improvement in energy status, and (b) significant and clinically relevant improvement was at the 5-year assessment independently of the BMI at diagnosis suggest that the change in lean body mass indices related more to improvement in the inflammatory state than to changes in absorption or food intake. The increase in skeletal muscle mass is of particular significance in a time of life where skeletal muscle mass would be expected to remain unchanged or fall. One could postulate that an increase in muscle mass might be beneficial both from not only from the point of view of disease recovery

but also the reduction in the risk falls and resultant fragility fractures in the longer term[421]. Consistent with our observations, previous prospective studies using inferential measures of fat indices also demonstrated an increase in fat mass and (to a lesser extent) fat free mass in a group with high rates of serological and histological response after 12 months[209].

There has been a considerable literature on the associations of reduced BMD with CD (reviewed in [422]) and the current study did show 27% patients at diagnosis to have osteopenia or osteoporosis. There are also several studies on the effects of a GFD on BMD. However, few have been prospective, dietary compliance has been variable, serological/histological correlation has often been lacking and there has been bias toward those with a low BMD[188,189,198,201]. The current study enabled prospective examination of bone mass over 5 years and for predictors of changes to be identified. As previously reported, bone mass did tend to improve overall, but this was largely restricted to those with reduced BMD at diagnosis. The improvement seemed to continue over the entire 5 years of observation, although two patients had a fall in bone mass at 5 years. Both of these participants were post-menopausal women and declining oestrogen may have contributed to this observation. In addition, both had low vitamin D levels throughout and one had ongoing villous atrophy. The changes were clinically significant as, at the five-year assessment, half of those with reduced BMD at baseline improved their classification (from either osteoporosis to osteopenia or from osteopenia to normal BMD). Thus, adherence to the GFD and mucosal improvement and healing are associated with continuing repair of reduced bone mass. Whether the improvement in BMD continues longer than 5 years and whether such improvement leads to clinical improvement via fewer fractures require further study.

While it is intuitive that improvement in body composition and BMD were causally related to the improvement or resolution of mucosal disease activity, such a relationship could not be measured in the present study, since adherence to the GFD and the mucosal response rates were very high. However, the 10% of the cohort who did have mucosal non-response at 5 years raise important questions regarding the definitions and management of refractory coeliac disease (RCD). Three quarters of these patients had negative antibodies and all had symptoms that were well controlled. None had further imaging or analysis of T-cell clonality via T-cell receptor gene rearrangement studies and cell surface markers to identify those at higher risk (RCD Type II) of progression to enteropathy associated T-cell lymphoma. However, the presence of symptoms underpins the current accepted definition of RCD[423] and symptomatology forms the basis of case identification. Management of asymptomatic individuals in this situation remains unclear[424].

The strengths of the study were its prospective nature, the size of the cohort, the fact that dietary education was delivered by a dietitian with considerable and recognised expertise in CD, and that adherence to the diet was so high. However, such higher compliance rates might be considered a weakness and source of bias. It did not permit evaluation of the effects of poor compliance. Compliance rates this high have not been previously reported and the tools used to assess compliance are relatively crude and subjective (i.e. food diary and dietitian interview). Nevertheless, these are the only validated tools for compliance and, until more accurate measures of compliance are available, research in similarly-designed prospective studies will be subject to this source of responder and recruitment bias. Coeliac antibodies, histology and gastrointestinal symptoms are poorly predictive of compliance and objective markers of compliance deserve further study[155]. The incomplete follow-up of many of the cohort related to the original design of the study (limiting year 1 follow up to the first half of the recruited patients due to funding issues) and to the lack of desire for many to have further invasive investigations at 5-years. However,

there is no indication that this biased the results. Those who had assessment at the three time points had very similar responses to the populations who were evaluated only at one or five years. Age- and gender-matched healthy controls would have strengthened the observations, particularly with regard to body compositional analysis and future studies examining these important disease outcomes should address this issue.

In conclusion, strict compliance with a GFD is associated with a very high chance of healing the intestinal lesion and correction of specific abnormalities in body composition. Both symptoms and coeliac-specific serology improve with time, but are poor predictors of the state of the duodenal mucosa. Even with strict dietary compliance and mucosal remission, symptoms and elevated coeliac antibodies can persist. Improvement of body composition occurs over the first five years. For fat mass, this increased early (within the first year) and seems to be restricted to those with a BMI <25 mg/kg^2 suggesting that it might relate to increased energy intake. For lean body mass indices, however, the improvement occurred in all patients and its slow time course was more consistent with improvement in disease activity than changes in dietary intake or nutrient absorption. Bone mineral density and bone mass continue to improve over the first five years in those with osteopenia or osteoporosis at diagnosis. Thus, these data support the contention that adherence to a strict GFD is an effective therapy in CD in achieving the major goals of intestinal healing and correction of body compositional abnormalities.

3.2 The impact of genotype on clinical phenotype

INTRODUCTION

Genetics clearly underpin the susceptibility for the development of coeliac disease (CD). More than 99% of those with CD express either the DQ2 or DQ8 haplotype or a combination thereof[10]. These genotypes can either be inherited in cis (on the same chromosome) or in trans (on different chromosomes). Up to 50% of the Australian population carry the susceptibility genotype[10,107] but there is variability in this incidence worldwide particularly in South East Asia, China and Japan[108,109].

A number of studies have addressed the relationship of DQ2 dosage to clinical presentation, histological severity of the duodenal lesion at diagnosis, immunopathology, and the likelihood of complicating of T-cell lymphoma. The findings have been heterogeneous, ranging from DQ2 homozygosity being associated with more severe presentation and duodenal histology[117-120] to no association at all[113,121,122]. In the only study addressing genotype dose in the context of gluten challenge, no dose-effect was seen[337]. That homozygosity for DQ2 is common amongst those with refractory coeliac disease (RCD) and predisposes to T-cell lymphoma is worthy of noting[123].

The effect of genotype on long term phenotype and disease outcomes in the longer term is unclear and the only previous prospective study assessed histological outcomes alone[118,119,212]. To date, all reports are relatively small case-control or population studies, and none has prospectively evaluated the effect of genotype on disease treatment and outcomes such as bone mineral density (BMD) and body composition.

We aimed to compare genotype dose to coeliac antibodies, histology and body composition in a group of patient with newly diagnosed coeliac disease and also to compare changes in these indices in response to treatment with a gluten free diet (GFD).

METHODOLOGY

The methods of patient selection in this study are outlined in the methods of Chapter 3.1. In brief, participants with newly diagnosed coeliac disease were examined prospectively over five years and data collected at 3 time points: Diagnosis, 1 year and 5 years (Figure 3.1). In addition to the methods described in Chapter 3.1, coeliac genotype was assessed by PCR amplification of DNA using sequence specific oligoneucleotide probes (Luminex-SSO®). Genotypes were then grouped according to whether there was a 'double dose' or 'single dose' as previously described (Table 3.6)[118,121]. Expression of DQ8 was considered equivalent to DQ2, as there is evidence to suggest coeliac disease related to DQ8 is equivalent in severity[114]. Genotype groupings were then compared to coeliac antibodies; nutritional markers (haemoglobin, albumin, and ferritin); histology (mucosal remission and response); gastrointestinal symptoms and body composition (anthropometry and DEXA).

STATISTICS

Statistical analyses were performed using GraphPad Prism (version 6.0 for Mac, GraphPad Software, San Diego California USA) and IBM SPSS version 20. Comparison of continuous data between groups was made using paired or unpaired t-tests with Welch's correction or Wilcoxon rank sign test according to distribution. Proportions were compared using Fisher's exact or chi-squared tests where appropriate. Pearson's or Spearman's correlation was used for parametric or

non-parametric data respectively. Binary logistic regression was used to identify variables associated with each genotype.

RESULTS

Associations at baseline

As detailed in Chapter 3.1, of the initial 99 participants consenting to involvement in the study, no follow-up data were available for 27 participants and were thus excluded (Figure 3.1). The baseline characteristics of the remaining 72 participants are presented in Table 3.6. At diagnosis, 38 (53%) patients were noted to express a 'double dose' of the susceptibility genotypes of DQ2 or DQ8 alone or in combination (Table 3.6). As detailed in Table 3.7 and Figure 3.7, a double dose of susceptibility genes was associated with a higher titre of tissue transglutaminase (tTG) antibodies and more severe histological lesion in these consecutively recruited patients. Further, a double dose was associated with lower weight (by an average of 6 kg) as well as lower bone mass and T-score for BMD at the lumbar spine (Table 3.8). Five patients were anaemic at diagnosis (all female, n=4 with double dose of genes; p=0.46), three of whom were iron deficient. No differences were noted in haemoglobin, ferritin or albumin according to gene dosage (Table 3.7). Eight patients were 'pure' DQ2 homozygotes. When compared to the rest of the group, there were no differences in antibody concentrations (mean [95% CI] tTG 86 [32-143] vs 107 [87-126]; p=0.46); 4 (50%) asymptomatic;), histological severity (Marsh 3C n=4, 3B n=3 and 3A n=1, p=0.66 Chi square) or clinical symptoms (severe symptoms n=3, moderate n=2, asymptomatic n=4, p=0.72) when this group was compared to the rest of the cohort.

Table 3.6 Baseline genotype

	Genotype	Allele		Number (%)	Female gender (% of genotype)	Mean age (range) y
Double Dose	DQ2.2 cis 2.5 cis	**DQB1*02**/DQA1*02	**DQB1*02/DQA1*05**	19 (26.4)	16 (84.2)	42 (20-71)
	DQ2.5 cis homo	**DQB1*02/DQA1*05**	**DQB1*02/DQA1*05**	8 (11.1)	5 (62.5)	36 (26-56)
	DQ2.5 cis DQ8	**DQB1*02/DQA1*05**	DQA1*03/**DQB1*03**	6 (8.3)	6 (100)	40 (31-52)
	DQ8 homozygotes	DQA1*03/**DQB1*03**	DQA1*03/**DQB1*03**	2 (2.8)	2 (100)	n/a (31,29)
	DQ2.2 cis DQ 8	**DQB1*02**/DQA1*02	DQA1*03/**DQB1*03**	3 (4.2)	1 (33.3)	42 (18-55)
			TOTAL	38 (52.7)	30 (78.9)	40 (18-71)
Single dose	DQ2.5 cis hetero	**DQB1*02/DQA1*05**	X/X	29 (40.3)	23 (79)	43 (24-69)
	DQ2.2 cis hetero	**DQB1*02**/DQA1*02	X/X	3 (4.2)	0 (0)	39 (39-40)
	DQ2.5 trans hetero	DQB1*02/X	DQA1*05/X	1 (1.4)	1 (100)	48
	DQ 8 hetero	DQA1*03/**DQB1*03**	X/X	1 (1.4)	1 (100)	52
			TOTAL	34 (47.2)	25 (73.5)	43 (24-69)

Table 3.7 Comparison of double dose and single dose genotype groups at baseline. All continuous variables are shown as mean (95% confidence intervals)

		Double dose (n=38)	Single dose (n=34)	p value
	Age (range)	40 (18-71)	43 (24-69)	0.33[a]
	Female gender (%)	30 (78.9)	25 (73.5)	0.78[b]
	Tissue transglutaminase (95% CI)	124 (99-151)	82 (58-105)	**<0.0001[a]**
Histology (%)	Marsh 3C	18 (47)	3 (9)	**0.004[c]**
	Marsh 3B	14 (37)	19 (56)	
	Marsh 3A	5 (13)	10 (29)	
	Marsh 1	1 (3)	2 (6)	
Gastrointestinal Symptoms	Severe	10	8	0.63[c]
	Moderate	16	18	
	Asymptomatic	12	8	
	Haemoglobin (g/L)	139 (134-143)	137 (132-141)	0.46[a]
	Albumin (g/L)	41 (39-42)	41 (40-42)	0.45[a]
	Ferritin (micrograms/L)	64 (41-86)	87 (48-126)	0.65[a]

[a] Mann-Whitney independent samples t-test

[b] Fisher's exact

[c] Chi-square

Figure 3.7 Coeliac antibodies at each time point according to gene dosage. (A) antibody titres at diagnosis and (B) change in antibody titres at 1 year and 5 years

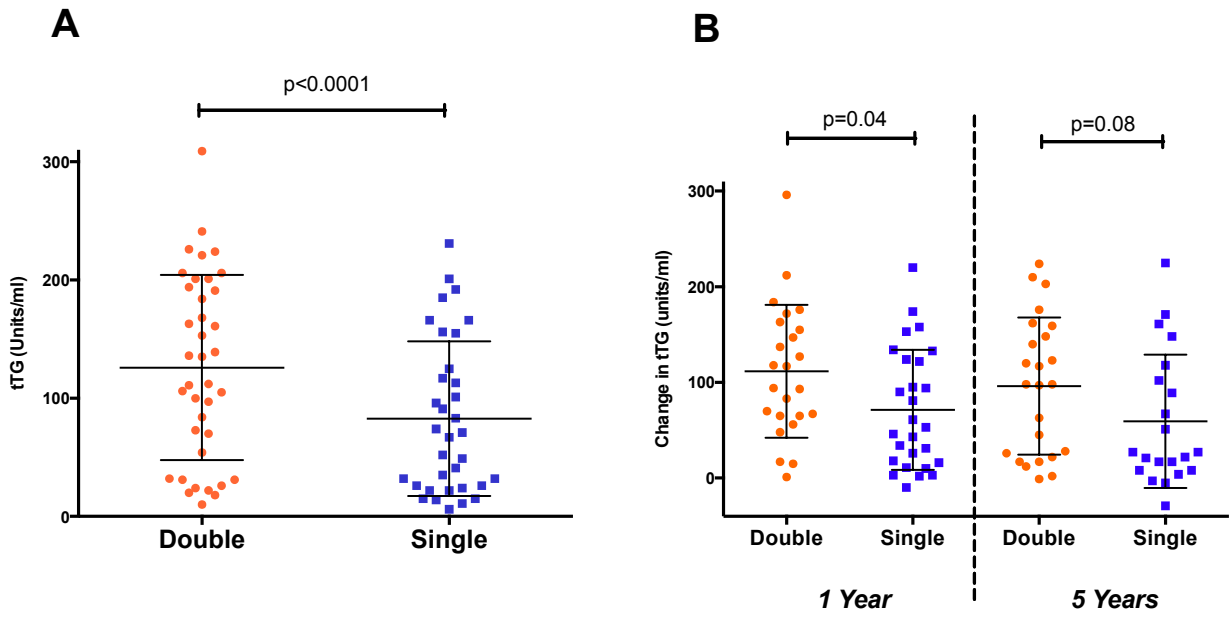


Table 3.8 Comparison of anthropometric and body composition data at baseline according to gene dosage. All data are shown as mean (95% confidence intervals)

		Double dose (n=38)	Single dose (n=34)	p value
	Weight (kg)	64 (60-69)	72 (67-78)	**0.03**[1]
	Body mass index (kg/m^2)	23.3 (22.3-24.3)	24.9 (23.2-26.6)	0.17[1]
	Waist to hip ratio	0.81(0.78-0.84)	0.83 (0.81-0.86)	0.07[1]
Lean Mass	Skeletal muscle mass (kg)	20.7 (18.9-22.4)	22.5 (20.5-24.5)	0.12[1]
	Fat free mass (kg)	46.8 (43.2-50.4)	46.1 (43.1-49.0)	0.82[1]
Fat Indices	Fat mass (kg)	19.9 (17.6-22.3)	23.9 (19.7-28.1)	0.26[1]
	Per cent fat	30.6 (27.9-33.2)	32.3 (28.4-36.1)	0.53[1]
Bone indices	Total bone mass (g)	2450 (2300-2601)	2821 (2609-3033)	**0.01**[1]
	Osteoporosis (% of total)	3 (8)	4 (12)	**0.02**[2]
	Osteopenia	16 (42)	6 (18)	
	Normal	19 (50)	24 (70)	

[1] Independent samples t-test

[2] Independent samples t-test comparing T-score at the lumbar spine

Associations over one and 5 years' follow-up

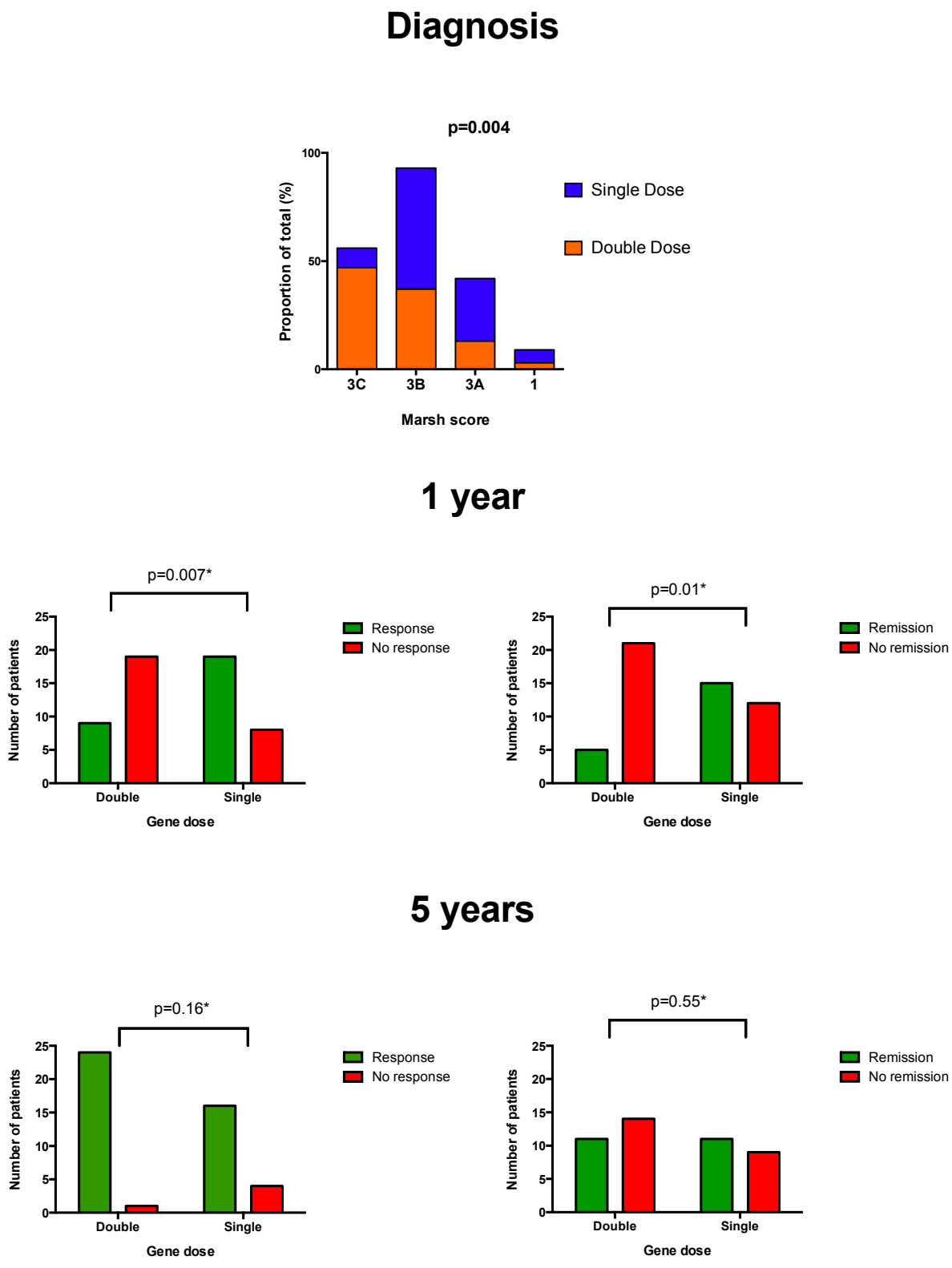
Changes in clinical, anthropometric and body composition indices at one- and five-year time points are shown in Table 3.9. At one (but not five) year(s), tissue transglutaminase antibodies reduced by more in those with a double dose (Figure 3.7), but there was no difference noted in the proportion of those patients in whom tTG normalised (noted in 13 (50%) of those with a double dose and 17 (63%) single dose; p=0.41, Fisher's exact). At 1 year, patients with a double dose were less likely to achieve mucosal response or remission (Figure 3.8). For body composition, changes in fat indices tended to be greater at both one and five years in association with gene double-dosage, but only for per cent body fat after one year was this increase statistically significant (Table 3.9). However, after Bonferroni correction, this was of doubtful significance. Also in participants with a double dose, there was a trend for fat mass to increase by more but there were no other significant changes to note at 1 year or 5 years. Bone indices were also unaffected by gene dosage. Genotype was not associated with either baseline ALT or change in ALT over time and nor was it associated with severity of symptoms at baseline, 1 year or 5 years. In addition, there were no themes in genotype when those with a low neutrophil count were analysed (discussed in detail in Chapter 3.4).

For those participants with data at all three time points (n=26), similar findings were noted with at 1 year regarding mucosal response (3/12 (25%) double dose versus 9/14 (64%); p=0.06, Fisher's exact) but not remission (2/12 (17%) versus 7/14 (50%); p=0.11).

Table 3.9 Change in clinical, anthropometric and body composition indices at 1 year and 5 years according to gene dosage

			Double dose	Single dose	p value
	Tissue transglutaminase (units/ml)	1 year	111 (82 to 140)	71.3 (46 to 96)	**0.04**
		5 years	96 (66 to 126)	59 (28 to 91)	0.08
	Weight (kg (95% CI))	1 year	2.9 (1.6 to 4.3)	2.0 (0.7 to 3.3)	0.24
		5 years	5.2 (3.1 to 7.3)	4.2 (1.8 to 6.5)	0.30
	Body mass index (kg/m2)	1 year	1.2 (0.6 to 1.7)	0.7 (0.3 to 1.2)	0.20
		5 years	1.9 (1.0 to 2.8)	1.5 (0.6 to 2.3)	0.19
	Waist to hip ratio	1 year	-0.003 (-0.02 to 0.01)	-0.006 (-0.02 to 0.01)	0.90
		5 years	0.05 (0.02 to 0.07)	0.06 (0.03 to 0.09)	0.53
Lean Mass	Skeletal muscle mass (kg)	1 year	0.4 (-0.02 to 0.8)	0.3 (-0.1 to 0.7)	0.56
		5 years	0.5 (0.08 to 0.3)	0.4 (-1.0 to 0.7)	0.62
	Fat free mass (kg)	1 year	0.16 (-0.34 to 0.66)	0.32 (-0.16 to 0.81)	0.52
		5 years	4.10 (1.09 to 7.17)	2.13 (0.49 to 3.76)	0.39
Fat Indices	Fat mass (kg)	1 year	2.9 (1.6 to 4.2)	1.7 (0.3 to 3.0)	0.06
		5 years	4.3 (2.1 to 6.4)	3.0 (0.6 to 5.4)	0.21
	Per cent body fat	1 year	3.0 (1.6 to 4.5)	1.6 (0.1 to 3.1)	**0.03**
		5 years	4.3 (1.9 to 6.5)	2.8 (0.2 to 5.4)	0.18
Bone indices	Total bone mass (g)	1 year	27 (-0.002 to 56)	30 (-0.03 to 93)	0.20
		5 years	83 (27 to 140)	25 (-74 to 124)	0.20
	Lumbar spine T-score	1 year	0.40 (0.19 to 0.62)	0.18 (0.04 to 0.33)	0.12
		5 years	0.12 (-0.17 to 0.40)	-0.05 (-0.42 to 0.31)	0.21
	Femoral head T-score	1 year	0.12 (-0.16 to 0.39)	0.04 (-0.08 to 0.15)	0.38
		5 years	-0.005 (-0.29 to 0.28)	-0.16 (-0.43 to 0.11)	0.15

Figure 3.8 Histological response and remission at diagnosis, 1 year and 5 years according to gene dosage. Chi-square was used for comparisons at diagnosis and Fisher's exact test for follow-up.



DISCUSSION

The study presented here provides further insights into the effect of gene dosage on the clinical phenotype of CD at presentation and histological outcomes with treatment. Importantly, we have also examined unique prospective data on clinical course of CD, particularly regarding body composition outcomes. At diagnosis, participants with a double dose of susceptibility genes were noted to have higher antibody titres, greater histological damage and a lower weight. Further, bone mass and lumbar spine (but not femoral head) T-scores were lower in the double dose group. During follow-up, the genotype dose was linked to slower rate of mucosal response or healing, but had little evidence of association with change in any other index.

Consistent with other populations examining patients at diagnosis, 15% of our population were 'pure' homozygotes (i.e. homozygous for either DQ2 or DQ8) and more than half expressed a 'double dose' of susceptibility genes[118,119]. To date the literature on gene dose has focused largely on the relationship of genotype to either the risk of developing CD or to the clinical phenotype at presentation of CD. Those who are homozygous for DQB1*02 (i.e., DQ2) have been shown to have a higher likelihood of developing CD than those who are heterozygous[112-114]. The most robust evidence for this observation has been derived from a paediatric population at risk for developing Type 1 diabetes[425]. When the clinical phenotype has been examined, there have been dichotomous conclusions. In both children and adults, a double dose of genes has been associated with a more classical presentation of CD and a more severe intestinal lesion in two studies[118,120] and not in two other assessing similar populations[121,212]. In the current study, we also found increased gene dose to be associated with a more severe clinical phenotype as evidenced by higher antibody titres and more severe mucosal inflammation. Coeliac-specific antibodies have been associated with gene dosage in prior studies of adults[212] but not in children[118].

There are no published data examining body compositional aspects and genotype dose. We found a double dose of genes to be associated with a lower weight than those with a single dose, while total bone mass and lumbar spine T-score were also lower for those with a double dose. Whilst we observed differences in objective measures of disease activity, we did not observe any differences in subjective measures, namely gastrointestinal symptoms at diagnosis. This is consistent with some previous studies[120,121] but at odds with another prospective study[119]. Contrary to prior studies[119], we did not find any relationship between age or haemoglobin and gene dosage, but in this cohort but only 4 participants were anaemic at baseline.

At the cellular level, genotype dose effects on clinical features has been proposed to relate to a higher affinity of presentation of T-cell epitopes in homozygotes[115] and, conversely, to the lower availability of DQ2 molecules in heterozygotes for presentation to antigen presenting cells [116]. Numbers of 'pure' DQ2.5 (DQB1*02/DQA1*05) homozygotes in our study are small (11%) but the concepts might be extrapolated to some of our observations. Higher affinity antigen presentation would be expected to result in more efficient generation of inflammatory cytokines, including coeliac antibodies, and consequent tissue damage.

There is a paucity of prospective studies examining the associations of gene dosage with intestinal healing, antibody levels and body composition. The only study that has examined healing found that gene dose was related to slower recovery time at 12 months[119]. In that series of 144 patients, the severity of the intestinal lesion at diagnosis was also greater. These findings were mimicked by those of the current study where a double dose of genes was associated with failure to achieve neither mucosal response nor remission at 1 (but not 5) year(s). As noted by

Karinen et al, this may relate to the more severe lesion taking longer to heal. To examine this issue, Marsh scores (an ordinal variable) do not readily lend themselves to covariate analysis and future studies in this area should make use of villous height to crypt depth ratio (a continuous variable).

Although gene dosage was associated with clinically relevant markers of disease severity, the effect on long term outcomes was less meaningful. A double dose of genes was associated with a greater increase in measures of fat mass at 1 year. The greater gain of body fat might represent correction of baseline individual deficits and a return to population norms as there were trends to lower fat mass indices at baseline for those with a double dose. There were also no differences noted in symptoms measured at 1 year or 5 years although relatively few had symptoms at 1 year and were thus a highly clinically responsive group.

Assessment of genotype has a clearly defined role in the assessment of those where the diagnosis of CD is in question[29] and is being adopted into diagnostic algorithms[10]. However, the results presented here question the relevance of genotype assessment in situations where there is no diagnostic doubt. HLA gene dosage had little impact upon long-term outcomes and thus would not have assisted treatment or longer term surveillance. For clinicians, efficient resource utilisation impels us to identify those who might be expected to have a more complex course of disease. If therapeutics in CD are to gain traction, it will be similarly important to target those who need therapy for a condition that affects up to 1.5% of the population in Western countries. Intuitively therapies should be administered to those who are identified as being most at risk of expressing a more severe clinical phenotype. Genome wide association studies (GWAS) have largely been devoted to refining our understanding of disease pathogenesis but robust

prospective studies applying similar tools might enable early identification of these high-risk groups that serve to benefit most from future therapies.

Limitations of this study include incomplete follow-up and sample size. As outlined in Chapter 3.1, the intention was only to follow-up the first 60 participants at 1 year due to funding constraints and as a result of contacting all 99 baseline participants, 46 agreed to participate at 5 years. Each of the follow-up cohorts were similar in their baseline characteristics (Table 3.1, Chapter 3.1). Although the sample size might be considered small, this cohort represents the largest prospective study comparing genotype to serological, histological and body compositional outcomes.

In conclusion, we have demonstrated that a higher genotype dose is associated with a more severe clinical presentation in those with newly diagnosed CD. But the effects on long-term clinical phenotype are less relevant, thus questioning the need for HLA genotype assessment when the diagnosis of CD is unequivocal. A double dose of susceptibility genes might be associated with ongoing intestinal inflammation but gene dosage does not appear to affect longer term outcomes in serology, histology or body composition. Larger prospective studies are needed with consideration for identification of non-HLA genes and their impact on response to treatment.

3.3 A prospective assessment of the pattern of liver function test abnormalities in coeliac disease over the first 5 years from diagnosis

INTRODUCTION

Abnormalities in liver function have been extensively described in association with CD and may be one of the most common extra-intestinal manifestations, but prospective longitudinal data are lacking in the adult population. Older studies suggest an incidence of LFT abnormalities of up to 42%[228,229] but more recent prospective collected data have placed this figure closer to 10%[230]. Whether those with CD have any increase in abnormal liver function tests at all has recently been questioned[231]. Conversely the incidence of CD within those with LFT abnormalities varies from 1%-10%[232-234].

Generally the reported abnormalities of liver function tests are mild, but more severe complications have been reported with the reversal of fulminant hepatic failure in four patients[235]. Histologically, the associated abnormalities in liver function have been attributed to a chronic periportal infiltrate of lymphocytes or a 'non-specific' picture[228,236]. Due to shared genetics and clustering of autoimmune condition, there is also an overlap with the biochemical, histological and autoantibody stigmata of primary biliary cirrhosis (PBC), autoimmune hepatitis and primary sclerosing cholangitis (PSC)[237-239].

The aetiology for liver function test abnormalities in CD has been attributed to many factors. As with routine evaluation, the isolated elevation of alkaline phosphatase has been associated with osteomalacia and vitamin D deficiency, whilst predominance of cholestasis or elevated transaminases should prompt evaluation for PBC and autoimmune hepatitis respectively [229].

Outside of these conditions, other contributors have been proposed to be steatohepatitis secondary to undernourishment, but malnutrition is now an uncommon presentation. Due to the increasing worldwide problem of obesity and the metabolic syndrome, steatohepatitis may explain an increasing proportion of LFT abnormalities in CD due to factors independent of coeliac disease but this has not been well studied. Only one prospective study has examined body mass index in concert with liver function tests and although ALT increased in a proportion, BMI did not significantly change[231].

The majority of the available literature reports numerical improvement in liver function tests[229-231,240] and autoimmune hepatitis has explained lack of normalisation[232]. One of the earliest studies in adults noted improvement in LFT's as early as 3 months but the improvement was not universal with 10% failing to normalise[229]. In a more recent prospective study of 130 adults, all with elevated transaminases at baseline (10%) returned to normal values, but there was a sub group whose ALT increased from normal values with treatment[231]. Prospective longitudinal data on the timecourse of improvement in liver function tests is lacking.

In the broader community, the obesity epidemic has been responsible for fatty liver disease being a major cause of abnormal liver function tests. Commencement of a GFD in coeliac disease has been recognised to result in an increase in body mass index (BMI) but thorough contemporaneous body composition analysis has not previously been undertaken in CD follow-up and no long term data are available.

In the present study, we aimed to evaluate the effect and time-course of treatment of patients with newly-diagnosed CD with a GFD on liver function tests in relation to mucosal healing, body

composition, levels of Vitamin D and coeliac serology in a population of patients with newly diagnosed coeliac disease followed prospectively for 5 years. Thorough assessment was undertaken at 5 years to identify secondary causes of the observed abnormalities.

METHODS

Patients and study protocol

The details of patient selection and study protocol are described in the methods for Chapter 3.1. Data described for dietary compliance, histology, and body composition analysis are also derived from the same methods. The relevant additional components to the study protocol for the present study are described below.

Routine blood tests

Antinuclear antibodies were assessed by direct immunofluorescence. Liver function tests were evaluated with the Roche utilising the Roche Cobas system (Roche Diagnostics). An abnormal liver function test was defined as a test outside the reference range (RR): alkaline phosphatase (ALP [RR 30-120 IU/L]), gamma-glutamyl transferase (GGT [RR 6-42 IU/L]), alanine-aminotransferase (ALT [RR 5-30 IU/L(female), 5-35(male)]) and albumin (RR 34-47g/L). Elevated levels of bilirubin (RR <22 micromol/L) are discussed separately. At the 5 year time-point participants with liver function tests results more than twice the upper limit of normal were invited to return for repeat evaluation that included a further blood test (for repeat liver function tests, smooth muscle and antimitochondrial antibodies, copper, caeruloplasmin and viral serology for hepatitis B and C) and a liver ultrasound.

Statistics

Statistical analyses were performed using GraphPad Prism (version 6.0 for Mac, GraphPad Software, San Diego California USA) and IBM SPSS version 20. Comparison of continuous data between groups was made using paired or unpaired t-tests with Welch's correction or Wilcoxon rank sign test according to distribution. Proportions were compared using Fisher's exact or chi-squared tests where appropriate. Pearson's or Spearman's correlation was used for parametric or non-parametric data respectively. A repeated measures ANOVA was used to test comparison of measures over time. A p value ≤ 0.05 was considered statistically significant.

RESULTS

Patients

As described in detail in Chapter 3.1, a total of 72 patients had follow-up data available for analysis (Figure 3.1). Of relevance to interpretation of the results of liver function tests, 51 participants had a reduced serum vitamin D at diagnosis but despite prescription to all, only 14 (27%) of these received vitamin D supplementation during the study.

Of the first 59 included participants invited to be part of the one-year follow-up study, complete data were available for 52. At 5 years, complete data were available for 46 participants, 26 of whom were also assessed at one year (Figure 3.1). At the 5-year time point, all participants underwent routine blood tests, 45/46 (98%) underwent endoscopy and 44/46 (96%) underwent repeat body composition analysis. Where data were missing, these patients were excluded from the relevant analysis only.

Liver Function Tests

At diagnosis, 21 (29%, 17 female, mean age 43 y) participants were noted to have at least one liver function test abnormality (Table 3.11). The most common abnormality was an elevated ALT (seen in 19 patients). Those with an elevated ALT or GGT (i.e. a pattern consistent with non-alcoholic fatty liver disease (NAFLD)) trended toward having a higher BMI (24.7[95% CI 23.1-26.3] versus 23.9[22.7-25.0]kg/m^2, p=0.054) but body fat percentage (31.1[28.4-33.7] versus 29.8[28.1-31.5]%, p=0.45), antibody titres (tTG 99[60-140] vs. 106[85-127]U/ml, p=0.67) and waist to hip ratio (0.83[0.78-0.87] vs. 0.82[0.79-0.84], p=0.98) were similar when compared to those with normal liver function tests. Logistic regression confirmed a lack of association between ALT elevation and any of these factors (data not shown).

Of the 5 participants with elevated alkaline phosphatase levels, vitamin D levels were reduced in all (median 45[range 25-61]nmol/L) and trended toward being lower than those with an ALP within the reference range (63[23-141]IU/L, p=0.09). Of these patients, four (80%) had either osteopenia (n=2) or osteoporosis (n=2). Amongst those with abnormal liver function tests 16/21(76.2%) had a severe intestinal lesion (defined as Marsh 3B or 3C) compared to 38/52(74.5%) with normal liver function tests (p=0.98).

A summary of the changes observed at each time point is presented in Table 3.11. After 1 year of treatment with a GFD there was a trend for average ALT levels to reduce (Figure 3.9). Body mass index increased between baseline and 1 year (from 24.1[23.1-25.0] to 25.0[23.9-26.2] kg/m^2) and there was there was no correlation between changes in ALT and change in any body composition index (Table 3.12).

Table 3.10 Liver function test comparisons between groups

Liver Function Test (reference range)		Diagnosis (95% CI)	1 Year	5 Years	p-value
Alkaline Phosphatase (30-120 IU/L)	1 year	79.4 (71.3-87.4)	67.3 (60.9-73.4)		**<0.0001***
	5 years	72.9 (66.9-79.0)		64.8 (59.2-70.4)	**=0.009***
	1 and 5 y	76.6 (67.7-85.4)	62.0 (55.2-68.8)[§]	64.8 (56.9-72.3)	**=0.0021∞**
Gamma-glutamyl transferase (6-42 IU/L)	1 year	20.4 (14.6-26.1)	22.4 (17.1-27.8)		=0.45*
	5 years	16.5 (13.8-19.3)		24.2 (16.3-32.1)	**=0.023***
	1 and 5 y	15.6 (12.1-19.1)	19.7 (13.0-23.4)	25.4 (11.4-39.5)	**=0.100∞**
Alanine aminotransferase (Female 5-30; Male 5-40 IU/L)	1 year	27.9 (25.1-30.7)	24.8 (20.2-29.4)		=0.15*
	5 years	25.3 (22.6-28.0)		21.2 (19.5-24.1)	**=0.019***
	1 and 5 y	25.2 (21.9-28.4)	23.6 (15.4-31.8)	22.6 (19.1-26.2)	**0.63∞**
Bilirubin (<22 micromol/L)	1 year	11.5 (10.4-12.6)	12.1 (10.5-13.6)		=0.35*
	5 years	11.7 (10.3-13.1)		8.5 (7.2-9.7)	**<0.0001***
	1 and 5 y	12.0 (10.2-13.9)	12.1 (9.4-14.8)	8.4 (6.9-9.9)	**<0.0001∞**
Albumin (34-47 g/L)	1 year	40.4 (39.6-41.3)	41.9 (41.1-42.7)		=0.002*
	5 years	41.1 (40.2-42.1)		42.2 (41.2-43.1)	**=0.065***
	1 and 5 y	40.6 (39.3-42.0)	42.0 (40.9-43.0)	42.0 (40.8-43.2)	**0.041∞**

* Wilcoxon signed rank

∞ one-way ANOVA

Table 3.11 Baseline liver function test abnormalities

Gender	Age	Marsh Score	tTG	BMI	ALP	GGT	ALT	Bilirubin	Albumin	Vitamin D
F	27	3B	71	26.2	58	11	48	7	41	65
F	29	3C	113	22.2	62	22	40	14	43	87
F	30	3B	32	28.8	107	23	40	4	42	44
F	31	3C	224	19.6	103	24	32	8	40	35
F	33	3C	161	24.5	102	49	34	11	39	93
F	34	3C	84	26.2	188	44	51	9	41	25
F	34	3A	163	28.5	39	8	32	9	38	101
F	36	3B	32	24.6	54	14	11	24	45	66
F	36	3A	25	15.7	56	34	34	8	39	50
F	39	3C	221	22.4	74	8	40	10	39	30
F	43	3B	139	19.1	83	11	4	23	39	50
F	46	3B	194	21.1	130	14	24	12	44	61
F	48	3B	226	26.7	105	20	33	6	38	28
F	48	3A	67	22.6	90	40	30	7	44	69
F	48	3C	201	29.9	72	19	35	7	39	36
F	50	3B	96	24.7	73	9	35	11	40	130
F	56	3C	28	24.4	124	6	36	18	41	48
F	56	3B	155	24.3	87	145	54	11	40	78
F	71	3C	3	22.7	170	15	42	10	37	45
M	39	3A	3	26.6	90	16	44	17	43	99
M	39	3B	32	26.7	89	41	41	13	46	57
M	57	1	46	26.7	96	37	42	13	43	88
M	67	3B	26	21.2	129	19	24	12	34	45

Figure 3.9 Liver function tests at 1 year

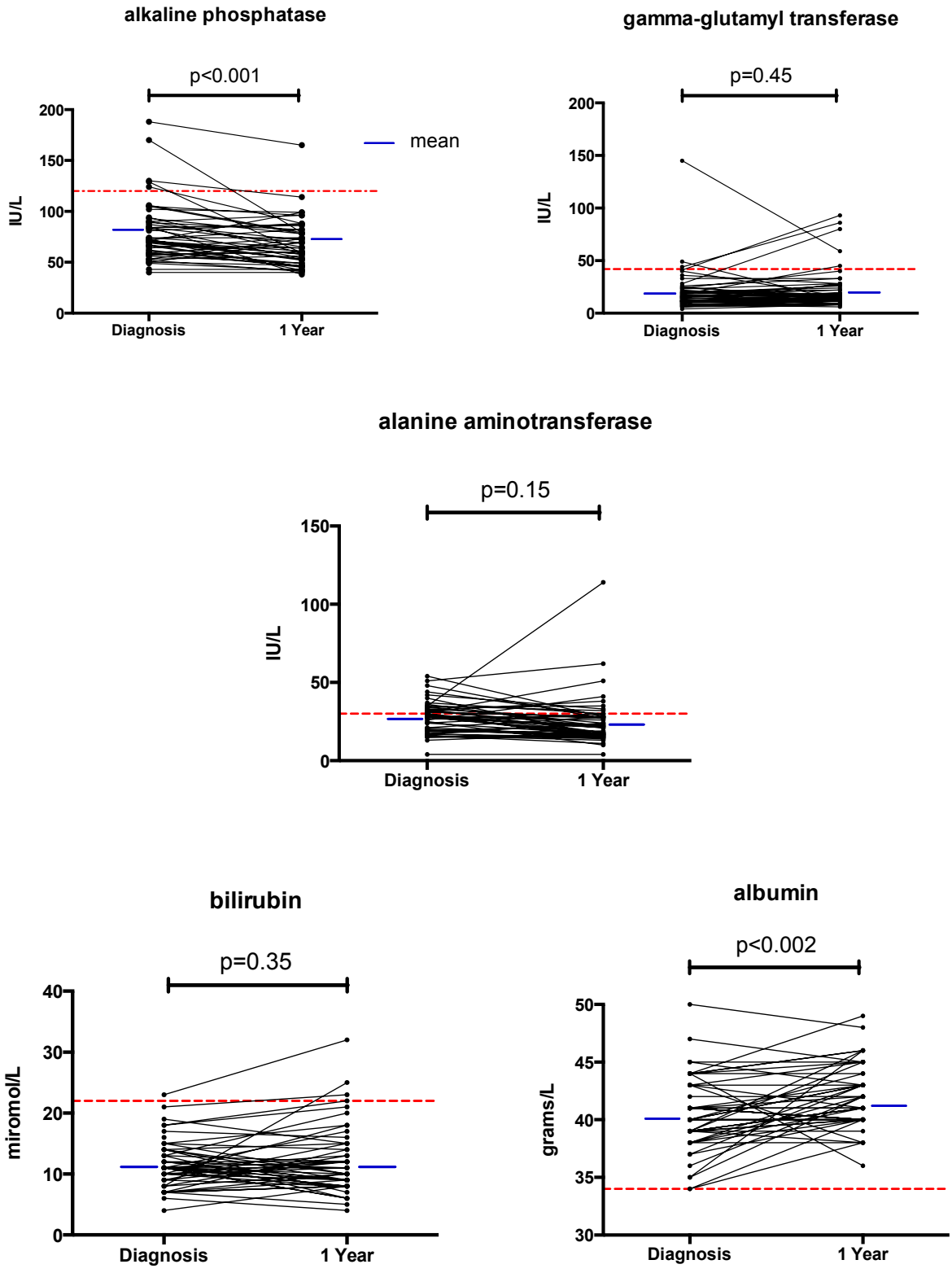


Table 3.12 Correlations with change in indices

		Correlation of change from diagnosis to change in liver function test [r(95% CI)]					
		Weight	**BMI**	**Waist to hip ratio**	**Fat mass**	**Percent body fat**	**Skeletal muscle mass**
ALT	1 year	0.12(-0.17 to 0.38)	0.16(-0.13 to 0.42)	0.23(-0.10 to 0.51)	0.17(-0.11 to 0.43)	0.14(-0.14 to 0.41)	-0.19(-0.45 to 0.09)
	5 years	0.20(-0.12 to 0.48)	0.23(-0.08 to 0.50)	0.23(-0.10-0.51)	0.22(-0.11 to 0.51)	0.12(-0.20 to 0.41)	-0.14(-0.44 to 0.18)
ALP	1 year	**-0.34(-0.57 to -0.06)***	**-0.33(-0.56 to 0.06)***	0.13(-0.15 to 0.40)	**-0.28(-0.52 to -0.01)***	-0.22(-0.47 to 0.06)	-0.14(-0.40 to 0.15)
	5 years	**-0.35(-0.59 to -0.05)***	-0.25(-0.51 to 0.07)	-0.04(-0.34 to 0.28)	-0.24(-0.51 to 0.08)	-0.28(-0.54 to 0.03)	-0.13(-0.42 to 0.19)
GGT	1 year	0.25(-0.04 to 0.49)	**0.35(0.08 to 0.57)***	0.26(-0.02 to 0.51)	**0.39(0.12 to 0.60)***	**0.37(0.10 to 0.59)***	-0.10(-0.37 to 0.19)
	5 years	**0.34(0.02 to 0.59)***	0.37(0.06 to 0.61)	0.03(-0.29 to 0.34)	0.22(-0.10 to 0.51)	0.23(-0.10 to 0.51)	0.27(-0.13 to 0.48)

- **p<0.05, Pearson correlation**

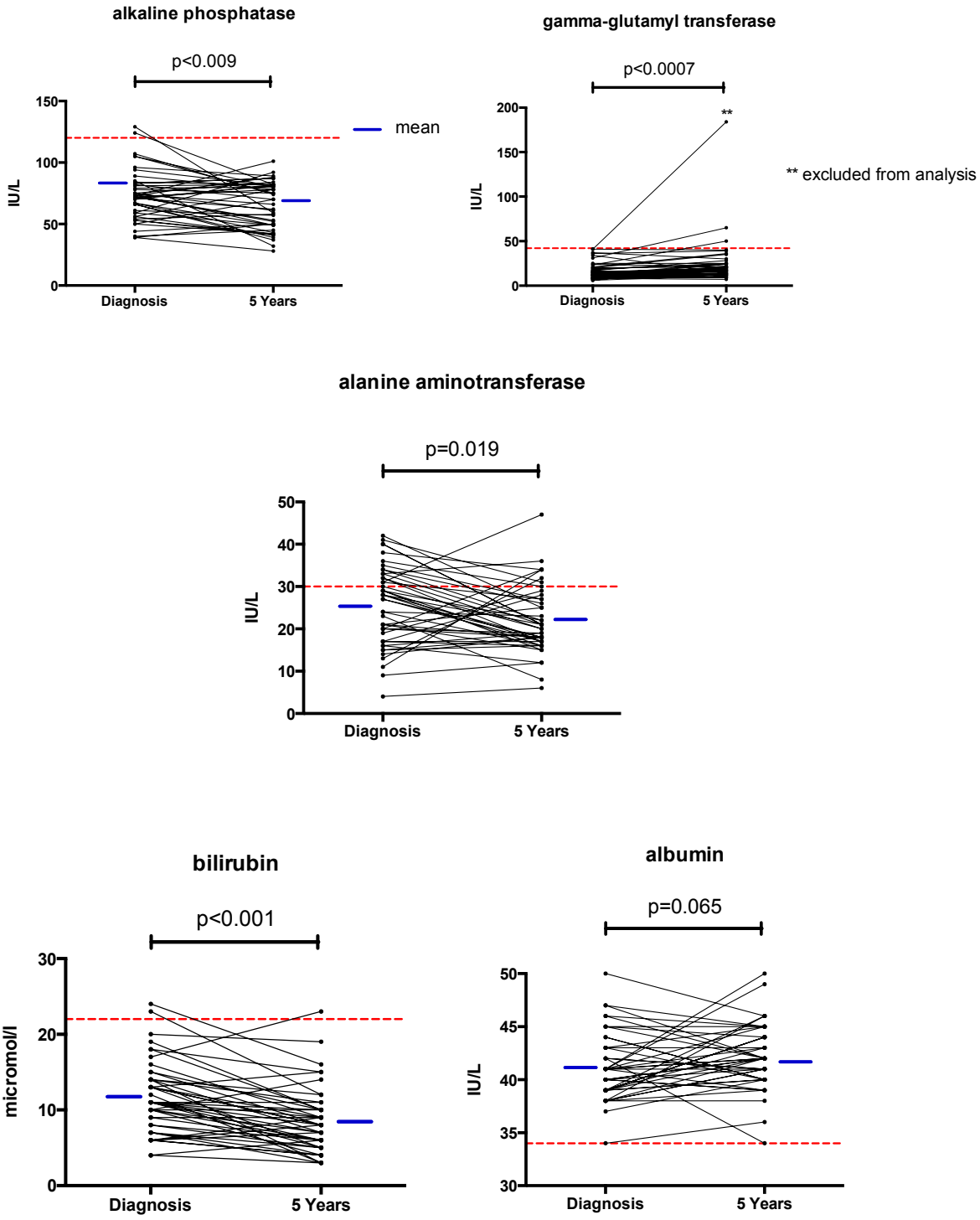
Table 3.13 Abnormal liver function tests at 1 year (baseline values)

Gender	Age	Marsh score	tTG	BMI at 1 year	Change in BMI	Change in body fat percentage	ALP	ALT	GGT	Bilirubin	Albumin	Vitamin D
F	31	0 (3C)	**48** (**224**)	20.0	0.4	2.6	43 (43)	**38 (32)**	40 (24)	13 (8)	44 (40)	**50 (35)**
F	33	1 (3C)	**24** (**161**)	27.7	3.2	7.9	99 (102)	**35 (34)**	12 (49)	6 (11)	38 (39)	88 (93)
F	34	1 (3C)	19 (**84**)	30.2	4.0	3.0	**165 (188)**	**62 (51)**	**86** (44)	15 (9)	40 (41)	**15 (25)**
F	35	3A (3A)	12 (**40**)	26.2	0.2	0.1	99 (71)	19 (18)	**80** (28)	13 (11)	40 (34)	**42** (75)
F	48	3A (3C)	**43** (**201**)	28.8	-1.1	-2.1	53 (72)	**32 (35)**	22 (19)	9 (7)	39 (39)	**64 (36)**
F	50	0 (3B)	6 (**96**)	26.2	1.5	2.5	82 (73)	**114 (35)**	28 (9)	17 (11)	43 (40)	79 (130)
F	56	3A (3B)	**33** (**155**)	24.7	0.4	0.1	72 (87)	28 (54)	**59 (145)**	8 (11)	40 (40)	**69** (78)
F	71	3A (3C)	2 (3)	26.3	3.6	12.0	79 (**170**)	**33 (42)**	19 (15)	8 (10)	43 (37)	**55 (45)**
M	40	3A (3B)	**30** (**97**)	25.4	1.9	5.6	74 (64)	**51** (31)	**45** (18)	11 (11)	40 (41)	**46 (69)**
M	43	0 (3B)	9 (**35**)	23.9	1.0	2.7	86 (84)	**41** (26)	27 (15)	25 (8)	49 (44)	**14 (41)**
M	45	3A (3B)	**47** (**112**)	27.3	1.0	0.5	49 (61)	28(31)	**93** (41)	7 (14)	44 (44)	96 (91)

An elevated ALT normalised in 6/13(46%) at 1 year (Table 3.13). In the remaining seven, the ALT increased further in three participants whilst in two patients with a normal ALT at diagnosis, levels increased above the reference range (both male aged 40 and 43, ALT 41 and 51 U/litre, Marsh 3A and 0, BMI increase by 1.9 and 1.0kg/m^2 respectively). Average levels of ALP decreased between baseline and 1 year (Table 3.13) and there was in inverse correlation between ALP and change in weight, BMI and fat mass (Table 3.12). No other liver function test demonstrated this pattern and total vitamin D levels actually reduced during the first year (65[57-74] to 56[48-65], p=0.0019). The observed change in ALP inversely correlated with change in bone mass (Pearson r= -0.50[-0.68 to -0.25], r^2=0.25, p=0.002) but there was no correlation with either mucosal response or remission(p=0.78 and 0.78 respectively). There was a trend for GGT to increase after 1 year and correlation was noted between the change in GGT and changes in BMI, fat mass and per cent body fat (Table 3.12).

Between diagnosis and 5 years there were changes in all liver function tests (Figure 3.10). Five (10.9%) participants had at least one abnormality (Table 3.14). All five had gained weight and body fat and ALP reduced (73[67-79] to 65[59-70]IU/L, p=0.009), as did ALT (25[23-28] to 22[19-24]IU/L, p=0.019) whilst GGT increased (16[14-19] to 24[16-32]IU/L). As noted at 1 year, change in ALP inversely correlated with change in bone mass (Pearson r= -0.47[-0.68 to -0.21], r^2=0.23, p=0.001) but there was no correlation to either mucosal response or remission. The outlier for GGT is discussed below and was excluded from this analysis. This increase in GGT correlated with weight change but not with BMI or fat mass indices (Table 3.12).

Figure 3.10 Liver function tests at 5 years



At 5 years, an elevated ALT at baseline resolved in 6/9 (67%) and an elevated ALP reduced in both with elevated levels at diagnosis. In one participant (54 year old female) an elevated PTH was noted (asymptomatic, normal calcium, ALP and vitamin D). No participant with any LFT abnormality had a detectable ANA titre.

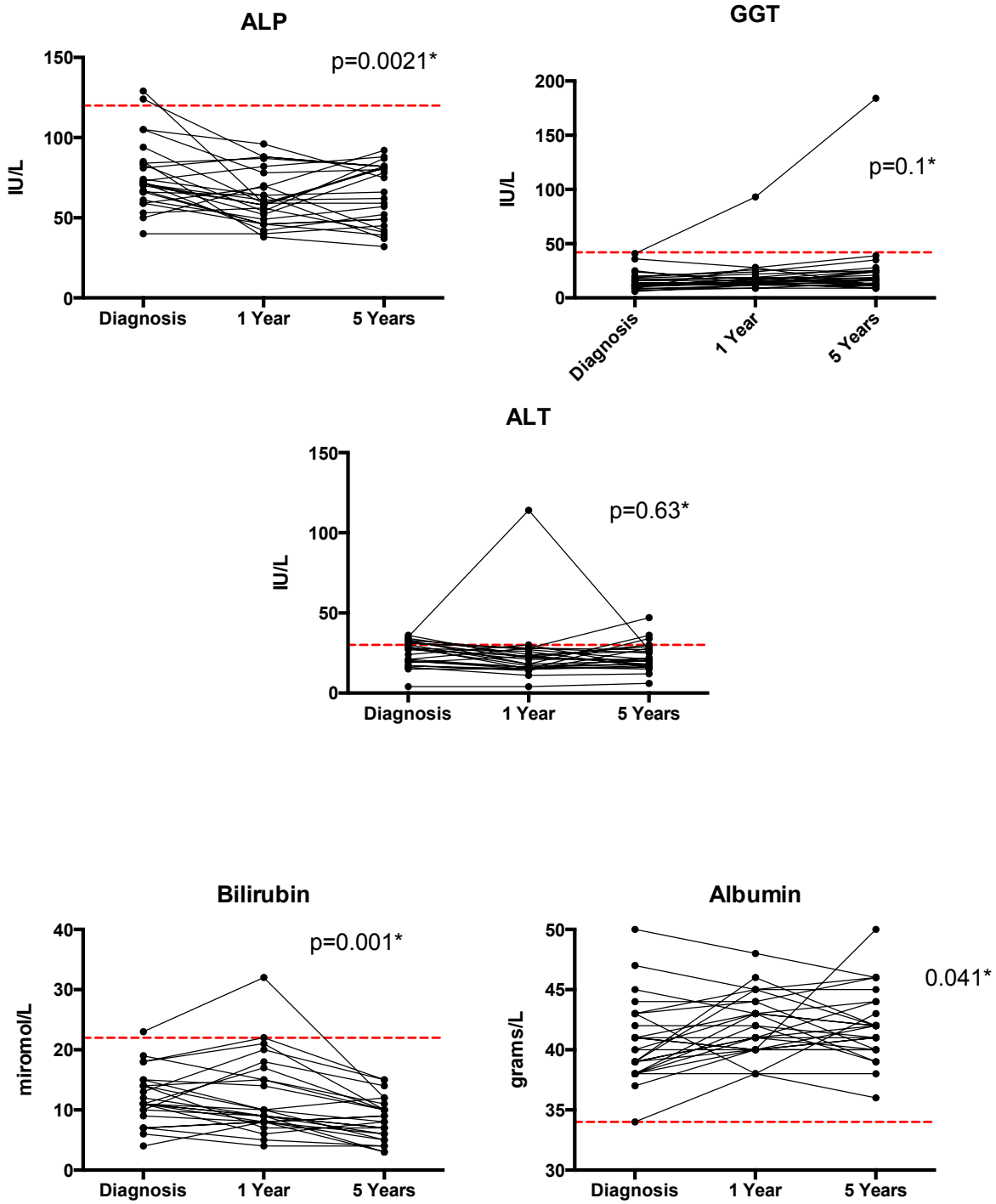
For the 26 participants with three data points, the time dependence of the above changes could be interrogated (Figure 3.11). Only ALP reduced significantly upon assessment of this group and this was principally contributed to by the improvement between baseline and 1 year ($p=0.0021$, one-way ANOVA). Fat mass increased in all but 2 participants whilst body fat percentage increased in all but three. Weight (by median 5kg) and BMI (by median 1.6 kg/m^2) increased in all at 5 years. As shown in Figure 3.12, the only liver function test displaying a relationship to a change in weight was GGT at 1 year (but not at 5 years).

The outlier GGT reading was a 50 year old male with a persistently elevated tTG at 1 and 5 years (47 and 87 IU/ml respectively) but normal DGP IgA and DGP IgG antibodies at 5 years. He was compliant with a GFD, a non-drinker, on no medications and had mucosal remission on small bowel biopsies at 5 years that was consistent with his excellent dietary compliance. No cause was found for this isolated finding after evaluation for other reversible causes (negative smooth muscle antibody, viral hepatitis screen, antinuclear antibody, copper studies and unremarkable liver ultrasound).

Table 3.14 Abnormal liver function tests at 5 years (baseline values)

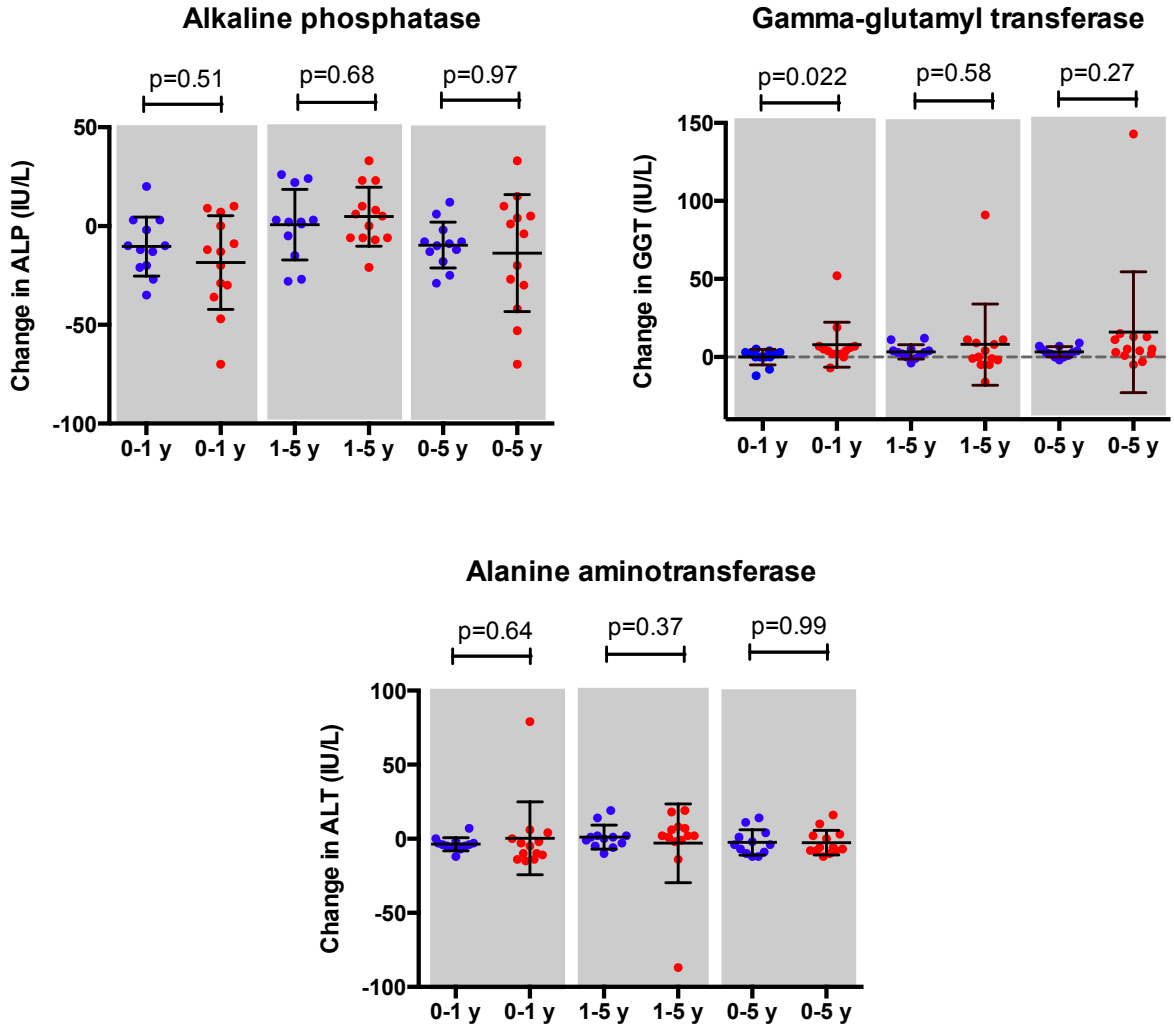
Gender	Age	Marsh Score	tTG (IgA)	DGP (IgG)	BMI	Change in BMI	Change in body fat percentage	Bilirubin	ALP	GGT	ALT	Albumin	Vitamin D
F	53	1 (3B)	16 (**226**)	7	31.1	4.4	3.8	4 (6)	75 (105)	35 (20)	**36** (**33**)	38 (38)	95
F	54	3A (3A)	14 (**41**)	**27**	27.0	4.3	10.1	8 (13)	78 (56)	22 (8)	**32** (13)	45 (41)	**59**
F	57	1 (3B)	6 (**231**)	6	24.4	1.3	1.0	4 (7)	81 (94)	19 (10)	**34** (20)	43 (43)	85
F	61	1 (3C)	6 (**28**)	13	30.6	6.2	11.4	10 (18)	82 (**124**)	11 (6)	**30** (**36**)	42 (41)	**66**
M	50	0 (3B)	**84** (**112**)	7	27.9	1.6	4.4	7 (14)	57 (61)	**184** (41)	**47** (31)	40 (44)	92

Figure 3.11 Liver function tests in those with data at baseline, 1 year and 5 years



* one-way ANOVA

Figure 3.12 Change in Liver function tests for those patients with data at all three data points (• weight gain <5kg, • weight gain ≥5kg)



Elevated bilirubin

At diagnosis, 1 year and 5 years an elevated bilirubin was noted in 2, 4 and 1 participant(s) respectively. Elevations were minor (range 22-32micromol/L), and in no patient was the elevation associated with any other liver function test abnormality. Average bilirubin levels increased between diagnosis and 1 year but then reduced overall at 5 years. No participant reported a past history of liver disease or jaundice. As total bilirubin was not significantly elevated in the majority of patients, levels were not fractionated and thus the contribution of unconjugated bilirubin to the total amounts could not be assessed.

DISCUSSION

We have described the pattern of liver function tests at diagnosis and the effect of treatment with a strict GFD over 5 years in a group of consecutively recruited participants with coeliac disease. In this well characterised population with excellent dietary adherence, at diagnosis almost one third (29%) were noted to have at least one abnormal liver function test and this fell to 21% at 1 year and 10.1% at 5 years suggesting an overall improvement with treatment. There was a higher incidence of liver function test abnormalities noted in our population at diagnosis compared to recent studies[230,426] but similar to a meta-analysis where up to 40% were found to have abnormal readings[427].

Although severe liver related complications are recognised in CD, as we have observed in the current study, the vast majority of abnormalities described are those relate to mild ALT elevations. In retrospective studies, so called 'transaminitis' has been noted to be associated with a higher BMI and more severe intestinal pathology at diagnosis of CD[426]. Although there was a trend in the present study for those with an elevated ALT to have a higher BMI,

this observation was not supported by body compartment subanalysis for body fat percentage or waist to hip ratio. These observations may support a role for an immune mediated process contributing to the abnormalities but we did not find any correlation to intestinal pathology or antibody titres. No participant evaluated at 5 years for abnormal testing was found to have a secondary cause and all five were negative for antinuclear and smooth muscle antibodies. Histological studies have identified a non-specific hepatitis underlying these abnormalities in CD[228,236] but the mild abnormalities in the current population did not justify liver biopsy to draw comparisons. Weight loss is also well recognised to cause abnormalities in liver function tests but we did not examine patterns of weight in the lead up to diagnosis.

In a younger population of patients with CD, ALP has been shown to decrease with treatment with a GFD [230]. In our cohort, there were 5 participants with an elevated ALP at baseline. All were Vitamin D deficient and 4 had reduced bone mineral density suggesting a possible role of osteomalacia and secondary hyperparathyroidism. But we did not assess PTH levels at this time point. Supporting this explanation was the observation that all 5 had either improved or normalised their ALP by 12 months and in one with persistent (but improved) elevation, vitamin D levels fell. Furthermore, the change in ALP correlated with an increase in total bone mass. Alkaline phosphatase can also be derived from the small intestine but there was no relationship between the changes in ALP and mucosal disease activity.

In short term follow-up studies of adults and in children, transaminitis has improved in the majority with elevated levels at diagnosis but long term prospective data has been lacking and there has been little correlation to thorough body composition analysis. Only one prospective study (limited to 1 year) has examined body mass index in concert with liver function tests in CD. Similar to our population, transaminases reduced on average and although ALT

increased in a proportion, BMI did not significantly change[231]. In the current study, BMI and weight increased during treatment but there was no relationship between the change in ALT and changes in overall or fat-compartment body composition indices. Levels of GGT increased and this may have been reflective of the increase in weight and BMI as evidenced by a weak relationship to BMI and fat indices at 1 year and weight at 5 years.

Most previous studies have report numerical improvement in liver function tests with commencement of a GFD[229-231,240] and conversely ALT has been observed to increase after gluten challenge [231]. Not all abnormalities resolved with a GFD in our cohort. Persistent elevations of ALT were noted in 12% at 1 year and 4% at 5 years, whilst in a small proportion liver function test values increased. This is somewhat consistent with a recent prospective study of 130 adults. All with elevated transaminases at baseline (10% of the total population studies) returned to normal values, but there was a sub group whose ALT increased from normal values with treatment[231].

The reduction in bilirubin over time is an interesting observation also noted, but not explained, by one previous study[230]. The observed reduction in our cohort was within the reference range and is therefore of limited clinical significance. A plausible explanation may be low-grade haemolysis given the recognised associations of CD with autoimmune conditions and hyposplenism, but these were not specifically assessed as part of the current study.

A weakness of our study was the lack of histological correlation. It would indeed have been informative to confirm whether the minor ALT elevations were associated with non-specific

the non-specific hepatitis noted by prior histological studies[228,236]. But the abnormalities we observed were very mild and in our view biopsy could not be justified. The resolution of the majority of abnormalities in the majority and the lack of correlation to body composition changes suggests an immune basis to the baseline abnormality. We did not find an association between antibody titres at diagnosis or at either follow-up time point but this may represent underpowering given the serological improvement in most of the cohort irrespective of baseline liver function tests.

The finding of one participant with predominant cholestasis at 5 years is interesting in the context of the recognised association with this pattern with both primary sclerosing cholangitis and primary biliary cirrhosis[237-239]. At subsequent follow-up this patient's liver function tests have normalised and no secondary cause was found at the time of the 5 year study visit (in particular anti-mitochondrial antibodies were negative).

The strengths of the study were its prospective nature, the size of the cohort, the fact that dietary education was delivered by a dietitian with considerable and recognised expertise in coeliac disease, and that adherence to the diet was so high. The incomplete follow-up of many of the cohort related to the original design of the study (limiting year 1 follow up to the first half of the recruited patients due to funding issues) and to the lack of desire for many to have further invasive investigations at 5-years. However, there is no indication that this biased the results. Those who had assessment at the three time points had very similar responses to the populations who were evaluated only at one or five years. Age- and gender-matched healthy controls would have strengthened the observations, particularly with regard to body compositional analysis and future studies examining these important disease outcomes should address this issue. This study was also limited by a lack of histological correlation. This was

representative of the very mild abnormalities noted and reinforced by improvement in the vast majority.

In conclusion, strict compliance with a GFD is associated with improvements in abnormal liver function tests when elevated at diagnosis in the majority of patients. Gamma-glutamyl transferase increased with measures of weight and fat whilst reduction in ALP over time was likely to be related to bone turnover. Transaminitis at diagnosis appears to be an extra-intestinal manifestation of CD as demonstrated by a lack of correlation to body composition indices and resolution of elevated levels in the majority of this population with a high proportion of mucosal response. Persistent abnormalities are found in a proportion and may be related to factors outside of CD pathogenesis. The data presented here support the hypothesis that liver function test abnormalities are common in CD and that resolution of these liver function tests occurs in the majority.

3.4 Neutropenia in newly diagnosed coeliac disease and response to treatment with a gluten free diet

INTRODUCTION

Coeliac disease is defined by small intestinal villous atrophy resulting from an abnormal immune response to ingested gluten in genetically susceptible individuals. The condition is common, affecting 1 in 100 of the population and, for reasons yet to be established, is increasing in incidence in parallel with other autoimmune diseases [8]. Manifestations beyond the intestinal lesion are being increasingly reported and include neurological disorders, infertility, osteoporosis and other autoimmune conditions [147,214,428-430]. It is therefore becoming increasingly evident that coeliac disease is a systemic condition with ramifications beyond the intestinal lesion.

A number of haematological manifestations have been previously described in CD including idiopathic thrombocytopenia purpura and venous thromboembolism [243,245,246]. In children there has been a reported increased incidence of leukopenia and more recently, links between CD and eosinophilic disorders have been described [431].

Following an observation that a number of adult patients with CD were being excluded from an unrelated clinical trial due to low neutrophil counts, we aimed to examine these indices in this prospectively evaluated cohort described in detail in Chapter 3.1 and to determine associations with other systemic and nutritional indices.

METHODOLOGY

Methods for recruitment are described in detail in Chapter 3.1 and Figure 3.1. At each of the three time points routine bloods included a white blood cell count as well as differential count. All cell counts were performed at the same laboratory (Eastern Health Pathology, Box Hill, Australia). As per the laboratory reference range, leukopenia was defined as a white cell count less than 4.0 x 10^9/L, neutropenia as less than 2.0 x 10^9/L and lymphopenia less than 1.0 x 10^9/L. Anaemia was defined as a haemoglobin less than 120 g/Litre in females and less than 130 g/L for males. Thrombocytopenia was defined as a platelet count less than 150 x 10^9/L. Abnormalities in white blood cell indices were then compared to demographic data, duodenal histopathology according to the Marsh grading, coeliac serology (tTG), evidence of other extra-intestinal manifestations (such as abnormal LFTs), nutritional status in terms of micronutrients and anthropometry, and body composition measured via DEXA to identify risk factors for the observations.

RESULTS

A detailed explanation and outline of the numbers at each time point are described in Chapter 3.1. As described in Table 3.15, at diagnosis 10 of 72 patients (13.9%) were noted to have leukopenia (median 3.7 (range 2.5 to 3.9) x 10^9/L) and 14 (19.4%) neutropenia (1.71 (1.32 to 1.97) x 10^9/L). Only one participant had neutropenia without leukopenia. Lymphopenia was noted in 4 (4.2%) patients, two of whom also had neutropenia and leukopenia. One female with neutropenia was noted to have mild anaemia with normal platelet and total white cell counts, and haematinic levels. Two patients (2.9%; both female aged 28 and 21 years) had mildly elevated eosinophil counts (0.51 and 0.52 x 10^9/Litre). Neither had a history of asthma or atopy. There were no patients at diagnosis with leucocytosis, neutrophilia or lymphocytosis.

Table 3.15 Clinically relevant characteristics of patients with low leukocyte, neutrophil and/or lymphocyte counts in peripheral blood at diagnosis of coeliac disease. Results outside the reference range are shown in red.

Age	Sex	Genotype	Marsh score	tTG (<20 U/mL)	Leuko-cytes (4.0-10.0 x 10^9)	Neutro-phils (2.0-7.0 x 10^9)	Lymph-ocytes (1.0-3.0 x 10^9)	Haemo-globin[1]	Platelets (150-410 x 10^9/L)	Albumin (34-47 g/L)	Ferritin (30-400 µg/l)	ALT[2]	BMI (kg/m^2)	Skeletal muscle mass (kg)	B_{12} (156-698 pmol/L)	Folate (10-42 nmol/L)
50	F	DQ 2.5 cis hetero	3B	96	**2.5**	**1.33**	**0.77**	130	177	40	20	**35**	24.7	22.7	433	**46**
33	F	DQ 2.5 cis hetero	3B	83	**3.1**	**1.97**	**0.79**	132	162	42	**11**	9	31.2	19.0	204	20
65	F	DQ 2.5 cis hetero	3B	117	**3.3**	**1.74**	1.06	148	232	39	65	17	20.3	17.2	971	**46**
48	F	DQ 2.5 cis hetero	3A	67	**3.8**	**1.87**	1.39	131	187	44	47	30	22.6	28.5	254	25
36	F	DQ 2.2 cis 2.5 cis	3A	10	**3.8**	**1.55**	1.79	149	209	41	**27**	17	15.7	19.6	263	27
49	F	DQ 2.5 cis DQ 8	3A	111	**3.8**	**1.95**	1.50	123	208	39	**29**	**34**	24.6	38.4	264	25
51	F	DQ 2.5 cis hetero	3B	156	**3.9**	**1.55**	1.79	125	214	41	30	28	28.5	26.2	313	20
27	F	DQ 2.5 cis hetero	3B	71	4	**1.84**	1.65	129	254	41	32	**48**	26.2	17.4	559	37
46	F	DQ 2.5 cis hetero	3B	194	4.1	**1.44**	2.21	146	313	44	199	24	21.1	18.4	203	12

Age	**Sex**	**Genotype**	**Marsh score**	**tTG** (<20 U/mL)	**Leuko-cytes** (4.0-10.0 x 10^9)	**Neutro-phils** (2.0-7.0 x 10^9)	**Lymph-ocytes** (1.0-3.0 x 10^9)	**Haemo-globin**[1]	**Platelets** (150-410 x 10^9/L)	**Albumin** (34-47 g/L)	**Ferritin** (30-400 µg/l)	**ALT**[2]	**BMI** (kg/m^2)	**Skeletal muscle mass** (kg)	**B_{12}** (156-698 pmol/L)	**Folate** (10-42 nmol/L)
56	F	DQ 2.2 cis 2.5 cis	3B	155	4.3	**1.87**	1.87	126	219	40	346	**54**	24.3	22.6	612	**46**
26	F	DQ 2.5 cis hetero	3B	6	4.4	**1.73**	2.02	142	223	40	55	20	23.1	21.6	458	27
39	F	DQ 2.5 cis homo	3A	221	5.7	**1.82**	2.73	**116**	361	39	70	**40**	22.4	20.4	261	21
36	M	DQ 2.2 cis 2.5 cis	3C	32	**3.7**	**1.93**	1.23	159	196	45	201	11	24.6	20.6	191	24
24	M	DQ 2.5 cis hetero	3A	6	**3.7**	**1.52**	1.58	148	**138**	47	40	**38**	24.7	20.8	224	35
56	M	DQ 2.5 cis hetero	3B	192	**3.9**	2.07	1.32	151	203	45	370	31	26.1	15.2	551	20
45	M	DQ 2.2 cis hetero	3A	22	5.7	4.20	**0.96**	132	274	39	35	33	19.1	24.4	229	41

[1] Reference range 120 to 150 g/L (female), and 130 to 170 g/L (male)

[2] Reference range 5 to 30 IU/L (female), and 5 to 40 IU/L (male)

No associations were found for the observed abnormalities in white cell counts or differentials. Patients with either leukopenia or neutropenia were of a similar age (mean 43 (95% CI 36-50) versus 41 (38-42) years; p=0.56) and had similar tTG antibody titres (101 (62-141) versus 105 (84-126) Units/ml; p=0.81), weight (68 (60-76) versus 68 (65-72) kg; p=0.73) and BMI (24 (22-26) versus 24 (23-25) kg/m^2; p=0.60) to those with normal white cell indices. Three female participants with neutropenia were found to have a low ferritin but were not anaemic. The remaining haematinic indices (B12 and folate) were normal in all. Further, no trends were noted in genotype or in the proportions with abnormal liver function tests, lean body mass, skeletal muscle mass and body fat indices or bone mineral density.

Response to treatment with a gluten-free diet

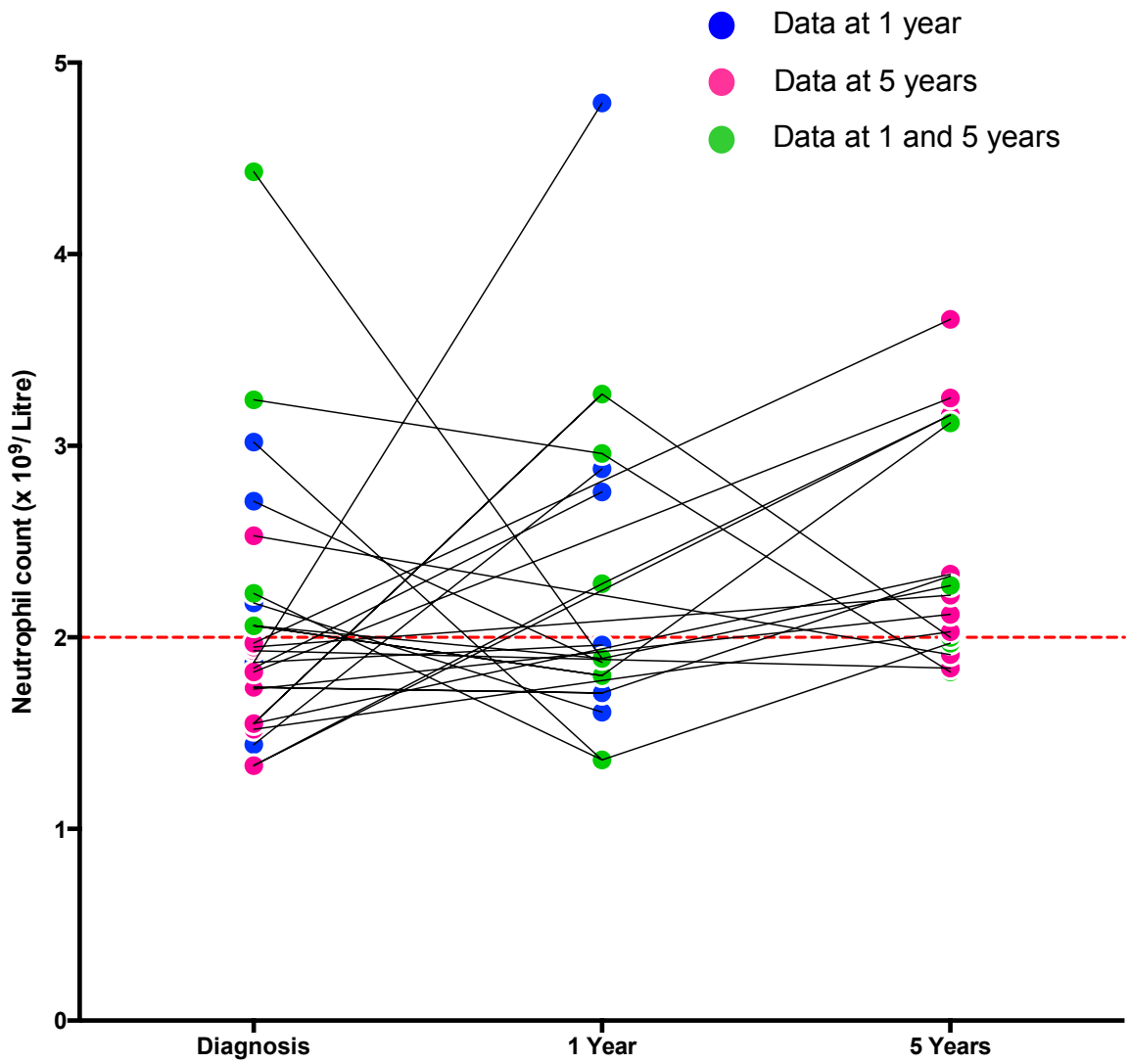
For those with data at 1 year (n=52), there was no difference between baseline total white cell count (mean 5.8 (95% CI 5.4 to 6.3) versus 6.1 $x10^9$ (5.5 to 6.7); p=0.89), neutrophil count (3.3 (2.9 to 3.6) versus 3.6 (3.1 to 4.1); p=0.54) or lymphocyte count (1.8 (1.7 to 2.0) versus 1.8 (1.6 to 2.0); p=0.98) between baseline and 1 year. Data was available for 9 patients with white blood cell abnormalities at 1 year, 12 patients at 5 years and for 5 patients at all 3 time points. At 1 year, the total white cell and neutrophil counts had returned to normal values in 3/6 (50%) and 6/7 (86%) respectively (mucosal remission n=4; mucosal response n=2). Of the patients with persistent leukopenia or neutropenia 2/3 (67%) had mucosal remission (one improved from Marsh 3B to 3A) and all had normal coeliac antibodies. Interestingly, 5 patients with previously normal white cell and neutrophil counts were noted to develop leukopenia (n=3) and neutropenia (n=5) at 1 year (Figure 1). All had markedly improved tTG concentrations (n=2 remained elevated at 21 and 42 Units/mL) at 1 year and four had improved Marsh scores (one remained static at Marsh 3A). Of those participants with

lymphocytosis at diagnosis, this persisted in 1/2 (50%). One participant with an elevated eosinophil count had a normal reading at 1 year.

Data were available for 46 patients at 5 years (Figure 3.1). Similar to data at 1 year, there was no difference in total white cells (5.4 (1.9 to 5.8) versus 5.5 (5.2 to 5.9); p=21) or neutrophils (2.9 (2.6 to 3.3 versus 3.1 (2.8 to 3.3); p=0.26) but total lymphocyte count significantly increased (1.7 (1.5 to 1.8) versus 1.8 (1.6 to 1.9); p=0.04). After 5 years of treatment with a GFD, 7/9 (78%) with leukopenia at baseline had returned to normal and 9/10 (90%) with neutropenia had a normal neutrophil count. Both with persistent abnormalities had mucosal remission and normal coeliac antibodies. In a similar trend to the observations at 1 year, 3 patients developed new leukopenia (n=1) and neutropenia (n=3) (Figure 1). All 3 had normal coeliac antibodies and were noted to have mucosal remission at 5 years. Data was available at 5 years for 2 of the 4 participants with lymphocytosis at baseline. Both had lymphocyte counts in the normal range. The remaining participant with an elevated eosinophil count had a normal count at 5 years.

For those with data at three time points, after 1 year, 2/4 (50%) with leukopenia and 2/3 (67%) with neutropenia at baseline had returned to normal and at 5 years these proportions were 3/4 (75%) and 3/3 (100%) respectively.

Figure 3.13 Pattern of change in neutrophil count for patients with neutropenia at any time point



DISCUSSION

In this study, we have described a novel observation in a group of well-characterised patients with newly diagnosed coeliac disease. We observed a high than expected incidence of both leukopenia (in 14%) and neutropenia (20%). The vast majority had normal haemoglobin and platelet indices (i.e. the abnormalities did not appear to be related to a pancytopenia) and, with the exception of iron deficiency without anaemia in a few, haematinics were normal. In CD, leukopenia has been observed in 9% of a selected paediatric population [243] and lower total white cell counts have been noted in adults [432]. The population incidence of neutropenia is less than 1% with higher rates observed in African Americans [242]. No patient in the current cohort was of African origin.

There are no firm population data on the incidence of lymphopenia but in newly diagnosed CD reduced proportions of total lymphocytes and T-cell subsets has been observed when compared to treated CD and healthy controls [432,433]. Also, higher levels of lymphocytes have been found in those with CD and hyposplenism [434]. In the present study, 6% were noted to have lymphopenia but we did not assess surface white blood cell markers and numbers were too small to make robust conclusions.

The incidence of peripheral eosinophilia has not been prospectively assessed in CD but a pathological case series identified a proportion of CD patients with eosinophilic infiltration of the lamina propria in the absence of peripheral eosinophilia[257]. We observed very mild abnormalities in a small proportion (2.8%), but did not specifically assess duodenal histology for eosinophilic infiltrates. For comparison with our cohort, the population incidence of peripheral eosinophilia may be as low as 0.1% [258]. As pointed out in a recent meta-analysis,

any link between CD and eosinophilic disorders is somewhat counterintuitive due to the unique pathophysiology [431].

Upon commencement of a GFD, the incidence of the observed abnormalities resolved in the majority. After 5 years, leukopenia and neutropenia had normalised in 78% and 90% respectively. Those with lymphopenia and eosinophilia also tended to normalise with time. This is suggestive of a treatment response but firm conclusions are difficult to draw. In addition to the persistence of neutropenia in a small proportion, a number of patients with normal white cell indices at diagnosis developed neutropenia and leukopenia at both 1 and 5 years. This was despite apparent disease response in the majority of patients. Further, factors that might be representative of disease burden at diagnosis (such as histological severity, coeliac antibody titres and body protein status) were not associated with white cell count indices. Treatment response for lymphopenia has previously been suggested in a case control study [432], but there are no previous prospective reports for comparison.

Of potential relevance to the above observations regarding leukopenia, CD is associated with a higher incidence of bacterial and mycobacterial infections[250-252]. This may in part be explained by the high incidence of functional hyposplenism in CD due to the reduced production of IgM memory cells important for defence from encapsulated organisms, and has prompted guidelines to recommend pneumococcal vaccination[29,253]. The incidence of functional hyposplenism in CD has been reported to be as high as 77% [254]. Unfortunately our patient cohort did not have a routine blood film performed and thus we were unable to comment on the presence or absence of functional hyposplenism. The increased rates of infection with non-encapsulated organisms in the above studies have not been adequately explained. Whether the observations in the present study point toward an impaired ability to

mount an effective neutrophil response to infection has not been evaluated. Interestingly, up to 28% of adults with CD have been noted to have neutrophilic infiltration in one histological study[435], but measures of intestinal inflammation that reflect neutrophil activity (such as faecal levels of calprotectin) have been disappointing in the evaluation and monitoring of CD[436].

The white cell indices observed in this study are mild and are at levels not necessarily associated with a higher incidence of infection in the broader community. However, neutropenia has not previously been reported in association with CD and these findings expand the growing list of extra-intestinal associations of CD. Exploring the aetiology of neutropenia was not an aim of this study but an immune basis would seem the most intuitive, particularly as no patient had reduced B12 or folate indices that might otherwise explain the observations. Similar manifestations are well recognised in other autoimmune conditions such as systemic lupus erythematosis and rheumatoid arthritis[437,438]. Conversely, coeliac disease should now be included in the list of causes for mild neutropenia and screening for coeliac disease should be part of the diagnostic workup of a person with unexplained neutropenia.

In conclusion, we have described neutropenia as a new extra-intestinal manifestation of CD, being present in up to 1 in 5 patients with newly diagnosed CD. Conversely, we have identified another cause for unexplained neutropenia; perhaps all such patients should have CD excluded. Underlying associations such as haematinic or protein deficiency, or other extraintestinal manifestations such as abnormal LFTs or BMD, were not identified. Although cell counts return to normal values in the majority upon treatment with a GFD, this was not universally so, even with complete healing of the duodenum and neutropenia can develop

during treatment. Similar treatment responses were observed for leukopenia and lymphopenia. These findings warrant further investigation in larger prospective case control studies. Whether they carry any clinical implications is not known, but potentially contribute to the increased incidence of sepsis in CD.

3.5 Point of care testing – an accurate tool for monitoring coeliac disease?

INTRODUCTION

Coeliac disease is an increasing public health problem with long term consequences that include an increased risk of malignancy, mortality and a myriad of extra-intestinal manifestations[9,12-14]. An evolving evidence base is demanding regular follow-up of patients with CD[439]. Ongoing mucosal inflammation is associated with an increased risk of osteoporosis, autoimmune disease and lymphoma[177,440] and as few as 8% achieve mucosal healing after 2 years and 66% after five years on a gluten free diet [100,157].

Coeliac antibodies form part of routine clinical practice in the monitoring of CD. They are a reliable marker of mucosal disease activity at the mucosal level and may provide insights into dietary compliance[441]. Owing to the need for 'batching' of samples to improve cost efficiencies in ELISA based assays, there can often be a delay of several days before receiving results, and in the ambulatory setting this often creates inefficiencies in service provision.

Qualitative Point of Care (PoC) testing has developed considerably over recent years, particularly as a population based screening tool for patients with undiagnosed CD[53,54]. Point of care testing provides a sensitive and specific means of antibody testing in real time during a patient consultation. Results are available promptly (within 10 minutes) and the test is well tolerated, requiring only a finger prick blood sample or microliter serum sample. There is a paucity of data evaluating qualitative PoC testing in the long term monitoring of treated adult CD.

We aimed to assess the sensitivity, specificity as well as positive and negative predictive values of a Deamidated Gliadin Peptide (DGP) IgA and IgG qualitative antibody PoC test (Simtomax®, Augurix SA, Plan-les-Ouates, Switzerland, described in [54]) in sera collected from a well defined population of patients diagnosed with CD being prospectively monitored on a gluten free diet for at least 5 years. We also set out to evaluate the inter-observer reliability between three qualified observers assessing these kits.

METHODOLOGY

As described in detail in section 3.1, participants enrolled in prospective study in 2006 evaluating newly diagnosed CD were invited to participate in 2011. The baseline study included subjects with CD defined as having at least Marsh 1 on duodenal histology in association with an HLA susceptibility genotype (HLA DQ2 or DQ8). Consenting participants had coeliac antibodies collected at baseline and were educated by a specialist dietitian regarding a gluten free diet at baseline and again at 12 months.

Each participant had blood drawn for coeliac antibodies assessed by ELISA for tTG IgA, DGP IgA and DGP IgG (INOVA Diagnostics; Reference range < 20 U/ml for both) and total serum IgA. Whole blood was also centrifuged and collected serum stored at -80 degrees for further analysis.

After bringing all stored serum samples simultaneously to room temperature, 20 microlitres of serum was applied to each DGP PoC test, followed by buffer solution as per the manufacturers instructions for serum and as described elsewhere [54]. Results were interpreted 10 minutes later by three observers blinded to both the serum results and other observers' interpretation. Each

observer was familiar with the kits and assessed the three lines of the PoC test (DGP (A), Total IgA (B) and Control (CT)) as Positive, Faint positive or Negative (see Figure 3.14). Observer 1 was the most experienced and was thus determined to be the gold standard for the purposes of assessing sensitivity, specificity and positive and negative predictive values.

STATISTICS

Descriptive statistics were used for the data set comparing cases to controls. A 'case' was defined as having any positive coeliac antibody (tTG IgA, DGP IgA or IgG >20U/ml). Fleiss' kappa was used to determine agreement between the 3 observers.

RESULTS

Of 99 eligible participants, 46 (median age 49 (range 23-72) years, 12 male) provided informed consent. None were excluded after enrolment. All had histologically confirmed CD and were prescribed a strict GFD at diagnosis. After 5 years (median 66 (range 61-69) months), 45/46 (98%) demonstrated good adherence to a GFD. One participant was noted to have fair compliance.

Serum results

Of the participants, 14 (30.0%) had elevated coeliac antibodies (Table 1). Three (3) subjects had elevated DGP IgA *and* IgG (DGP IgA/IgG values of 44/22, 31/34 and 21/30 U/ml respectively). A further 6 subjects had elevated IgG alone (median 28 (range 22 to 57) U/mL) as assessed by ELISA. No patient was IgA deficient (mean serum IgA 2.3 (range 0.8-4.5) g/L). Five subjects with negative DGP IgA and IgG were found to have an elevated tTG (median 42 (20-84) U/ml).

Table 3.16 Summary of results (Observer 1)

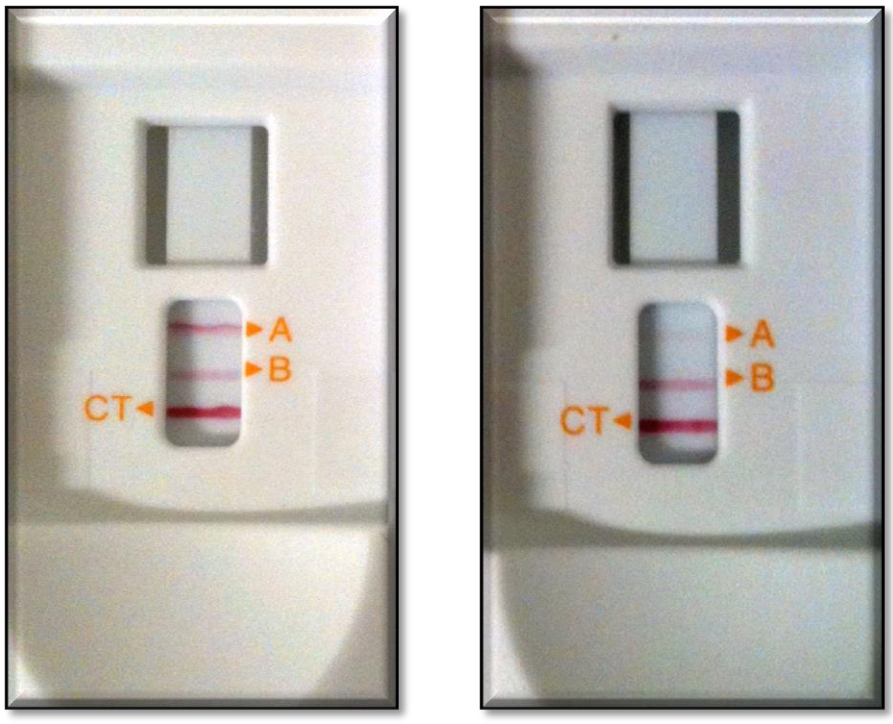
		Serum ELISA (DGP IgA or IgG >20 U/ml)		
		Positive	Negative	Total
DGP PoC test	Positive	9	2	11
	Faint positive	1	6	7
	Negative	0	28	28
	Total	10	36	46

Sensitivity (%)	100
Specificity	77.8
Positive predictive value	55.6
Negative predictive value	100

DGP PoC Test Performance

Across the 46 samples, all PoC tests performed well with no device failure (as assessed by the presence of internal control line (CT – Figure 3.14). The IgA line ('B') was present in all subjects showing a 100% concordance with serum results. All 9 positive DGP ELISA results were interpreted as positive by the PoC test yielding 100% sensitivity (Table 1). Nine ELISA-negative kits were interpreted as either PoC Positive (n=3 (DGP=7, 3 and 18 U/ml)) or Faint positive (n=7 (DGP median 11 (3-19) U/ml)) by Observer 1 (Table 3.16) resulting in a specificity of 78% and a positive predictive value (PPV) of 56%.

Figure 3.14 DGP Point of Care examples showing the 3 interpretation lines: Control line ('CT'), total IgA line ('B' positive in both), combined DGP IgA and IgG line ('A') as labeled.



Positive 'A' line Faint positive 'A' line

Inter-observer agreement

Interobserver agreement was excellent (Fleiss' kappa=0.85 (95% CI (0.73 to 0.97)) across the 46 sera. When only positive or faint positive results were analysed, the inter-observer agreement was less robust (0.68 (0.48 to 0.88)) placing concordance at the lower end of 'strong agreement'. For the 13 kits where at least one observer noted a faint positive result, inter-observer agreement was poor (0.19 (-0.05 to 0.43).

DISCUSSION

Due to the recognised complications of poor mucosal healing in CD, there is a clinical need for the long term monitoring of CD[439]. In particular, it behoves the clinician to ensure monitoring for mucosal healing given the now well recognised risks of poor mucosal healing[8,177,440]. Although imperfect[165], coeliac antibodies are the most reliable non-invasive means for assessing disease activity at the mucosal level[441]. Traditional antibodies such as whole gliadin and endomysial antibodies are being superseded by more sensitive and specific ELISA based antibodies that are less operator dependent. More recently antibodies to deamidated gliadin peptide have become widely accepted and allow for assessment of both DGP IgG and IgG antibodies.

Qualitative PoC testing is relatively new to the field of CD diagnosis and has been demonstrated to be a useful tool in the population screening. In this context point of care testing has demonstrated sensitivities and specificities ranging from 79-93% and 95-96% respectively and a PPV of 71% [53,54]. The Simtomax DGP test is well suited for a screening application as it detects both IgA and IgG anti-DGP antibodies, as well as total IgA for diagnosing all CD patients, including those suffering from an IgA deficiency. Delays in obtaining coeliac antibodies in the

clinic setting can make clinic appointments inefficient and thus PoC testing may be an attractive means of real time antibody assessment. Few studies have evaluated the application of PoC testing to CD monitoring.

The perfect (100%) diagnostic sensitivity of the DGP PoC found in the current study reflects and confirms its applicability as a screening tool. However, with such a high sensitivity, samples at or near the assay cut-off levels were interpreted as positive or faint-positive higher rate of false positive results (24%) and a resultant impact on specificity and PPV (77.8% and 55.6% respectively). The negative predictive value of 100% can be assessed as reassuring the clinician that serum ELISA results will be negative, however if faint or positive results appear, formal pathology tests or endoscopy should be awaited. The finding of poor inter-observer reliability in assessing faint-positive results is interesting and has potentially important implications for both the context of this study and for CD screening. For reasons that are unclear previous studies have identified Type I diabetes as a risk factor for false positive PoC results[53] but Type I diabetes was not present in our cohort. To overcome this issue investigators have used a colorimetric scale do define a cut-off for positivity of the point of care test. This technique may prove a cumbersome barrier to the implementation of an otherwise easy to use technology but a Rann scale cut-off of 2 accurately separated diseases from the controls[54].

Another finding of this study was the relative inaccuracy of coeliac antibodies in predicting dietary compliance. After at least 5 years on a gluten free diet, at all except one participant were considered to have good compliance as assessed by food diaries and specialist dietitian assessment. Despite this reported compliance the incidence of elevated coeliac antibodies was high (30%). Certainly there may be bias in the reported compliance in a clinical trial setting, but

these findings are consistent with previous reports[165] and there should be caution in attributing elevated antibodies to dietary misadventure.

The strengths of this study are that the participants were well characterised and that they had been monitored prospectively whilst on a gluten free diet for at least 5 years. All had been educated regarding a gluten free diet by a specialist dietitian at diagnosis – advice that was reinforced and at 12 months. Unsurprisingly, compliance was high at this 5 year time point. The PoC tests were conducted under identical conditions for all three observers and blinding was maintained. Also, a standardised ELISA kit in a single laboratory was used to assess all sera to provide the measured standard. A drawback of this study is the use of serum rather than whole blood. Whilst whole blood would have provided a more real life assessment of these kits, other investigators have similarly used serum and there are few intuitive reasons to suggest why the results would have been significantly different utilising this technique[53,54].

It is pertinent that PoC testing was not specifically designed for monitoring CD. As demonstrated in this study, for a test relying on antibody binding to activate a visual cue, the rate of false positive results increases when the results are at near the quoted cut-off. Nevertheless, as demonstrated in this well characterised and largely compliant population, antibody positivity is a common occurrence and certainly more common than encountered in population screening. Thus, the pre-test probability is significantly higher and more accurate non-invasive tests would be advantageous for the reasons outlined.

CONCLUSION

In this cohort of patients treated with a GFD for 5 years or more the DGP PoC test produced results of similar accuracy and reliability to those found in population screening but with a lower positive predictive value. It is reliably negative but poorly predicts low levels of continuing positivity of DGP in this cohort. Thus a positive PoC result in this setting warrants further investigation with ELISA and/or small bowel biopsies however a negative PoC result can be assumed to be reliable and predictive of serum results. Although greater accuracy is desirable, the DGP PoC test may be a practical tool to avoid delays in assessment of an increasingly monitored outpatient population.

Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution
Dr Evan Newnham was involved in study conception, study design, patient recruitment, conducting cognitive tests and study visits, data analysis and preparation of the manuscript.

Extent of contribution: 40%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Irene Lichtwark
Nature of contribution	Irene Lichtwark was involved in conducting cognitive tests and study visits, interpretation of the cognitive tests, data analysis and preparation of the manuscript.

Extent of contribution (%) for student co-authors only: 40%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature [redacted]

Date 16/12/[redacted]

Main Supervisor's Signature [redacted]

Date

Chapter 4: Cognitive impairment in coeliac disease improves on a gluten free diet and correlates with histological and serological indices of disease severity

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INTRODUCTION

Coeliac disease (CD) is an inflammatory autoimmune disorder that affects at least 1% of the adult population in many countries, and a strict, life-long gluten free diet (GFD) is the only recommended treatment [442,443]. While the disease is primarily an intestinal disorder that is histologically characterised by intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy, there is increasing support for a broader concept of a systemic inflammatory disease. Support for this view comes from clinical observations of extra-intestinal manifestations such as dermatologic, hepatic, osteologic, endocrine and neurological signs. Reported neurological manifestations include amnesia, ataxia, acalculia, epilepsy, chronic neuropathies, confusion and personality changes[219,220,222,444]. Some of these severe neurological symptoms can improve upon treatment with a GFD[185,219,445].

A milder form of cognitive impairment frequently reported by people with CD has been colloquially termed 'brain fog'. Brain fog can include difficulty concentrating, problems with attentiveness, lapses in memory, word-finding difficulties, temporary loss in mental acuity and creativity, and confusion or disorientation[446]. In our experience, patients often report that brain fog dissipates after treatment on a GFD or returns after inadvertent gluten exposure.

Few studies have investigated such subtle cognitive deficits. In a group of 8 'cognitively normal' participants with CD and idiopathic cerebellar ataxia significant impairments were found in immediate recall from episodic and semantic memory, and there was a trend towards deficits in phonemic verbal fluency and executive function[446]. Further, a study of 15 elders with cognitive decline that began within two years of the onset of CD symptoms suggested a link might exist between cognitive impairment and CD [219]. The possibility of such a link has been strengthened

by the findings of a retrospective study of elderly patients in which CD was diagnosed after the age of 60 years[447]. Of the 7 patients identified, two presented with cognitive decline that had been attributed to Alzheimer's disease but was ameliorated after the initiation of a GFD, while a third patient had peripheral neuropathy that completely resolved after the initiation of a GFD.

The present pilot study investigated relationships between levels of cognitive functioning and mucosal healing in people commencing on a GFD after being diagnosed with CD. It was hypothesised that the level of cognitive function would improve concomitantly with mucosal healing.

METHODOLODY

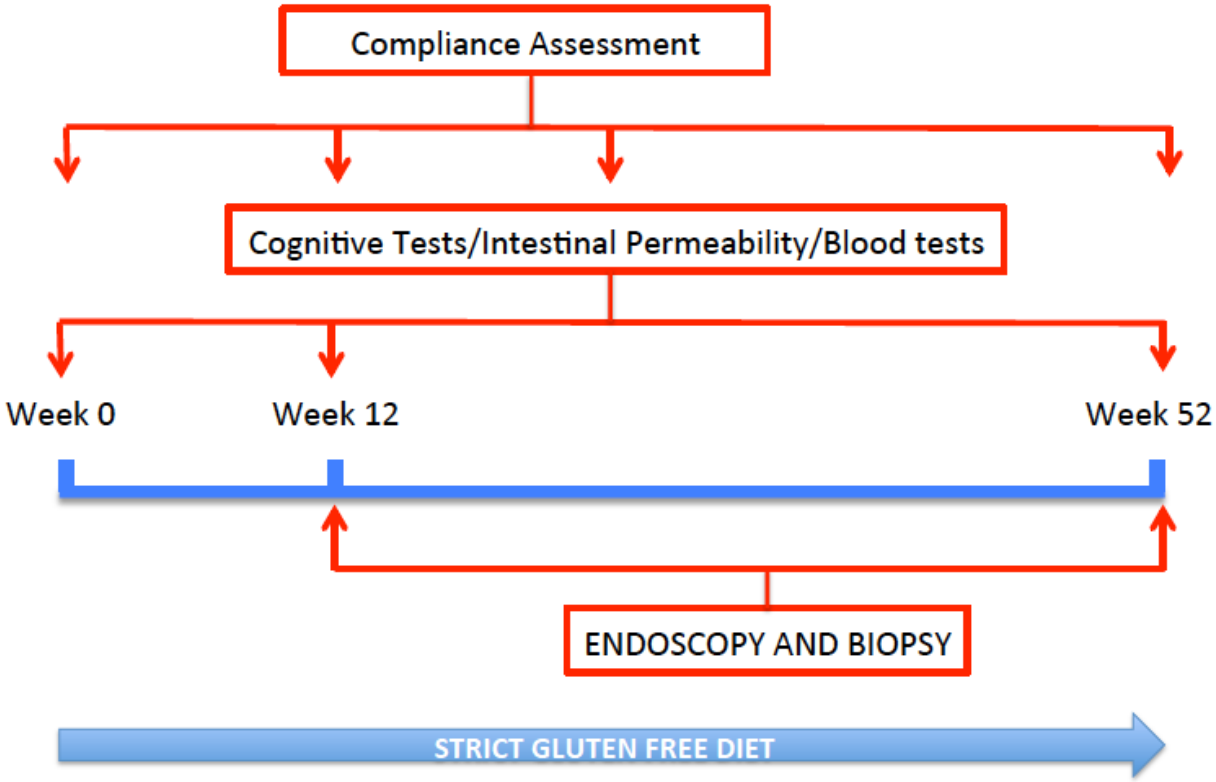
Participants

Participants were recruited via the Box Hill Hospital Coeliac Clinic (Melbourne, Australia), advertisements in local Coeliac Society publications, and from a local private dietetic practice. Patients were eligible for the study if they were aged between 18 and 40 years, had been diagnosed with CD and had not commenced a GFD more than 4 weeks prior to enrolment. The diagnosis of CD was made on the basis of duodenal biopsies showing villous atrophy (at least Marsh 3a) or Marsh 1 or 2 with positive CD-specific antibodies. All participants were required to express the HLA-DQ2 and/or DQ8 haplotype. Exclusion criteria comprised other significant gastrointestinal or inflammatory diseases, pregnancy, a history of neurological or psychiatric events or of brain injury resulting in unconsciousness for more than 30 minutes, existing neurodegenerative disease, and an inability to speak and write in English or to give written informed consent.

Protocol

The timeline for interventions in the study is summarised in Figure 4.1. During screening and at defined time points over the following 12 months, patients were assessed clinically by a gastroenterologist, had blood and intestinal permeability tests, underwent cognitive testing, and completed prospective 7-day food diaries to assess dietary compliance. Duodenal biopsies were performed at 12 and 52 weeks. The study protocol was approved by the Human Research and Ethics Committees at Monash University and Eastern Health in Melbourne, Australia. The longitudinal design alleviated issues associated with the small sample size. For instance, inter-participant variability was eliminated because the participants served as their own control through their baseline measures.

Figure 4.1 Timeline of the study protocol. Compliance of the 11 participants to the GFD was assessed via 7-day food diaries and interview by a dietitian. Intestinal permeability was assessed using a dual sugar methodology. Blood tests examined biochemical, nutritional and serological markers.



Compliance measures

At baseline screening and at weeks 12 and 52, participants completed a food diary for 7 consecutive days, recording all food and drink consumed as well as ingredients for home-prepared meals. The diaries were assessed by an experienced dietitian, with face-to-face consultations provided if concerns were identified by the participant, clinician or dietitian. Adherence was judged by an arbitrary scale comprising 'excellent' when no sources of gluten ingestion were identified, 'good' if minor gluten intake (estimated <0.3 g gluten per episode) occurred no more than six times in the 12 months, 'fair' if minor gluten intake (<0.3 g) occurred up to one episode per week, and 'poor' if gluten intake was more frequent than once a week.

Biological measures

Duodenal biopsies were obtained during routine upper gastrointestinal endoscopy at weeks 12 and 52 of the study. Two biopsies were taken from the first part of the duodenum and at least four from the second part. Histological assessment was performed by an experienced pathologist (PH) who was blinded to the baseline histology. Marsh scores were assigned using the most severe lesion found in the biopsies. The index biopsies were reviewed by the same (blinded) pathologist and scored retrospectively in a similar way.

Intestinal permeability (IP) was assessed by a dual-sugar permeability test. After an overnight fast, the bladder was emptied, and 1 g L-rhamnose and 5 g lactulose dissolved in 120 ml water was ingested. Urine was collected for 5 h after ingestion. Concentrations of both sugars were measured by high performance liquid chromatography and the lactulose:rhamnose (L:R) ratio was calculated.

Venous blood samples were collected at baseline (screening) and at weeks 12 and 52. Sera were assessed for tissue transglutaminase (tTG) IgA antibodies (Inova QUANTA Lite ELISA), haemoglobin, vitamins D and B_{12}, iron studies and liver function tests were performed as routine pathology tests. Thyroid function tests were assessed at baseline and weeks 26 and 52.

Cognitive measures

Cognitive tests are outlined in detail in Table 4.1. They were administered in a consistent order to keep the testing time to a minimum whilst ensuring that there was adherence to the temporal requirements of tests with multiple components (e.g., delayed memory components). The Subtle Cognitive Impairment Test (SCIT) was administered, followed by Trail Making Test A & B, Controlled Oral Word Association Task (COWAT), Rey-Osterrieth Complex Figure (ROCF) immediate recall, Rey Auditory Verbal Learning Task (RAVLT) (trials 1 to 5, foil trial and immediate recall trial), Anxiety and Depression subscales of the State-Trait Personality Inventory (STPI), Grooved Pegboard Task, RAVLT delayed recall trial, and finally the ROCF delayed recall trial. All cognitive testing was conducted during morning visits. Cognitive test performances were scored according to their respective published manuals.

Data analysis

Most datasets violated the assumption of normality (Shapiro-Wilkes analyses) and, since Marsh scores are an ordinal measure, the Friedman test, a nonparametric alternative to the repeated measures ANOVA, was used to investigate whether significant changes in any of the variables had occurred over the duration of the study. Wilcoxon signed rank tests were used as *post hoc* tests to determine when significant changes may have occurred. Given the exploratory nature of this study and to avoid rejecting potential true effects that could be followed up in a larger study,

$p<0.1$ was considered to represent statistical significance. To investigate whether significant changes had occurred for tTG, IP and serum ferritin over the duration of the study, a one-way repeated measures ANOVA was used. Correlations between measures across time points were calculated using Spearman's rho, and the False Discovery Rate (FDR) was used to adjust for type 1 errors[448,449]. The significance levels for the correlations were kept at $p<0.05$ to provide a manageable and meaningful number of correlations.

Table 4.1 Description of the psychological tests used in this study.

Test	Purpose	Procedure	Read-out
Subtle Cognitive Impairment Test (SCIT) (Yelland, 2004)[450]	Measures the speed and effectiveness (efficacy) of information processing from iconic memory to short term memory.	Participants respond to a simple asymmetrical stimulus appearing on the screen for varying duration from 16 to 133 ms by pressing the left or right mouse button corresponding to the shorter side of the stimulus. Each of the two versions of the stimulus is presented six times and response time and error rate averages are calculated over 96 trials. Data for the four shortest exposure durations (16 to 66 ms) are pooled to provide two scores from the head of their respective curves: response time (SCIT-RT_H) and error rate (SCIT-$E_{H)}$. Data from the four longer presentation durations (83 to 133ms) are pooled to provide two scores from the tail of the data curve for response time (SCIT-RT_T) and error rate (SCIT-E_T).	Response time (SCIT-RT_H and SCIT-RT_T) Error rate (SCIT-ER_H and SCIT-ER_T).
Trail Making Test A & B (Reitan, 1955)[451]	Measures speed of processing, attention, sequencing, flexibility, visual search and fine motoric function.	The participant connects letters and numbers in sequential order from letters and numbers randomly dispersed on a page.	The time needed to complete the task in seconds.

Test	Purpose	Procedure	Read-out
Rey-Osterrieth Complex Figure (ROCF) (Knight, 2003)[452]	Evaluates visuospatial ability and visuospatial memory.	A copy of a complex figure is drawn by the participant and is then repeated from memory 3 minutes and 30 minutes later. Maximum obtainable score is 36.	Presence and correct placement of the each element of the drawings.
Controlled Oral Word Association Task (COWAT) (Strauss, 1991)[453]	Measures word fluency as an indicator of frontal lobe functioning.	The participant is asked to produce as many words as possible in one minute, starting with a given letter. Each word produced is scored.	Sum of the number of words in three trials, each with a different letter
Rey Auditory Verbal Learning Task (RAVLT) (Rey, 1964)[454]	Evaluates verbal learning and memory.	The participant is asked to recall a 15-word list several times: first directly after it has been read out loud, then after a 3-minute and a 30-minute delay.	Number of correctly recalled words per trial.

Test	Purpose	Procedure	Read-out
Grooved Pegboard Task (Klove, 1949)[455]	Evaluates manual dexterity.	Participant inserts small pegs with little keys on one side into a pegboard where the holes have randomly placed slots in which to fit the keys. This is a timed activity and each hand is tested separately (dominant and non-dominant hands).	Sum of time to completion in seconds, pegs inserted, and number of dropped pegs.
Anxiety and Depression subscales of the State-Trait Personality Inventory (Spielberger, 1995)[456]	Evaluates state (at the moment) and trait (in general) anxiety and depression.	Self-report questionnaire in which participant is asked to rate forty statements on a four-point scale, ranging from 'not at all' to 'very much so'.	Four separate scores each for state and trait.
Wechsler's Test of Adult Reading (WTAR) (Wechsler, 2001)[457]	Estimates premorbid IQ.	Participant reads aloud 50 words that are irregular in their grapheme-to-phoneme translation.	Number of correctly-pronounced words.

RESULTS

Characterisation of the participants

Sixteen participants enrolled for the study. Five withdrew: three due to an inability to meet the time commitments, one due to pregnancy before the week 12 endoscopy and one due to a clinical diagnosis of depression at week 4 based on analysis of the HADS from the screening visit (symptoms that predated the diagnosis of CD). Thus, 11 participants with a mean age of 30 (range 22-39) years completed the 12-month study. Demographic, clinical, laboratory and histopathological characteristics of the patients at baseline are summarised in Table 2. Participants commenced a GFD 4-26 days (mean 14 days) before study enrolment. Based on dietary history and food diary analysis, adherence to the GFD was considered to be excellent in all participants across all time points in the study. One participant with Marsh 3c histology and DQ2 haplotype was antibody-negative. Both participants with Marsh 1 lesions had an elevated tTG and were HLA-DQ2 positive.

Changes in biological measures over time

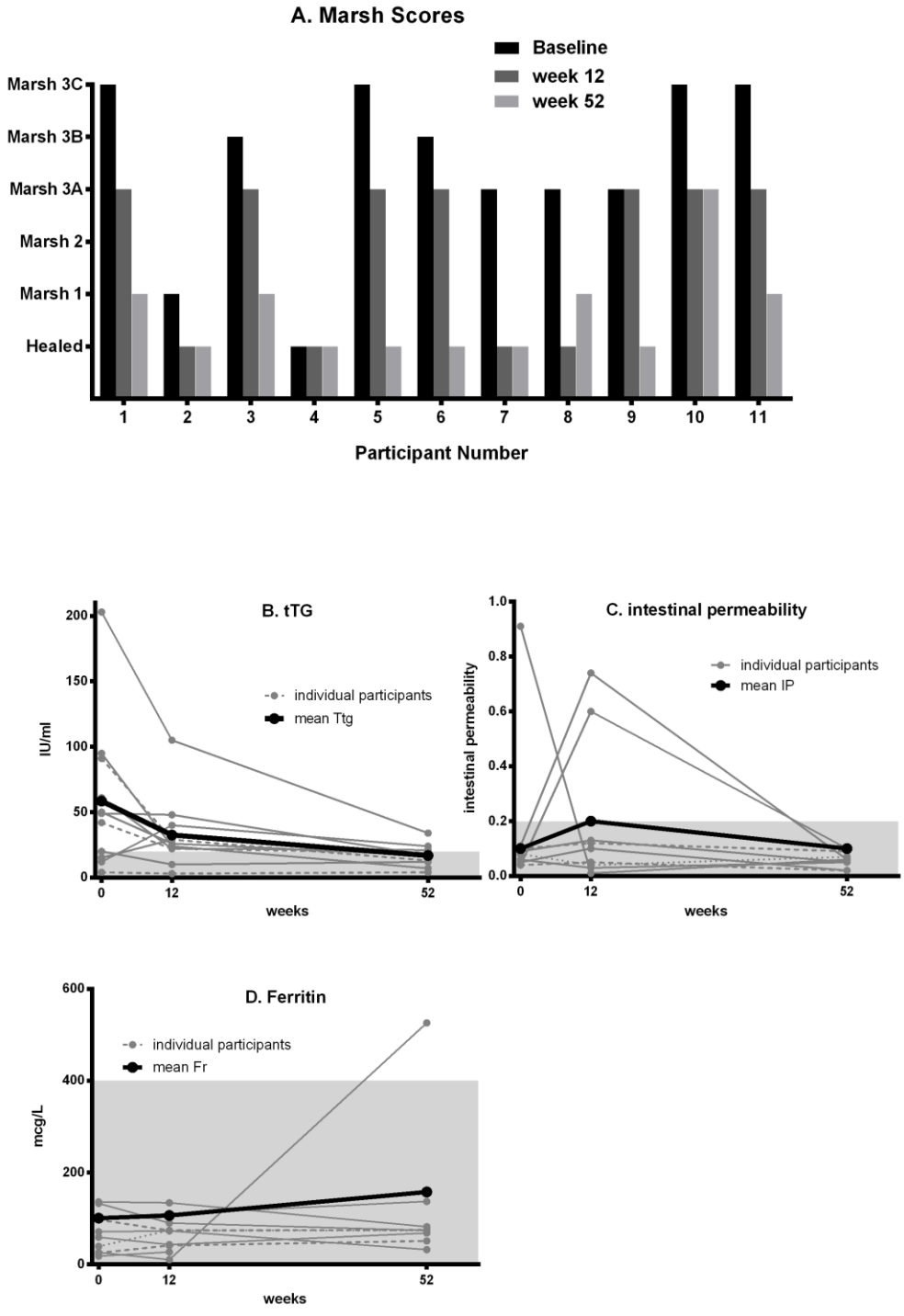
At baseline (week 0), the median Marsh score was 3b (range 1-3c), at 12 weeks it was 3a (0-3a) and by week 52 it had decreased to 1 (0-3a). Marsh scores improved across the three time points ($\chi^2_F(2)=15.70$, p=0.001; (Friedman's test), Figure 4.2). Histological improvement was marked in the first 12 weeks (T=1.15, p=0.01), but improvement between weeks 12 and 52 was not significant (p=0.371). At 52 weeks, mucosal remission was achieved in four and mucosal response (Marsh 1) in five, while one stayed at Marsh 3a and biopsy data was not obtained for the last participant.

Table 4.2 Characteristics at baseline of the 11 patients studied. Data are shown as (median) where applicable.

Age in years	22-39 (33)
Female gender	8 (73%)
Body Mass Index in kg/m^2	23.0-28.6 (25.3)
Marsh score of duodenal biopsy	
1	2
2	0
3a	3
3b	2
3c	4
Anti-tissue transglutaminase IgA (<20 U/ml)[a]	4-203 (49)
Haemoglobin (120-150 g/L) [a]	112-156 (142)
Ferritin (30-400 μg/L) [a]	18-399 (71)
B_{12} (156-698 pmol/L) [a]	215-708 (297)
Vitamin D (>75 nmol/L) [a]	35-165 (71)
Thyroid stimulating hormone (0.27-4.2 mlU/L) [a]	0.75-2.58 (1.33)
Intestinal permeability: lactulose:rhamnose ratio (<0.20) [a]	0.04-0.91 (0.06)
Intelligence Quotient (IQ)	90-112 (114)

a normal reference range in parenthesis

Figure 4.2 The effect of 12 months' gluten-free diet on selected physiological measures. A. Median Marsh scores for each participant (n= 11) improved significantly across the three time points (p=0.001; Friedman's test). B. Tissue transglutaminase antibodies concentrations (shown as mean±1 *SEM)* (n= 11) fell significantly across the three time-points (p=0.02). C and D: Intestinal permeability and serum ferritin are shown as mean±1 SEM (n= 11). Neither measure differed significantly between the time-points.



Serum tTG levels significantly decreased in all patients from a mean of 58.4 at baseline to 32.5 at 12 weeks and 16.8 U/ml at 52 weeks ($F(1.14, 10.26) = 6.8$, $p<0.023$, Figure 4.2). Further, differences between weeks 0 and 12, as well as between weeks 12 and 52, were also significant ($F(1, 9)=6.15$, $p=0.03$ and $F(1,9) = 5.42$, $p= 0.04$ respectively). tTG had normalised in two patients by 12 weeks and in seven patients at 52 weeks. Normalisation of serum tTG concentrations was associated with mucosal healing in the two patients with normalised tTG levels at 12 weeks, and for five patients at 52 weeks. For the remaining two patients in whom tTG concentrations fell over time, the duodenal mucosa either did not improve from the diagnostic biopsy at Marsh 3a or deteriorated from Marsh 0 to 1 by 52 weeks. Furthermore, Marsh scores improved in two of the four patients who did not achieve normal tTG levels.

One participant had abnormally high vitamin B_{12} levels as a result of supplementation. Mild iron deficiency anaemia (haemoglobin concentration 112 g/L) was present in 1 participant who remained mildly anaemic throughout the study. Two other participants were found to be iron deficient at baseline, but with normal haemoglobin concentration. Only one of the three iron-deficient patients was given iron repletion therapy via a total dose iron infusion between weeks 12 and 26. The serum ferritin levels over the 12 months are (Figure 2D) did not differ between the three time-points ($F(1.08, 8.67) = 0.64$, $p=0.46$). Vitamin D concentrations were low in six participants at baseline. These remained low in all but one participant despite oral vitamin D replacement therapy (administered to all six). In two further participants, the initially normal vitamin D levels fell below normal at 12 and 52 weeks. No patient had abnormal thyroid function and no changes were noted in thyroid function tests or liver function tests (data not shown). Intestinal permeability was elevated in three participants, one of these at baseline the other two at week 12. All IP levels were within the normal range at 52 weeks (Figure 2C).

Changes in IP across the three time-points were not statistically significant ($F(2,18) = 0.90$, $p= 0.42$).

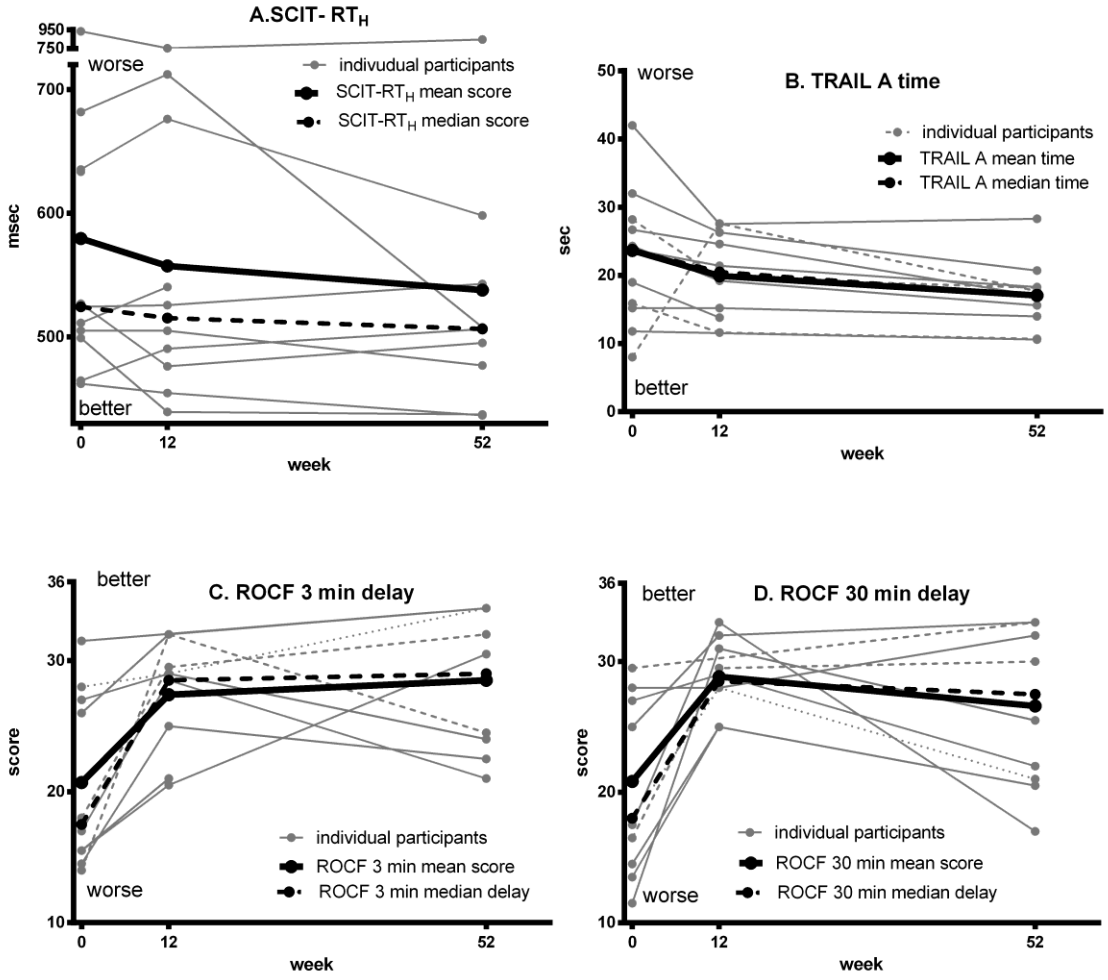
Cognitive changes over time

Four of the eight cognitive tests demonstrated a significant improvement in performance between time 0 and 52 weeks (Figure 4.3). The time taken to perform the TRAILS A part of the Trail Making Task improved from 23.7 s in week 0 to 17.0 s in week 52 ($T= 6.50$; $p= 0.032$). Mean performance on SCIT-RT_H improved from 580 ms in week 0 to 538 ms in week 52 ($T= 7.00$; $p= 0.066$). Similarly, the scores for accurately recalling the complex figure of the ROCF after 3 min improved from 21.05 to 27.65 ($T= 52.00$; $p= 0.012$), and the scores for accurately recalling of the ROCF after 30 min improved from 20.50 to 26.45 ($T= 49.0$; $p= 0.028$). The changes in performance between weeks 0 and 52 on all other cognitive tests were not significant at $p<0.1$ and are not included in Figure 4.3.

Correlation between cognitive outcomes, other indices and Marsh scores

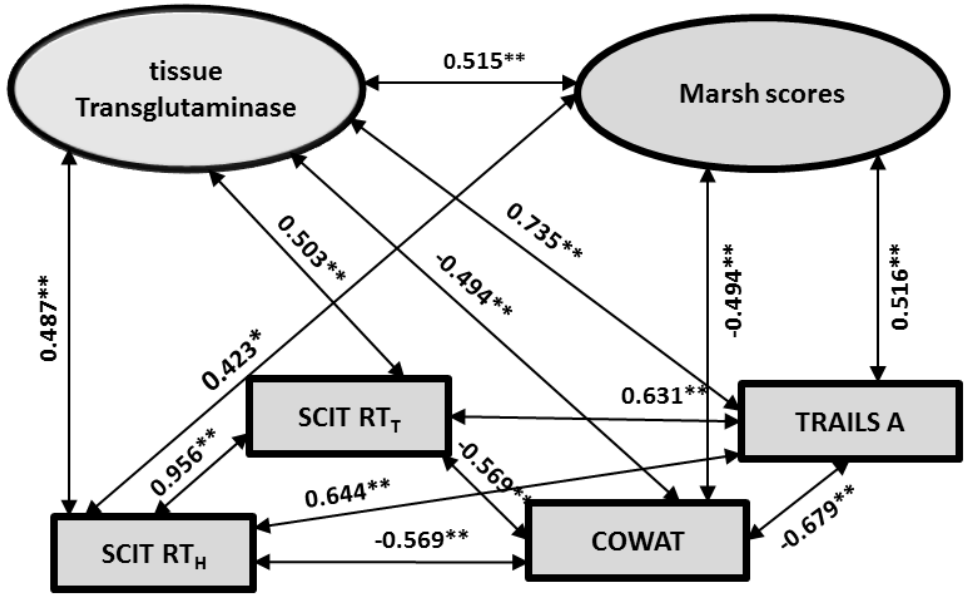
Performance on the following cognitive tests and physiological measures did not correlate significantly with Marsh scores across the three time points: SCIT-E_H and -E_T, Trail making task parts B and B minus A, ROCF, RAVLT immediate recall, and recall at 30 minutes, STPI, haemoglobin, IP, and serum levels of ferritin and vitamins B_{12} and D. Performance on the following tests correlated significantly with Marsh scores and tTG levels: TRAILS A and COWAT at $p< 0.01$, and SCIT-RT_H, SCIT-RT_T, RAVLT 3 min at $p< 0.05$. As shown in Figure 4.4, the correlations (Spearman's r) were large and ranged from 0.377 to 0.735.

Figure 4.3 Changes in selected cognitive measures during 12 month on a GFD. Results are shown as mean ±1 *SEM, (n= 11).* Data for SCIT-RT, TRAILS A and tTG are plotted with improvement indicated by a downward trend. The difference in performance on SCIT-RT_H between weeks 0 and 52 was significant at p=0.06, for TRAILS A at p= 0.03, ROCF 3 min p = 0.01 and ROCF 30 min p= 0.028. COWAT differences were not significant at p=0.184.



Performance on the following cognitive tests and physiological measures did not correlate significantly with tTG serum titre across the three time points: SCIT-E_H and -E_T, all of the ROCF measures, RAVLT immediate recall and recall at 30 minutes, STPI, haemoglobin and serum levels of ferritin and vitamins B_{12} and D. Performance on the following tests correlated significantly with tTG levels: SCIT-RT_H, SCIT-RT_T, TRAILS A, B and COWAT (all at $p<0.01$) as well as TRAILS B minus A, RAVLT1-5, grooved pegboard (dominant and non-dominant hands) and IP (at $p<0.05$). No meaningful patterns could be found for the relationships between the cognitive test results and the serum concentrations of ferritin, haemoglobin, vitamin B_{12} and vitamin D, or between cognitive test outcomes and IP for the participants with elevated IP.

Figure 4.4 Participants' (n= 11) performance on four cognitive tests showed significant correlations with each other and with histological scores and tTG levels (**p<0.001, *p<0.05; Spearman's Rho).



DISCUSSION

This pilot study examined the relationships between cognitive function and mucosal healing in patients who had been recently diagnosed with CD, and who adhered to a GFD over the first year of treatment. The study demonstrated the presence of cognitive impairment that improved with therapy and was correlated with histological evidence of mucosal recovery and/or healing.

The present study provides the beginnings of an evidence-base for the ill-defined, yet frequently reported symptoms of brain fog in CD. While participants improved on all nine cognitive tests, only TRAILS A, ROCF and SCIT-RT_H demonstrated a significant improvement over the course of the study. Two of these tests are strongly dependent on processing speed: and include speed of response in the SCIT-RT_H, and visuomotor speed in the TRAILS A test. ROCF is mainly a test for visuospatial short-term memory. Although improved performance on the TRAILS A test might be due to practice effects, the published test-retest reliability over 3-12 months is high, suggesting that any practice effect would be minor. Alternative versions were used for the ROCF and COWAT, and the SCIT does not have learning or practice effects [450,458].

It is notable that the level of impairment on the SCIT-RT_H at pre-GFD baseline was similar to that in people with a blood alcohol level of 0.05g/100 ml[450], which is the upper legal limit for driving in Australia. It is also equivalent to level of impairment in SCIT-RT_H observed in participants with severe jetlag (Yelland & Robison, unpublished data). Further, the 8% relative change in SCIT performance in people with untreated CD to their paired test after being adherent to the GFD, was similar to that demonstrated between 0.05 and 0.02 blood alcohol levels and to the recovery from jetlag over a 24-hour period. In people with undiagnosed CD, such cognitive deficits might result in impaired performance in driving and at work[459]. If these findings are

confirmed in a larger study, they may have important health and safety implications. When viewed together, the present results indicate that short-term memory, movement and processing speed are impaired in untreated CD and that they improve during adherence to a GFD. These impairments, although subclinical, may contribute to the sensation of brain fog that is commonly reported by people with CD.

There are three reasons why cognition might be impaired in patients with untreated CD. First, nutrient deficiencies involving, for example, iron, vitamin D and folate have been associated with cognitive impairment[460-462]. As part of the malabsorption that might occur in patients with CD, such micronutrient deficiencies do occur. In this study however, changes in iron levels were not associated with cognitive performance, and the persisting low levels of vitamin D in six participants argues against a link to the cognitive improvement found over the duration of the study.

Second, cognitive impairment in patients with untreated CD may be due to the high levels of circulating cytokines associated with systemic inflammation[460-462]. Elevated concentrations of circulating cytokines have been associated with changes in behaviour, mood and cognition[463-469]. The brain possesses receptors for circulating cytokines, whilst cytokine activation of neurons and subsequent central signalling may also be important[469].

Third, the cognitive improvement may be related to reduced exposure to gluten *per se*. Animal studies have shown dietary gluten may reduce brain tryptophan concentrations. Since tryptophan is the precursor of the neurotransmitter, serotonin, it has been speculated that gluten may impair cognitive function by lowering brain serotonin levels[470]. Alternatively, opioid peptides derived

from partially digested gluten, so-called "exorphins", can have several effects on higher brain function in rodents[471], although no studies have been reported in humans. Furthermore, changes to the diet can alter the gut microbiota, and this has been found to strongly influence behaviour in rats [472-475]. Humans might be similarly affected, since a recent brain imaging study reported that a four-week intake of probiotic fermented milk by healthy women affected the activity of brain regions that control central processing of emotion and sensation[476].

The present study demonstrated that improvements in cognition in CD are significantly correlated with the extent of intestinal healing, as indicated by Marsh scores. Indeed, the strength of these correlations was as good as those obtained for serum tTG. The fact that significant correlations between disease activity measures and cognitive function were obtained with a sample of just 11 participants, underscores the magnitude of the effect sizes. The strength of these correlations raises the interesting possibility that a small battery of cognitive tests might offer a non-invasive means to regularly screen CD patients for intestinal healing. Currently there is need for an accurate biomarker of healing that can lessen the requirement for repeated intestinal biopsies. A larger prospective study will be needed to determine whether cognitive tests have sufficient reliability to serve as a proxy of intestinal healing.

In conclusion, this pilot study indicates that cognition is impaired in people with untreated CD. Cognitive function improves after commencement of a strict GFD, and this improvement is correlated with a normalisation of histopathological markers of disease severity. These results support patient reports of brain fog in untreated CD, and demonstrate that impairments in cognitive performance are an additional extra-intestinal or systematic manifestation of CD. Our

findings introduce the possibility that cognitive tests have the potential to provide a non-invasive, cost-efficient marker of intestinal healing.

Monash University

Declaration for Thesis Chapter 5.1

Declaration by candidate

In the case of Chapter 5.1, the nature and extent of my contribution to the work was the following:

Nature of contribution
Dr Evan Newnham was involved in patient recruitment, study visits, collection and analysis of samples, data analysis and preparation of the manuscript.

Extent of contribution: 40%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Dr Jessica Biesiekierski
Nature of contribution	Dr Biesiekierski was involved in study design, patient recruitment, study visits, data analysis and preparation of the manuscript.

Extent of contribution (%) for student co-authors only: 40%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature

Date 16/12/14

Main Supervisor's Signature

Date 16.12.14

Chapter 5: Non-coeliac gluten sensitivity

5.1 Gluten causes gastrointestinal symptoms in subjects without coeliac disease: a double blind randomised placebo controlled trial

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INTRODUCTION

In clinical practice, some patients have symptoms of irritable bowel syndrome (IBS) that respond well to a gluten-free diet but they have no markers of coeliac disease. The published scientific literature is largely devoid of the so-called "non-coeliac gluten intolerance" and "wheat intolerance", yet they are widely believed to be very common[381-383]. In the evaluation of exclusion diets, wheat has been found to be one of the most common factors inducing gastrointestinal symptoms [384], but it is not known whether gluten is the responsible agent, since wheat, the major cereal removed from the gluten-free diet, contains other components that include other proteins, lipids and carbohydrates. Of particular importance are fructans which are poorly absorbed carbohydrates and can induce symptoms themselves[385,386].

The role of gluten in coeliac disease is clear. The toxic peptide sequences have been defined [25,477], the genetic susceptibility loci identified and the pathological processes comparatively well known. Deamidation of these gliadin epitopes by tissue transglutaminase (tTG) enables them to be presented with high affinity to MHC Class II T-cells in genetically susceptible individuals (HLA-DQ2 or -DQ8 being expressed in 99.4% of patients with coeliac disease) [478]. This process initiates a cascade of events resulting in mucosal inflammation, small intestinal villous atrophy[88], increased intestinal permeability[310], malabsorption of macro and micronutrients[479] and resultant complications of coeliac disease. To date, the literature regarding the effect of gluten outside of coeliac disease has been limited to experiments in cancer cell lines and to uncontrolled clinical studies[302,381,383,392-395]. Whether gluten itself can contribute to gastrointestinal symptoms and/or induce injury to the proximal small intestine in non-coeliac patients has never been directly assessed.

The aims of the current study were to examine the hypotheses that gluten can cause gastrointestinal symptoms in patients without coeliac disease and to preliminary screen for potential mechanisms of whether gluten does so by causing intestinal injury and/or inflammation in such subjects. To do this, a randomised, double-blinded, placebo-controlled, dietary rechallenge trial was conducted in subjects with IBS who had coeliac disease excluded by best practice methods and who reported a symptom response to a gluten-free diet.

METHODS

Patients

Patients were recruited between July 2007 and December 2008 via advertisements in e-newsletters and community/state newspapers in metropolitan Melbourne, and by referral in private dietetic

practice. The inclusion criteria were age greater than 16 years, symptoms of IBS fulfilling Rome III criteria that have improved on a gluten-free diet, and adherence to the diet for at least six weeks immediately prior to screening. Coeliac disease was excluded by either (a) absence of the HLA-DQ2 and DQ8 haplotype or (b) a normal duodenal biopsy (Marsh 0) performed at endoscopy whilst on a gluten containing diet in those expressing the HLA-DQ2 or DQ8 haplotype. Patients with significant gastrointestinal disease (such as cirrhosis or inflammatory bowel disease), excessive alcohol intake, intake of non-steroidal anti-inflammatory agents, and unable to give written informed consent were excluded.

Study protocol

Patients were randomised according to a computer-generated list of random numbers held by an independent observer to either gluten or placebo treatment group. Baseline symptom data and a seven-day food diary were collected during a two-week run-in period. Participants continued on a gluten-free diet throughout the study, but were asked to consume one study muffin and two study slices of bread containing gluten (total intake of 16 g/day) or not (see below) every day for six weeks. Both the patient and the investigators evaluating the patient were blinded to the study treatment. At the end of each study week and for three weeks after completion of the study intervention, symptoms were evaluated, as previously applied[386]. Patients were asked to complete a symptom questionnaire containing the question for the primary outcome detailed below, and a 100 mm visual analogue scale (VAS), with zero representing no symptoms, assessing overall symptoms, bloating, abdominal pain, satisfaction with stool consistency, nausea, and tiredness. At weeks zero and six, serum, urine and stool samples were collected for analysis, and intestinal permeability was measured. Food diaries were maintained by the patients during the third and sixth study weeks, and unused muffins and bread were returned at the end of weeks three and six of the treatment period for counting. Patients unable to continue the study due to intolerable

symptoms were permitted to cease the study food after data were collected as per week six (symptom assessment, blood, urine and stool samples collected). The protocol was approved by Eastern Health Research and Ethics Committee.

End-points

The primary outcome was the proportion of patients answering "no" on more than half of the symptom assessments to the question "Over the last week were your symptoms adequately controlled?". This question was asked at the end of each study week or at withdrawal if premature. Secondary outcomes were the change in overall and individual gastrointestinal symptoms as assessed by the VAS, and changes in biomarkers (see below). Compliance with the study treatment was assessed by an unused food count at weeks three and six. Compliance with the gluten-free diet was judged on food diary entries and on specific questioning at review.

Study food preparation

The muffins and bread were prepared and baked commercially in gluten-free ovens and conditions. The base mixes were gluten-free. For the gluten group, commercially available, carbohydrate-deplete wheat-gluten (Gemtec 1160, Manildra Group) was added prior to baking at the amount of 8 g per muffin and 4 g per slice of bread. Analysis of the baked products using a commercially available assay (Biokits Gluten Assay Kit; Tepnel Biosystems, Flintshire, UK; AOAC 991·19 Method) confirmed preservation of intact gluten and in the amount expected. The gluten used contained 91.7% protein, 1.1% crude fibre, 1.9% lipid, 1.8% starch, and 3.5% ash shown on reversed-phase high-pressure liquid chromatography (HPLC). To assess FODMAP content, the gluten was analysed as previously described[480] and was shown to be free of the short-chain carbohydrates, fructans, fructose, glucose, lactose, sorbitol, mannitol, raffinose, stachyose,

nystose, and kestose. Based on size-exclusion HPLC, the protein content had the distribution of 2.3% non-gluten protein (albumin/globulin), 45.7% glutenin, and 52.0% gliadin. Preliminary testing in ten healthy people showed that the muffins and bread containing gluten could not be differentiated from those that did not on the basis of taste or texture.

Measurement of biomarkers

All markers were measured after randomisation. Serum was analysed for antibodies to tissue transglutaminase (tTG IgA) and whole gliadin (IgA and IgG) by ELISA using commercially available assays (INOVA Diagnostics Inc., San Diego, USA). The manufacturer's reference ranges were used to determine the classification of the serological result. Endomysial antibodies were examined by immunofluorescent staining of distal monkey oesophagus (Chemicon Australia, Boronia, Victoria, Australia).

Highly-sensitive C-reactive protein (hsCRP) was measured using an immunoturbidimetric assay (Tina-Quant CRP Roche Diagnostics, Basel, Switzerland). Intestinal permeability was measured using a dual sugar test. After an overnight fast, patients emptied their bladder and consumed a solution of lactulose (5 g) and rhamnose (1 g) dissolved in 120 mL of water. All urine over the next five hours was collected in containers containing boric acid as a preservative and samples stored at -80 ^{o}C until assayed by HPLC as previously described[481]. The urinary lactulose-to-rhamnose ratio was calculated. Faecal lactoferrin was measured by ELISA using a commercially available kit (*IBD Scan*, Techlab®, Virginia). Two dilutions of each sample were assayed and the results expressed in units of mg/ml faeces.

Statistical analyses

Power calculations were based upon a placebo effect using a similar end-point and re-challenge methodology of about 20% [386] and an estimate of 60% response to gluten since there were no previous data upon which this could be judged. This indicated 30 patients were needed in each group to achieve a power of 80% and *P*-value 0.05. The study was terminated early due to difficulty with recruitment of patients in whom coeliac disease had been definitely excluded (see above).

To determine the relationship between tolerable symptoms ("yes" / "no") over the six weeks, a generalized estimating equation (GEE), was utilised (primary outcome). Change in symptom severity was calculated as the scored difference between commencement and one week and was tested via the independent samples t test for within group comparisons and ANCOVA between groups. A linear mixed effects model assessed symptom severity scores across the treatment period (longitudinal data). Correlation between measured symptoms and their model residuals was assessed using the Pearson's correlation coefficient. Changes in biomarker levels after therapy within each dietary group were assessed by paired t test using log-transformed data. Comparison of change in biomarker levels between each group were assessed using ANCOVA and by change in the indices using an independent samples t test. Blinding was assessed by using the Kappa agreement statistic, where a value of 1 indicated complete agreement, and 0 indicated no agreement. All statistical analyses were conducted using the R Statistical Software Package (R Development Core Team, R: A Language and Environment for Statistical Computing, Vienna, Austria). Two-tailed *P*-values at or below 0.05 were considered statistically significant.

RESULTS

Less than one-third of respondents to the advertisements were deemed suitable for screening. Of those 103 subjects, only 39 met inclusion criteria and were enrolled. Subject flow is shown in Figure 5.1. Following randomisation, five patients had to be withdrawn. Thus, 34 patients completed the study as per protocol; 19 received gluten and 15 received placebo. The details of those patients are shown in Table 5.1. All patients were negative for tTG and endomysial antibodies and there were no differences for whole gliadin antibodies between gluten or placebo groups including those within the DQ2/8 positive group.

Figure 5.1 Recruitment pathway and reasons for screen failure and withdrawals.

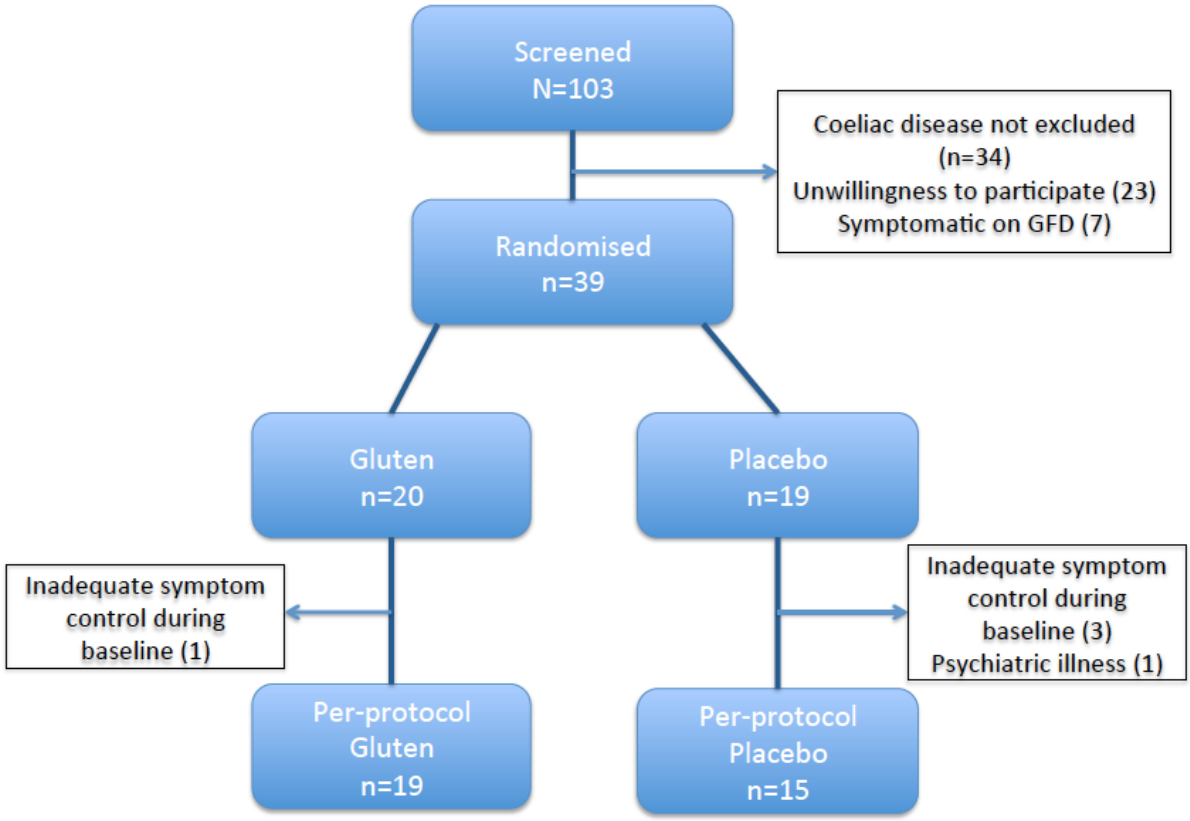

Table 5.1 Patient characteristics according to the dietary treatment group. There were no significant differences between dietary groups for any index (independent samples t-test, Chi-square test)

Patient characteristic		Gluten	Placebo
Number of patients		19	15
Median age (range) in years		40 (29–55)	49 (33–51)
Men		16%	7%
Median body mass index range (range)		23 (18–41)	22 (18–33)
Number with predominant bowel habit:	Constipation	16%	20%
	Diarrhoea	58%	33%
	Alternating	26%	47%
HLA type:	DQ2 or DQ8 positive	53%	60%
	DQ negative	47%	40%
Elevated serum coeliac antibodies (percentage of patients (mean (SEM) U/mL)):	Tissue transglutaminase (IgA)	0	0
	Tissue transglutaminase (IgG)	0	0
	Endomysium (IgA)	0	0
	Whole-gliadin (IgA)	27% (39 (9))	30% (33 (9))
	• DQ2/8 positive	5% (33 (0))	7% (24 (0))
	Whole gliadin (IgG)	25% (25 (1))	0
	• DQ2/8 positive	5% (23 (0))	0

All patients adhered to the gluten-free diet during the study. Alcohol intake did not differ during the treatment period, and did not differ between groups. Nearly all food supplements (95% and 96%) were consumed in the placebo and gluten groups, respectively. The blinding technique was successful, supported by a kappa score of 0.24 (low agreement between actual treatment and participant guessing).

Nine patients ceased the study diet prematurely due to intolerable symptoms. Six were in the gluten arm and they withdrew after a median of 7 (range 2–18) days, while three in the placebo arm withdrew after 16 (11–21) days. There were no statistical differences between the groups in frequency and timing of withdrawal. Serum, urine, and stool samples were collected from all of these patients upon cessation of the diet as per week six.

Significantly more patients in the gluten group (68%; n=13/19) reported the answer, 'no' (the primary outcome question) compared to those on placebo (40%; n=6/15) for more than half of the study therapy duration (P=0.001; GEE). As shown in Figure 2, the changes in symptoms from baseline to end of week one as scored on the VAS after one week's therapy were significantly greater in those patients who consumed the gluten diet for overall symptoms, pain, bloating, satisfaction with stool consistency, and tiredness, but not for wind or nausea. Over the entire study period, the severity scores of pain, satisfaction with stool consistency, and tiredness were significantly higher for those consuming the gluten (Figure 5.2). Correlation between model residuals to estimate symptom score redundancy was assessed. Correlation coefficients ranged between 0.3 and 0.9, with the highest correlations between overall score and pain.

As shown in Table 2, neither treatment group had significant changes from baseline for any of the biomarkers measured. Similarly, no significant differences were observed in the magnitude of changes between the groups, whether assessed using raw data (ANCOVA) or by comparing changes in the indices (t test). Faecal lactoferrin was below the detectable level prior to and following treatment in all but one patient in the placebo arm whose level was 36 mg/mL at both weeks zero and six. Removal of this patient's data from the analysis had no effect on the results (data not shown).

Symptomatic responses to gluten did not significantly differ in those expressing HLA-DQ2 and/or DQ8 (n=10) with those who did not (n=9; data not shown). Likewise, no differences in the response of biomarkers to gluten exposure were noted according to HLA-D status (data not shown).

Figure 5.2 (Overleaf) Change in symptom severity from baseline in the gluten and placebo-treated groups over the 6-weeks of the study. Data shown represent the mean change for the subjects remaining on study therapy at each time point. The differences were compared at week one by an independent samples t-test, in which overall symptoms (*P*=0.047), abdominal pain (0.016), bloating (0.031), satisfaction with stool consistency (0.024) and tiredness (0.001) were statistically significant but wind (0.053) and nausea (0.120) were not. The differences were also compared over the entire study period using a linear mixed effects model, in which abdominal pain (0.02), satisfaction with stool consistency (0.03), and tiredness (0.001) were statistically significant but overall symptoms (0.15), wind (0.08) and nausea (0.69) were not.

Figure 2

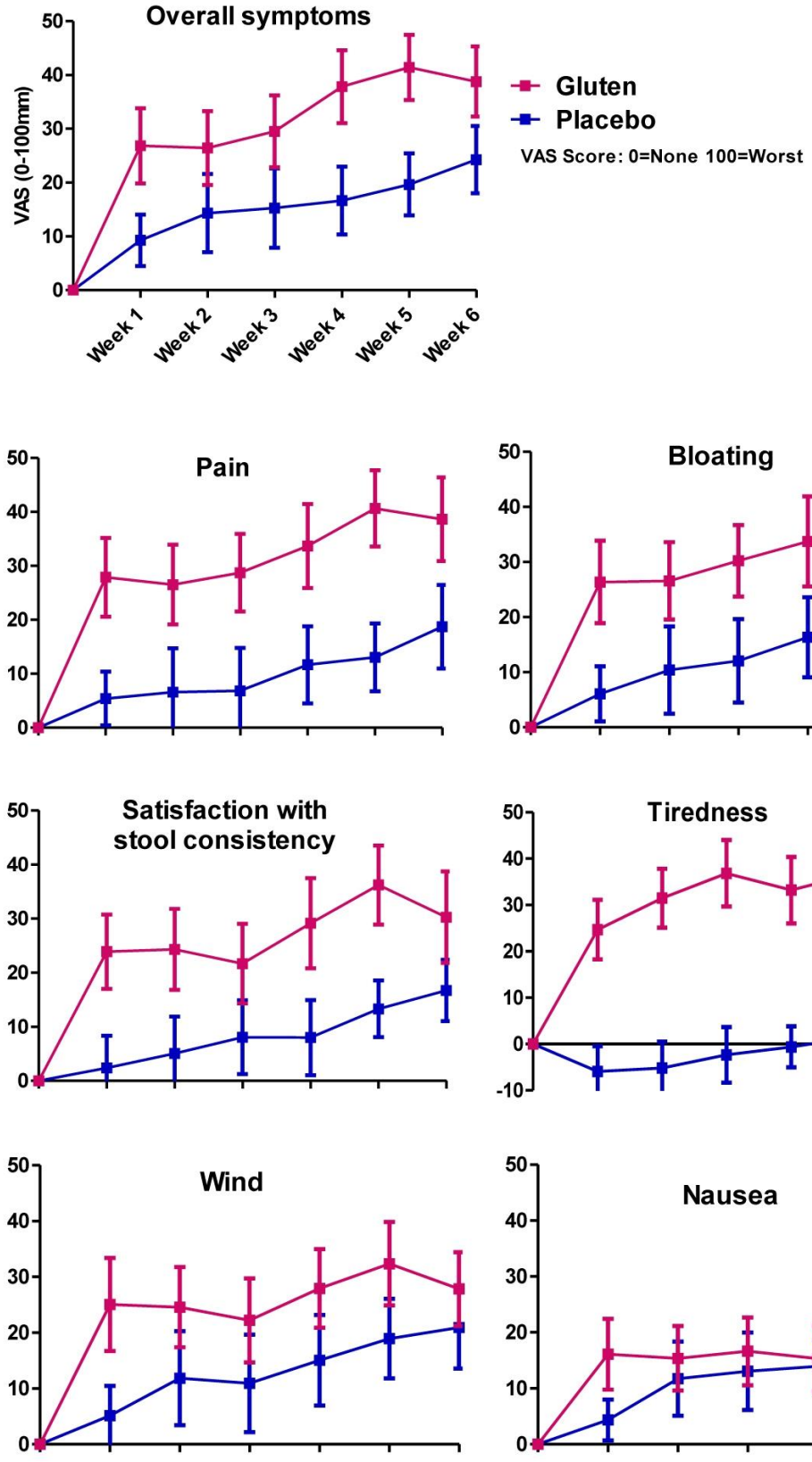
Overall symptoms
VAS (0-100mm)
50
40
30
20
10
0
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Gluten
Placebo
VAS Score: 0=None 100=Worst
Pain
Bloating
Satisfaction with stool consistency
Tiredness
-10
Wind
Nausea

Table 5.2 Coeliac serology, intestinal permeability and C-reactive protein results before and during therapy with gluten or placebo, shown as median (range), and changes in those indices, shown as mean (SEM). There were no statistically significant differences within each dietary group (paired t-test on log-transformed data) or between treatment groups whether evaluated using baseline and treatment data (ANCOVA) or the changes in indices (independent samples t-test; all $P \geq 0.1$).

	Gluten (n=19)			**Placebo** (n=15)		
Biomarker	**Baseline**	**With therapy**	***Change***	**Baseline**	**With therapy**	***Change***
Coeliac serology (U/mL)						
• Tissue transglutaminase (IgA)	3.0 (2.0-7.0)	4.5 (2.0-7.0)	*0.6 (0.3)*	3.0 (2.0-10.0)	3.5 (2.0-10.0)	*0.4 (0.5)*
• Whole gliadin (IgA)	10.8 (3.5-241.5)	4.6 (0.1-51.3)	*-29.7 (19.2)*	6.6 (0.1-36.6)	5.9 (0.1-36.6)	*-4.3 (2.5)*
• Whole gliadin (IgG)	14.6 (12.1-31.5)	15.5 (11.4-50.6)	*2.5 (2.0)*	11.9 (10.9-14.6)	11.9 (10.6-15.7)	*0.2 (0.3)*
Intestinal permeability (L:R ratio)	0.02 (0.01-0.6)	0.01 (0.01-2.4)	*0.09 (0.1)*	0.04 (0.01-0.15)	0.02 (0.01-0.18)	*-0.01 (0.02)*
Highly sensitive C-reactive protein (mg/L)	1.4 (0.3-5.3)	0.3 (0.4-19.8)	*2.1 (1.4)*	1.1 (0.2-8.2)	1.2 (0.3-13.1)	*0.5 (0.9)*

DISCUSSION

Gluten intolerance in people without coeliac disease is a controversial issue and has recently been described as the "no man's land of gluten sensitivity" [482]. The evidence-base for such claims is unfortunately very thin with no randomised controlled trials demonstrating that the entity does actually exist. Most published descriptions involve patients with positive serology associated with coeliac disease or with intraepithelial lymphocytosis in the duodenum. In other words, evidence of immunological responses seen in coeliac disease has been present[381] and this may just represent coeliac disease not fulfilling ESPGHAN criteria for diagnosis. The current double-blind, randomised, placebo-controlled rechallenge trial in patients who claim considerable improvement of gut symptoms with the institution of a gluten-free diet does indeed support the existence of non-coeliac gluten sensitivity. Gluten specifically induced symptoms including bloating, dissatisfaction with stool consistency, abdominal pain and tiredness.

Recruitment of patients for this study was not easy due mainly to the failure of most to have coeliac disease effectively ruled out. Although the final number (n=34) of participants recruited was less than the priori power calculations suggested and relatively small, our interim power analyses confirmed that the reduced number was adequate to infer a statistically robust result, with unequivocal significant separation of the two groups. All patients developed exacerbation of symptoms in response to gluten and did so within the first week of rechallenge in contrast to the placebo group where symptom induction occurred more slowly and the level of symptoms reached was less severe. This occurred across the relevant abdominal symptoms of bloating, pain and satisfaction with stool form whereas no differences between the treatment groups were shown for the less relevant symptom of nausea. Interestingly, the symptom that quantitatively differentiated the treatment groups to the greatest extent was tiredness, mainly due to placebo having no

apparent effect on this end-point. Tiredness is a common symptom of IBS[483] and its induction by gluten may provide insights into a mechanism of action.

A key question is by what mechanism symptoms were induced by the ingestion of the gluten. It might be anticipated that some patients reporting symptomatic improvement from the GFD have undiagnosed coeliac disease. Coeliac disease can be patchy[80] and, although unlikely, it is therefore possible some patients with undiagnosed coeliac disease were included. However, there were no significant changes in coeliac antibodies seen in either group. About one half did not carry HLA-D genes believed to be essential for the development of coeliac disease. While the study was not powered to determine differences in responses between genotypes, no clear differences were noted.

Simple non-invasive studies were performed to look for a signal that inflammation and/or intestinal damage were being induced. This was particularly suspected since evidence of an immune basis for at least a proportion of patients with functional gastrointestinal disorders has already been shown[329,484], and gluten had a prominent effect on tiredness in this population, suggesting a more systemic process. Change in hsCRP is considered a marker for systemic circulation of cytokines from a localised site, but no effect on this was observed. Faecal lactoferrin levels rise in the presence of intestinal inflammation due to transepithelial migration of neutrophils to the lumen and the inability of gut bacteria to degrade lactoferrin[485]. However, levels were not increased by the interventions. Finally, intestinal permeability, as examined using a dual sugar absorption test, is believed to be a sensitive marker of intestinal injury, but this also did not change overall, and there were no differences between the gluten and placebo groups. Clearly these

markers may not have the required sensitivity to detect subtle inflammation and/or intestinal damage. Examination at the tissue level is warranted to better address this issue.

Other potential mechanisms by which a dietary product can induce functional gut symptoms include induction of intestinal distension via the fermentation of poorly absorbed gluten peptides. However, passage of excessive flatus was not a prominent feature (as it is for carbohydrate sources [486] and malodorous flatus might be anticipated due to sulphide production, but was not reported by the patients in the study. Indeed, if hydrogen sulphide production was increased this might potentially alter visceral sensitivity[487]. Alternatively, gluten may mediate cholinergic activation as has been shown in murine models of gluten sensitivity[398]. This may lead to increased smooth muscle contractility and indirectly have effects on luminal water content. Other functional gut symptoms might also be induced by stimulation of the enteric nervous system either directly by the supply of neuroactive molecules or by indirect release of neurotransmitters from, for example, mast cell activation. Neurally active peptides from gluten digestion might potentially gain access to enteric nerve endings, but these are not known to occur and their absorption might seem less likely given normal intestinal permeability. Newer techniques such as examining basophil activation in response to the gluten used might be instructive in this way[488].

The other key issue is whether symptoms are being induced by peptide(s) derived from gliadin proteins or non-gliadin parts of gluten, or by a contaminant of the gluten. There is ample evidence *in vitro* that gluten can induce injury and changes in epithelial cells by non-DQ2-restricted mechanisms. For example, gliadin is able to increase epithelial permeability and alter protein expression of components of the tight junction[302], induce apoptosis[394,489], and increase oxidative stress[395] in Caco-2 (human colon adenocarcinoma) monolayers, a surrogate model for the human gut epithelium. In addition, gliadin may inhibit RNA and DNA synthesis[393]. Of non-gliadin

components, carbohydrates would be considered a likely candidate, especially as fructans are present in wheat, are poorly absorbed in the small intestine and do induce functional gut symptoms[386]. However, the gluten used was devoid of FODMAPs. Wheat proteins are commonly implicated in food hypersensitivity and it must be considered that the induction of symptoms by the gluten in the present study might be a wheat-specific phenomenon, and not gluten-specific. Such a finding would have implications for the dietary restriction that would be necessary in such patients to attain good symptomatic control.

The prevalence of non-coeliac gluten intolerance amongst patients with functional gut disorders is unknown. Patients in the present study were highly selected due to the frequent failure of investigative work-up by health professionals for coeliac disease or from self-administered therapy without any investigations at all. Methods to identify these patients are needed. Currently, they are restricted to ruling out coeliac disease, followed by trial of a gluten-free diet, followed by rechallenge. Better diagnostics will only derive from understanding the mechanism of action and what part of the gluten soup is actually inducing the symptoms.

In conclusion, this double-blind, randomised, placebo-controlled rechallenge study of patients with IBS without coeliac disease who have reached satisfactory levels of symptom control with a gluten-free diet shows that gluten is indeed a trigger of gut symptoms and of tiredness. No evidence for intestinal inflammation or damage, or for latent coeliac disease was found to offer a mechanistic explanation for symptom deterioration caused by gluten. How common non-coeliac gluten intolerance is, how it can be reliably identified and what its underlying mechanisms are, warrant further evaluation.

Monash University

Declaration for Thesis Chapter 5.2

Declaration by candidate

In the case of Chapter 5.2, the nature and extent of my contribution to the work was the following:

Nature of contribution
Dr Evan Newnham was involved in study design, patient recruitment, study visits, data analysis and preparation of the manuscript.

Extent of contribution: 30%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Dr Jessica Biesiekierski
Nature of contribution	Dr Biesiekierski was involved in study design, patient recruitment, study visits, food preparation, analysis of blood samples, data analysis and preparation of the manuscript.

Extent of contribution (%) for student co-authors only: 35%

Name	Miss Simone Peters
Nature of contribution	Miss Peters was involved in study visits, food preparation, analysis of blood samples, data analysis and preparation of the manuscript.

Extent of contribution (%) for student co-authors only: 30%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature [redacted]

Date 16/12/14

Main Supervisor's Signature [redacted]

Date

5.2 No effects of gluten in patients with self-reported non-coeliac gluten sensitivity after dietary reduction of fermentable, poorly absorbed, short-chain carbohydrates

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INTRODUCTION

There is an emerging belief that gluten might mediate the symptoms of at least some pateints with irritable bowel syndrome (IBS) [97], where the avoidance of wheat- and gluten-containing products continues to increase worldwide[490]. The clinical entity of 'non-coeliac gluten sensitivity' (NCGS) has been defined as those without coeliac disease but whose gastrointestinal symptoms improve on a gluten-free diet (GFD) [11,222]. Since its original description in 1980[491], reports of NCGS have not taken into account the presence of other components of wheat, particularly fructans, that might have been pathogenically responsible for the symptoms. The first evidence that gluten might specifically induce symptoms in patients with IBS derived from a randomized, placebo-controlled trial of a single dose of carbohydrate-deplete gluten in 36 patients remaining on their habitual GFD in parallel groups[396]. While there is some evidence of the effects of gluten in animal models or cancer cell lines[302,381,394], little else is known regarding this entity. For example, mechanisms have not been identified and dose dependence has not been demonstrated.

In order to further evaluate this concept of NCGS, the current study aimed to examine the hypotheses that, in subjects who report to have NCGS, gluten induces dose-dependent, reproducible gastrointestinal and systemic symptoms. To do this, a randomized, double-blind, crossover controlled feeding trial of three diets differing in gluten content was conducted in patients with IBS fulfilling the definition of NCGS, followed by a rechallenge trial in the same patient cohort. In order to control other potential triggers of gut symptoms, all diets had reduced content of fermentable, poorly-absorbed short-chain carbohydrates (FODMAPs) [492] and, in the second, dairy products and food chemicals were additionally controlled.

METHODS

Patients

Patients were recruited between January 2010 and January 2011 via advertisements in e-newsletters and community newspapers in metropolitan Melbourne, and by referrals from private dietetic practice or gastroenterology clinics. The inclusion criteria were (a) age >16 years; (b) symptoms of IBS fulfilling Rome III criteria that self-reportedly improved with a GFD; (c) symptoms well controlled on a GFD; and (d) adherence to the GFD for at least 6 weeks immediately before screening as assessed at an interview by a trained nutritionist (JRB). Coeliac disease was excluded by either (i) absence of the HLA-DQ2 and HLA-DQ8 haplotype or (ii) a normal duodenal biopsy (Marsh 0) performed at endoscopy while on a gluten-containing diet in individuals expressing the HLA-DQ2 or HLA-DQ8 haplotype. Patients with significant gastrointestinal disease (such as cirrhosis or inflammatory bowel disease), excessive alcohol intake, intake of non-steroidal anti-inflammatory agents, use of systemic immunosuppressant

medication, poorly controlled psychiatric disease and unable to give written informed consent were excluded.

Study Protocol

The first study was a randomized, placebo-controlled, double-blinded crossover trial. After an initial one-week baseline period where the subjects recorded their usual diet and symptoms, participants entered a two-week run-in period, at the beginning of which all were educated on a diet low in FODMAPs[492]. They were continued on a GFD low in FODMAPs throughout. Patients then received one of three diet treatments (high-gluten, low-gluten or placebo) for one week, followed by a washout period of at least two weeks and until symptoms induced during the previous dietary challenge resolved, before crossing over to the next diet. Patients were randomized at recruitment according to a computer-generated order, held by an independent observer. Patients unable to continue a treatment due to intolerable symptoms were permitted to cease the study food of that particular arm, but continue to collect data as per day six (symptom assessment, physical activity studies, blood and stool samples collected) and collect symptom and food diaries when not on the study diet. Patients then resumed any remaining treatment arms following the allocated washout period.

All participants were invited to return to take part in a rechallenge trial. This was designed and conducted after the initial trial was analysed. A 3-day challenge period was chosen on the basis of the kinetics of symptom induction in the first trial and a stricter background control of potential triggers of gut symptoms was employed (see below). As the time between participation of the two trials varied from 8 to 17 months, inclusion/exclusion criteria (as above) were confirmed. Participants were randomly allocated (as for the first study) to receive one of the

three dietary treatments (see below) for 3 days, followed by a washout period of minimum 3 days (or until symptoms induced during the previous dietary challenge resolved), before crossing over to the next diet. Patients unable to continue a treatment due to intolerable symptoms were permitted to cease the study food of that particular arm but continue to collect data as per day three (symptom assessment) and go onto resume any remaining treatment arms following the allocated washout period.

Both trials were approved by Eastern Health Research and Ethics Committee and the 7-day protocol also registered with Australia and New Zealand Clinical Trials Register (ANZCTR): ACTRN12610000524099. All authors had access to the study data and had reviewed and approved the final manuscript.

End-points

The primary end-point was the change in overall symptom score measured on a visual analogue scale (VAS) from the run-in period to that at the end of the treatment period. Secondary end-points comprised (a) the proportions of participants demonstrating an increase of at least 20mm on the VAS in overall and individual symptom scores; (b) the change in individual symptom scores compared with run-in; (c) changes in biomarkers and by-products of protein metabolism; (d) the magnitude of gluten-specific T-cell responses following gluten challenge; (e) change and comparison in scores on fatigue scales and activity levels; and (f) the reproducibility of gastrointestinal symptom scores between the 7-day trial and 3-day rechallenge.

Study food preparation

For the initial 7-day trial, the background diet was gluten-free and low in FODMAPs, a major trigger of gut symptoms. During the three treatment periods, the background diet had the following incorporated: 16g/day whole-wheat gluten ('high-gluten' arm), 2g/day whole-wheat gluten per day and 14g/day whey protein isolate ('low-gluten' arm) or 16g/day whey protein isolate ('placebo' arm).

For the 3-day rechallenge trial, the background diet was gluten-free, and not only reduced in FODMAPs, but also dairy-free and low in naturally occurring and artificially added food chemicals (salicylates, amines, monosodium glutamate, as well as preservatives benzoates, propionate, sulphites, nitrites, sorbic acid, plus added antioxidants and colours), which are all putatively capable of triggering symptoms in some patients[492,493]. During the three treatment periods, the study diets had the following incorporated: 16g/day whole-wheat gluten ('gluten' arm), 16g/day whey protein isolate ('whey' arm) or no additional protein ('placebo' arm).

All main meals were supplied to the subjects. Detailed food lists of low FODMAP fruit and vegetables were supplied to the participants so they were able to purchase fresh perishable items themselves. The meal plan was adequate in macronutrients, micronutrients and provided 8MJ energy daily. Volunteers with smaller energy requirements were given smaller portions, but the same proportion of gluten was added. Volunteers with larger energy requirements were provided with additional low FODMAP, gluten-free meals and snacks.

Meals in each trial were similar across the three diets in texture, taste and appearance, confirmed with preliminary testing in five healthy people where the food containing the gluten could not be

differentiated from those that did not. The gluten used was commercially available, carbohydrate-depleted wheat gluten (Vital Wheat Gluten; Penford Australia Ltd, Tamworth, Australia) and contained 75% protein, 1.8% crude fiber, 6.9% lipid, 15.6% starch and 0.6% ash, as shown on reversed-phase high-performance liquid chromatography. On the basis of size-exclusion high-performance liquid chromatography, the protein content had a distribution of 6.6% non-gluten protein (albumin/globulin), 53.4% glutenin and 40.0% gliadin. The whey protein isolate (RESOURCE® Beneprotein Instant Protein Powder; Nestle Healthcare Nutrition, Inc., Minneapolis, USA) was lactose-free and low-FODMAP, as measured following methodologies described previously[480,494].

The investigator (JRB) and University research chef, assisted by two hospitality students, prepared all food in commercial kitchens. Meals were provided as frozen complete meals with instructions to thaw and warm either via microwave or oven. They were free of charge and delivered to participant's homes weekly.

Measurements

Medical history, examination and, if not already done, HLA genotyping were completed at baseline. For the 7-day trial, dietary adherence was assessed by entries into a tick-box diary completed during the week and by an unused food count at the end of each treatment. A description of any additional food consumed was written in the diary and discussed with one of the investigating team (JRB). Adherence to the GFD was assessed at entry by specific questioning and using a flow chart to give a numerical score[495]. This was crosschecked with assessment of participants' baseline 7-day food diary. Gastrointestinal symptoms were assessed by the participant completing daily diary cards via a 100mm VAS to score the presence and

severity of overall abdominal symptoms, abdominal pain, bloating, wind, satisfaction with stool consistency, tiredness and nausea, as previously applied[386,396]. Gastrointestinal symptom cards were completed daily throughout both trials. Clinically significant change of symptoms was defined as a change of at least 20mm. Severity of fatigue was evaluated by the Daily-Fatigue Impact Scale (D-FIS) [496], a questionnaire containing eight items that evaluates the impact of fatigue on cognition, physical functioning and daily activities. Accelerometry was used to objectively assess physical activity and sleep patterns[497,498]. The participants were asked to wear the accelerometer (ActiGraph GT3X Accelerometer, LLC, Fort Walton Beach, Florida, USA) for seven consecutive days, at all times during the baseline week and during each treatment arm.

Gliadin-specific T-cells in the peripheral blood were assessed by an enzyme-linked immunospot (ELISpot) assay in which the immunological readout is IFN-γ, as previously described[19] using commercially available kits (Mabtech, Nacka Strand, Sweden). Blood was taken from patients on day 0 and day 6 of each treatment week.

Sera from baseline and on day 6 of each treatment week were examined for antibodies to whole gliadin (IgA and IgG) and deamidated gliadin (IgA and IgG) by enzyme-linked immunosorbent assay (ELISA) using commercially available assays (INOVA Diagnostics, San Diego, USA). All tests were performed in conjunction with a total-IgA level. Serum from day 6 of each treatment week was analysed for human eosinophil cationic protein (ECP) by ELISA (Cuasbio Biotech Co., Ltd, Newark, USA) and for IgE antibodies to wheat by radioallergosorbent test (RAST; Phadia AB, Uppsala, Sweden). Assays were performed in duplicate according to manufacturers' instructions.

All faeces passed from day 5-7 were collected during every randomized dietary arm. Volunteers were asked to collect all output during this three-day period, avoiding urine contamination. The date and time of collection was noted on each container, which was then placed immediately into a -20°C portable freezer that was supplied. The faecal samples from each patient were thawed, combined and weighed. The pH of approximately a 20g aliquot warmed to room temperature was measured using a pH electrode probe and portable meter (Mettler Toledo InLab® pH Combination Electrode, and AG FiveGo™ Duo reader, Schwerzenbach, Switzerland). The remainder of the faeces were freeze-dried to obtain a dry weight. The concentration of ammonia was measured enzymatically (Megazyme Ammonia *Rapid* Kit; Megazyme International Ireland Ltd, Wicklow, Ireland), and human ß-defensin-2 (HßD-2) (Immundiagnostik AG, Bensheim, Germany) and calprotectin (Bühlmann Laboratories AG, Schönenbuch, Switzerland) were analysed by ELISA.

For the 3-day trial, only gastrointestinal symptoms (via the VAS as above) and severity of fatigue by the D-FIS were measured.

Statistical analyses

Power calculations were based on previous data[6] and allowed for dropout, missing data and error rate, and assumed a measure of variance from that score (0.29). This indicated that 37 patients were required to achieve a power of 80%, at a two-sided 5% significance level (if the true difference is 0.2).

Per-protocol analyses were performed. Comparisons of symptom severity scores and measured parameters across treatment periods were assessed by repeated measures ANOVA or Friedman

test, as appropriate. Paired t-tests were used to compare the normally distributed data and Wilcoxon signed rank test to compare the non-parametric data. Spearman's correlations were used for associations between symptom severity and biomarkers. The reproducibility was assessed by the test-retest reliability, by calculating the correlation between measured symptoms using the Pearson's correlation coefficient. High test-retest correlations indicate a more reliable sale. Two-tailed *p*-values at or below 0.05 were considered statistically significant.

RESULTS

Study population

Subject flow is shown in Figure 5.3. Following randomisation for the 7-day trial, three patients were withdrawn due to poor symptom control during the run-in period. Thus, 37 patients completed the 7-day trial as per protocol. Twenty-two subjects returned to complete the 3-day rechallenge. The details of those patients are shown in Table 5.3

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Dietary adherence

For the 7-day trial, all 37 patients adhered to the GFD during the study and undertook all three treatment arms. Nearly all (98%) of the main meals during the interventional periods were consumed. Two patients ceased a study diet treatment arm prematurely because of intolerable symptoms. One patient was in the high-gluten arm and withdrew after 4 days, whereas the other was in the placebo arm and withdrew after 3 days. Serum and stool samples were collected from these patients upon cessation of the diet as per day 6. Average consumption of each diet is detailed in Table 5.4. Five participants continued to consume their usual milk products (lactose-containing) as they had previous negative lactose breath hydrogen tests. There was a significant

decrease in dietary fibre and FODMAP intake during the run-in and also an average decrease in energy content from 7.9MJ per day during baseline to 7.3MJ per day during the run-in.

For the 3-day rechallenge, all 22 volunteers undertook the three randomized treatment arms. One patient ceased the whey arm prematurely because of intolerable symptoms after lunch on the second day. Data continued to be collected as per day three. Nearly all meals (96-99%) were consumed in the dietary arms. All patients adhered to the gluten-free, low FODMAP diet during the study. There were seven participants who consumed snacks high in natural food chemicals (e.g., one banana per day), but this did not differ across the treatment arms within participants.

Figure 5.3 Recruitment pathway and reasons for screen failure. Recruitment survey was a 23-item questionnaire about symptoms, diet and investigations for coeliac disease previously described. [499]

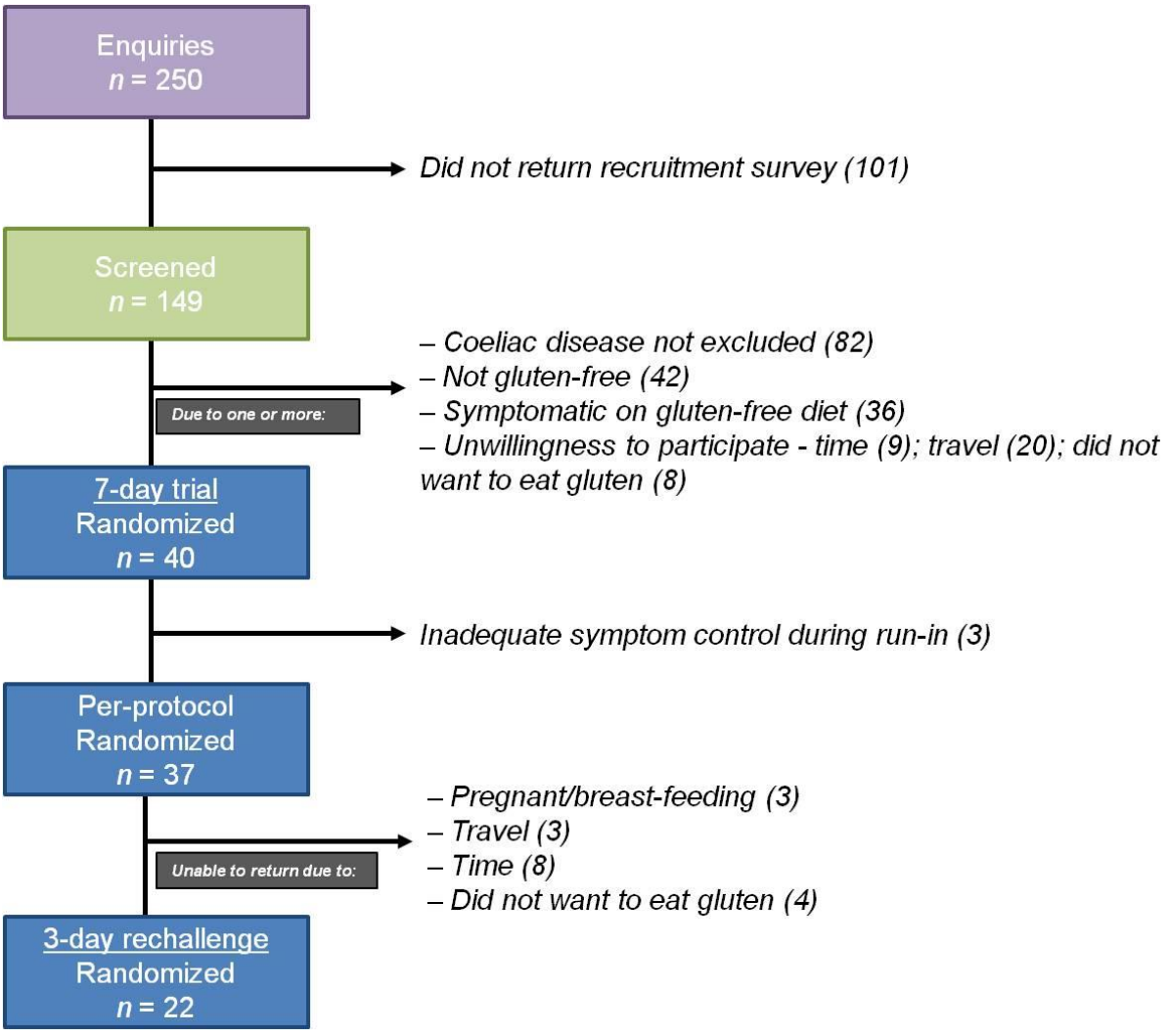


Table 5.3 Study subject characteristics at baseline.

Characteristics	7-day trial	3-day rechallenge
Number of patients	37	22
Gender	6 male	5 male
Median age (range)	45 (24 – 61) years	48 (24 – 62) years
Median body mass index (range)	23 (17 – 39) kg/m^2	23 (17 – 32) kg/m^2
Predominant bowel habit		
Diarrhoea	43%	36%
Constipation	35%	46%
Mixed/Alternating	22%	18%
HLA[a] type		
DQ2 or DQ8 positive	57%	55%
Coeliac serology: mean (± SEM) concentrations (U/ml) (% elevated)		Not tested
Whole-gliadin (IgA)	27 ± 3 (37%)	
Whole gliadin (IgG)	8 ± 1 (5%)	
Deamidated gliadin (IgA)	17 ± 1 (13%)	
Deamidated gliadin (IgG)	9 ± 2 (5%)	

a HLA, human leukocyte antigen

Reference ranges for coeliac antibody assays: negative <20 U/ml; weak positive 20-30 U/ml; strong positive >30 U/ml.

Table 5.4 Actual daily dietary intake during each phase of the 7-day trial

Dietary component	Baseline	Run-in	*P* value	High gluten	Low gluten	Placebo	*P* value
Energy, *MJ*	7.9 ± 0.3	7.3 ± 0.3	.003	7.9 ± 0.2	8.1 ± 0.2	8.0 ± 1.8	NS
Protein, *g*	83 ± 3.2	84 ± 3.9	NS	76 ± 1.8	78 ± 2.1	77 ± 1.9	NS
Total fat, *g*	69 ± 3.0	67 ± 3.6	NS	75 ± 1.8	76 ± 1.9	75 ± 1.9	NS
Total starch, *g*	118 ± 5.9	113 ± 6.3	NS	134 ± 3.4	135 ± 2.6	135 ± 3.1	NS
Dietary fiber, *g*	23 ± 2.3	19 ± 1.9	<.0001	26 ± 0.6	26 ± 0.6	26 ± 0.6	NS
Carbohydrates	210 ± 8.7	183 ± 9.2	.001	215 ± 5.3	221 ± 5.5	220 ± 5.3	NS
Monosaccharides, *g*							
Glucose	18 ± 1.6	15 ± 1.1	.017	21 ± 1.0	23 ± 1.5	21 ± 1.2	NS
Fructose	15 ± 1.5	9.5 ± 0.7	.001	12 ± 0.7	13 ± 0.9	12 ± 0.7	NS
Disaccharides, *g*							
Sucrose	28 ± 3.3	21 ± 2.7	.001	24 ± 1.2	26 ± 1.6	25 ± 1.4	NS
Lactose	14 ± 2.0	9.8 ± 1.1	.030	2.4 ± 0.7	3.3 ± 1.1	3.5 ± 1.2	NS
Sugar polyols, *g*							
Sorbitol	1.1 ± 0.2	0.4 ± 0.07	<.0001	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.02	NS
Mannitol	0.4 ± 0.05	0.2 ± 0.03	.011	0.4 ± 0.02	0.4 ± 0.02	0.4 ± 0.02	NS
Oligosaccharides, *g*							
Fructans	1.5 ± 0.1	1.2 ± 0.1	.011	0.9 ± 0.03	0.9 ± 0.03	0.8 ± 0.03	NS
GOS	1.1 ± 0.2	1.0 ± 0.1	NS	0.5 ± 0.1	0.5 ± 0.09	0.4 ± 0.08	NS
Total FODMAPs, *g*	19 ± 2.0	12 ± 1.1	.003	4.3 ± 0.7	5.2 ± 1.1	5.4 ± 1.2	NS
Alcohol, *g*	12 ± 5.8	6.7 ± 1.4	NS	2.6 ± 0.8	3.7 ± 1.1	2.9 ± 0.9	NS

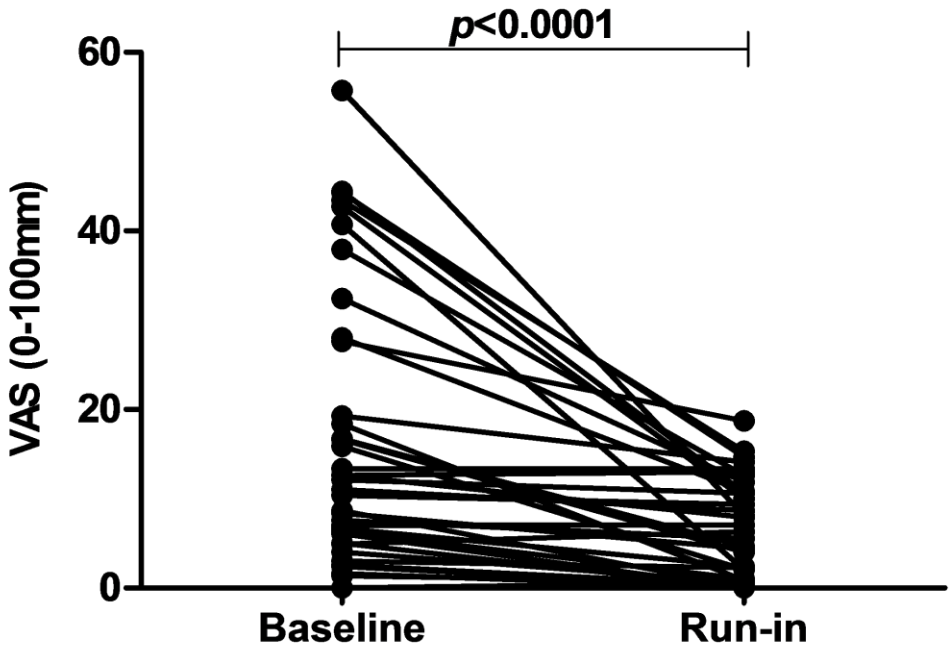
NOTE. Total FODMAPs was calculated as the sum of excess fructose (fructose minus glucose), lactose, sorbitol, mannitol, fructans, and galacto-oligosaccharides (GOS). Foods were analysed directly as described previously.[12,13] Results from laboratory analysis were added to the Foodworks database and are expressed as mean ± SEM. Comparisons were made using Wilcoxon signed rank or Friedman test.
NS, not significant.

Effect on gastrointestinal symptoms

Seven-day trial

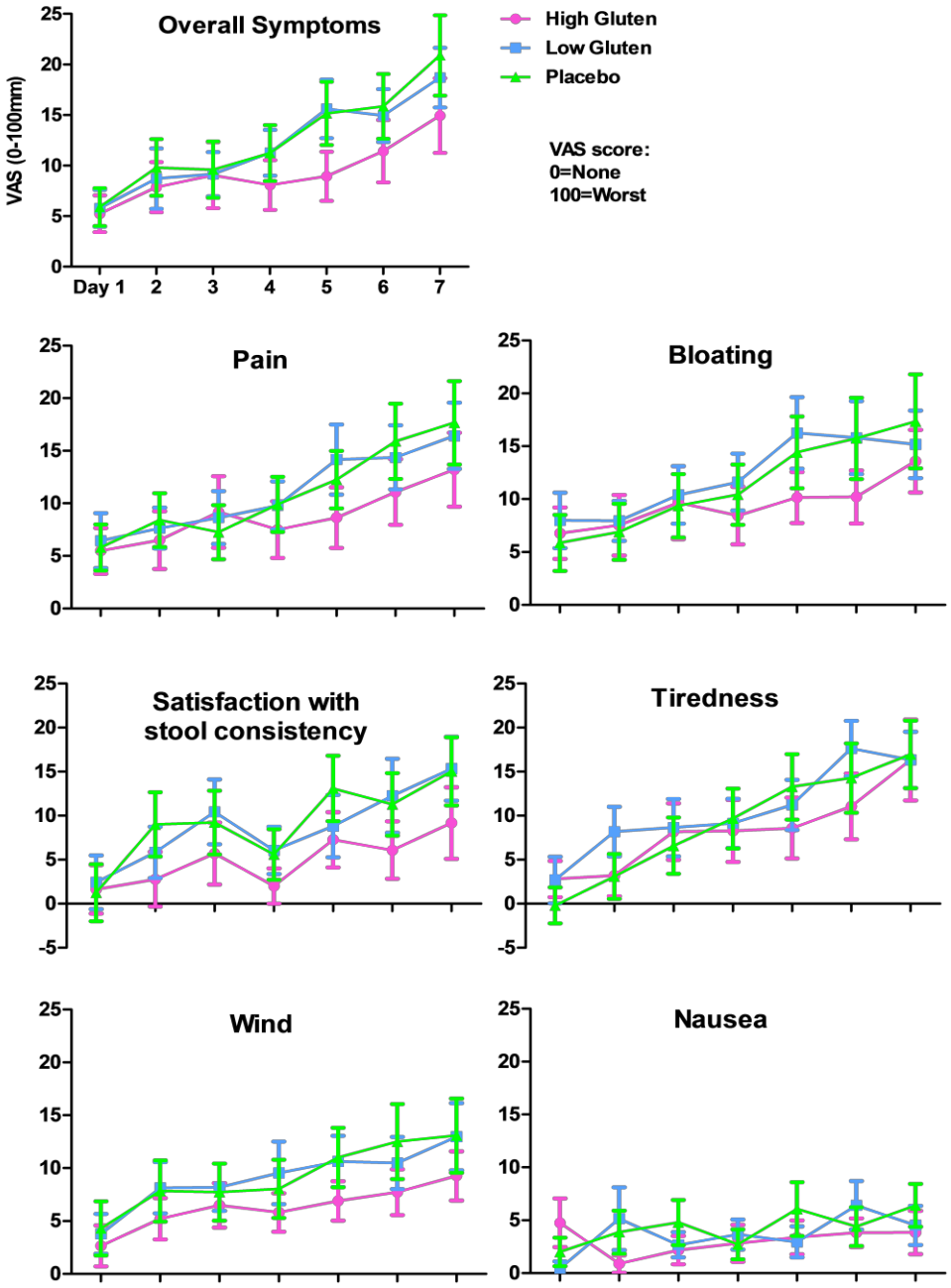
Gastrointestinal symptoms during the baseline period varied across the patients with a median (range) of average overall symptom scores being 12.1 (0-55.7) mm. The average of symptoms from the second week of the low FODMAP run-in period generally improved compared with the baseline. This included overall symptoms (see Figure 5.4), abdominal pain, bloating, satisfaction with stool consistency, wind and tiredness (all $p<0.0001$; Wilcoxon signed rank test), but not nausea ($p=0.149$). Eight participants (22% of total cohort) had an average improvement on the VAS for overall abdominal symptoms of more than 20mm during the low FODMAP run-in period from their baseline level.

Figure 5.4 Individual responses in mean overall symptom severity score during the run-in period, where low FODMAP diet was commenced, compared with those in the baseline period, where participant's usual gluten-free diet was consumed during 7-day trial. Scores were significantly greater during the baseline period (p<0.0001, Wilcoxon signed rank test). VAS, visual analogue scale.



Overall symptoms and pain significantly worsened compared with average scores during the last week of run-in during each dietary treatment period, irrespective of the diet, as detailed in Figure 5.5. Bloating and tiredness significantly worsened during low-gluten and placebo treatment arms only.

Figure 5.5 Change in symptom severity from run-in for each dietary treatment over 7-day study period. Data shown represent mean ± SEM. The differences across the treatment arms were compared by Friedman test, in which overall symptoms (p=0.001), bloating (p=0.016), satisfaction with stool consistency (p=0.008), and wind (p=0.003) were statistically significant, but abdominal pain (p=0.085), tiredness (p=0.305) and nausea (p=0.486) were not. VAS, visual analogue scale.



Only six participants (16% of total cohort) had an average increase in overall abdominal symptoms of more than 20mm on the high-gluten arm compared to those during the run-in period. Three of these patients were HLA-DQ2 positive and three HLA-DQ2/8 negative. Only one of these also had a positive response to the low-gluten arm. Three also had a positive response to the placebo arm. One responded in all three arms. Thus, a dose effect of gluten was not observed and gluten-specificity of symptomatic responses was observed in only three subjects (8% of the total cohort). Eleven participants (30%) had a positive response in overall symptom severity in the placebo arm, eight of whom also reacted in the low-gluten arm. Only one of these eight responded to the high-gluten arm. Thus, seven subjects (19% of the total cohort) had whey-specific symptomatic responses.

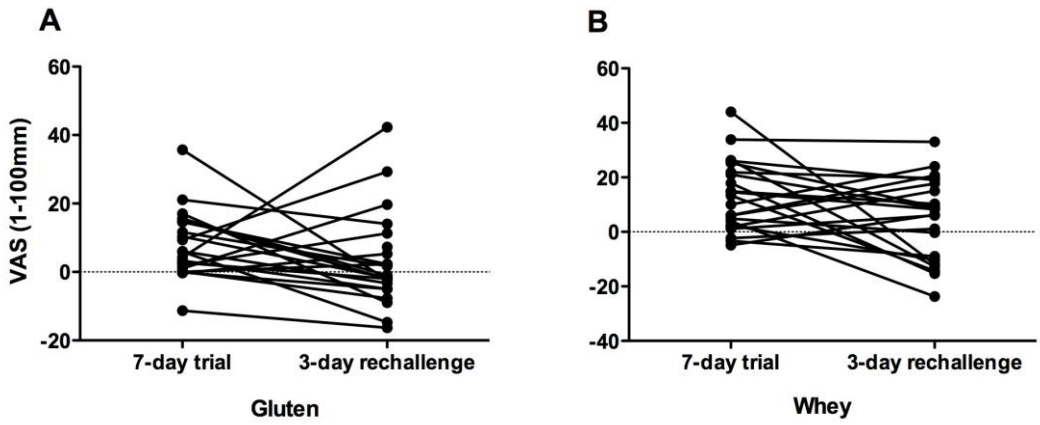
Three-day rechallenge trial

There were no differences across the dietary treatment arms for change of overall symptoms on day 3 compared with the average over the baseline period. Changes in individual symptoms (bloating, satisfaction with stool consistency, wind, pain, tiredness and nausea) were similar across the three dietary periods (all $p>0.209$; data not shown).

The reproducibility of participants' response to gluten (16g/d) and whey (16g/d) between the 7-day challenge and the 3-day rechallenge was evaluated by comparing the change in severity of overall symptoms. There were no significant differences (shown in Figure 5.6) and those identified with a positive symptomatic response to gluten and whey differed between the two trials. The two participants who had an average increase on the VAS for overall abdominal symptoms of more than 20mm on the gluten (16g/d) arm in the 7-day trial were not the same two participants who had a positive response to the gluten (16g/d) arm in the 3-day rechallenge

(Figure 5.6**A**). Thus, gluten-specificity was not reproduced in any subject. Six participants had a positive response in overall symptom severity in the whey (16g/d) arm in the 7-day trial, one of whom also reacted to the whey (16g/d) arm in the 3-day rechallenge. Three different participants also had a positive whey response in the 3-day rechallenge (Figure 5.6**B**). Thus, only one subject reproduced their whey-specific symptomatic response. Re-test reliability in average change in overall symptom severity score (mm) between the two challenges showed no correlation for either gluten (Pearson r=-0.04, p=0.858) or whey (Pearson r=0.08, p=0.748) treatment arms.

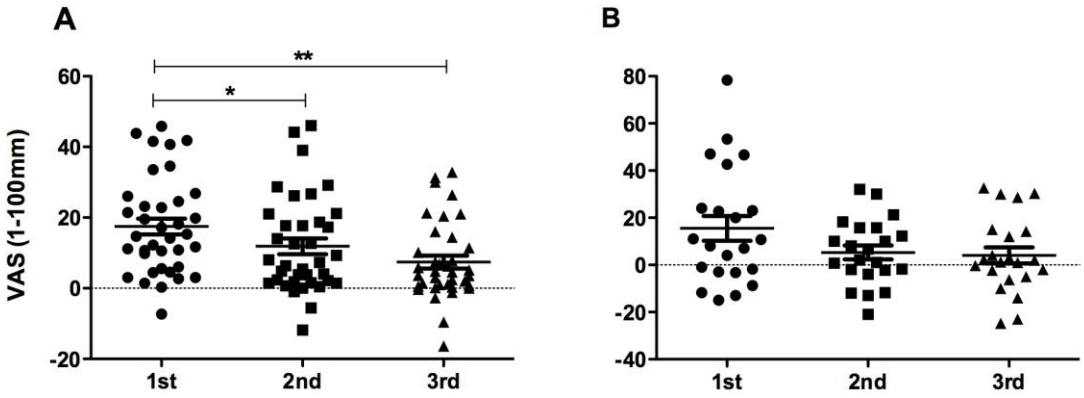
Figure 5.6 Reproducibility in change in overall symptom severity for (A) gluten (16g/d) and (B) whey (16g/d) treatment arms. The 7-day trial used average data from the 7-day treatment period and the 3-day rechallenge used data from the third day of the 3-day treatment period. VAS, visual analogue scale.



For both studies, several patient-related factors were examined in terms of their association with symptomatic responses to the diets in the 7- and 3-day dietary challenges. The predominant bowel habits, BMI, age, sex, duration of GFD and HLA-DQ status did not predict the responses to any of the diets (Spearman's correlation and chi-square analysis, data not shown).

In both studies, the order of the dietary interventions was associated with the degree of symptomatic response. In the 7-day study, the first intervention significantly induced greater symptomatic changes than subsequent challenges, regardless of what it contained (Figure 5.7**A).** Likewise, there was a significant difference across the three groups (p=0.044; repeated measures ANOVA) in the 3-day rechallenge (Figure 5.7**B**), with the first intervention being associated with greater symptomatic changes (mean 15.5mm) than the second (mean 5.3mm) or third (mean 4.0mm) challenges, regardless of its content.

Figure 5.7 Average change in overall symptom severity grouped in order of treatment arm received during (A) 7-day trial and (B) 3-day rechallenge. The differences were compared by repeated measures ANOVA (7-day trial: p=0.001; 3-day rechallenge: p=0.044). Differences were also compared between each group by a paired t-test (7-day trial: *p=0.026, **p=0.001; 3-day rechallenge: all p>0.058). VAS, visual analogue scale.



Effect on fatigue, physical activity and sleep

For the 7-day trial, the low FODMAP run-in period was associated with the lowest average D-FIS score (mean±SEM, 1.95±0.53), which was significantly less than that in the baseline period (5.04±0.87; p=0.0006, paired t-test). There were no differences in levels of fatigue across or during the dietary treatment arms, but there was a significant increase compared with the run-in period for high-gluten (2.19±0.76; p=0.005), low-gluten (2.87±0.77; p=0.004) and placebo (2.41±0.81; p=0.003) for the 7-day trial. There were no differences for gluten (2.05±1.44), whey (1.85±1.03) and placebo (2.42±1.45) for the 3-day rechallenge. There were no apparent effects of dietary treatment on activity levels or any sleep measure analysed by accelerometry (data shown in Table 5.5).

Table 5.5 Physical activity and sleep characteristics of study participants in 7-day trial

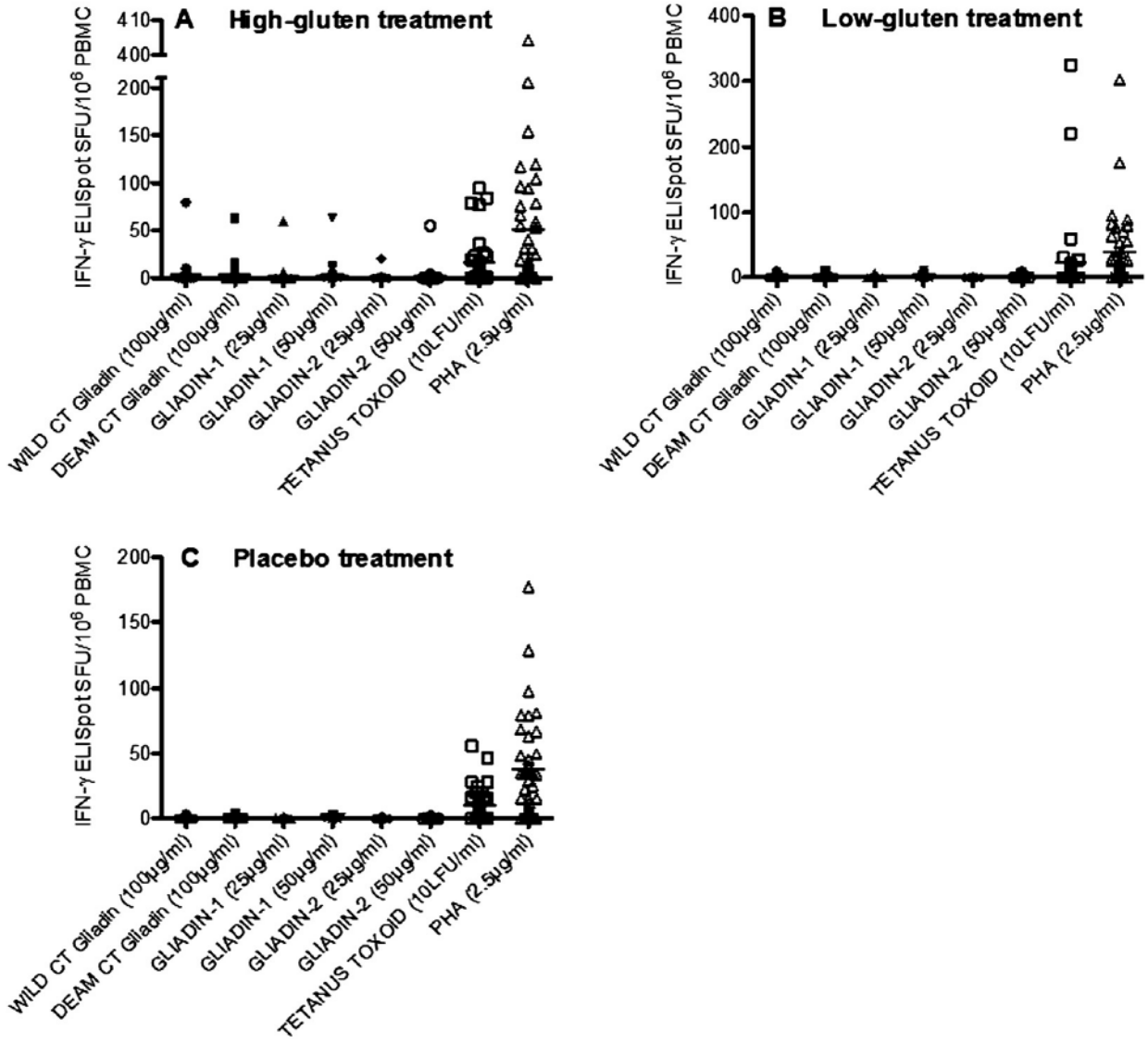
Characteristic	Baseline	High gluten	Low gluten	Placebo
Activity, *kJ/d*	1732 ± 146	1766 ± 134	1678 ± 121	1640 ± 121
% of Time spent at each activity level while accelerometer worn				
Sedentary	76 ± 1.0	75 ± 0.9	76 ± 1.0	76 ± 1.0
Light intensity	16 ± 0.7	17 ± 0.7	16 ± 0.6	16 ± 0.8
Lifestyle	5.7 ± 0.4	5.6 ± 0.2	5.8 ± 0.4	5.6 ± 0.4
Moderate intensity	2.2 ± 0.3	2.3 ± 0.2	2.1 ± 0.2	2.3 ± 0.2
Vigorous intensity	0.1 ± 0.06	0.1 ± 0.05	0.1 ± 0.04	0.1 ± 0.06
Bout				
No. of bouts	4.4 ± 0.8	4.5 ± 0.7	4.5 ± 0.8	3.8 ± 0.7
Time in bout, *min*	13 ± 1.2	13 ± 1.3	12 ± 1.3	12 ± 1.3
Sleep characteristics				
Latency,[a] *min*	3.6 ± 0.5	3.8 ± 0.6	3.5 ± 0.5	3.2 ± 0.6
Efficiency	95 ± 0.5	95 ± 0.5	95 ± 0.5	94 ± 0.5
Time in bed, *min*	499 ± 10	488 ± 7.7	491 ± 9.0	498 ± 8.6
Total sleep time, *min*	471 ± 9.3	461 ± 7.8	466 ± 8.5	469 ± 8.1
No. of awakenings	8.1 ± 0.9	7.5 ± 0.7	7.4 ± 0.8	7.4 ± 0.7
Mean awakening length, *min*	3.8 ± 0.2	3.9 ± 0.3	3.8 ± 0.3	4.1 ± 0.3

NOTE. Values are mean ± SEM. There were no significant differences for diet difference on any measure, compared by repeated-measures analysis of variance.
[a]Latency is the time taken to fall sleep.

Effect on gliadin-specific T-cell responses

All subjects responded to one or both of the positive controls (tetanus toxoid and PHA; Figure 5.8). Only one participant elicited a positive T-cell response following the high-gluten (16g/d) challenge, where her day 6 response was more than a three-fold change from day 0 (Figure 5.8**A**), a response similar to those reported in patients with coeliac disease[337].

Figure 5.8 Interferon-γ (IFN-γ) ELISpot responses of peripheral blood mononuclear cells from study participants after a gluten-free diet for ≥2 weeks in all study participants (n=37) on day 6 after commencing a 7-day treatment in a random order of (A) high gluten (16 g/day), (B) low gluten (2 g/day) and (C) placebo (0 g/day). SFU, spot forming units.



Effect on other biomarkers

There were no significant differences across the treatment periods for serological or other blood markers, ECP or RAST, for the whole sample (n=37), the gluten responders (n=6) or the placebo responders (n=11) (Table 5.6). Likewise, faecal wet and dry weight, pH, and concentrations of HßD-2, calprotectin and ammonia levels were similar across the treatment groups. No correlation existed between average overall symptom score on high-gluten and any of the markers. There was no apparent trend for those patients who had elevated scores on any biomarker with those who demonstrated a gluten- or whey-specific symptom response. No differences in the response of biomarkers to high-gluten exposure were noted according to HLA-D status.

Table 5.6 Coeliac serology, biomarker and fecal characteristic results during treatment periods. Data shown as mean ± SEM (% elevated) unless otherwise indicated. There were no significant differences for diet difference on any measure (compared by repeated measures ANOVA or Friedman test as appropriate).

Biomarker	High-gluten	Low-gluten	Placebo
Coeliac serology (units/mL)			
Whole gliadin (IgA)	19 ± 3.6 (21%)	19 ± 3.4 (13%)	17 ± 1.6 (13%)
Whole gliadin (IgG)	11 ± 2.9 (8%)	9.4 ± 1.8 (5%)	11 ± 2.1 (8%)
Deamidated gliadin (IgA)	16 ± 1.4 (8%)	15 ± 1.4 (3%)	14 ± 1.2 (5%)
Deamidated gliadin (IgG)	8.7 ± 1.5 (8%)	8.8 ± 1.6 (11%)	9.3 ± 1.5 (11%)
Human ß-defensin-2 (ng/mL)	35 ± 4.9 (21%)	33 ± 4.8 (24%)	34 ± 5.6 (29%)
Eosinophil cationic protein (ng/mL)	3.6 ± 0.6 (3%)	3.5 ± 0.6 (3%)	3.4 ± 0.5 (3%)
Radioallergosorbent test (kU/L)	0.09 ± 0.05 (0%)	0.07 ± 0.02 (0%)	0.07 ± 0.02 (0%)
Fecal characteristic			
Median frequency, times/d (range)	1 (0-4)	1 (0-3)	1 (0-4)
Output			
g wet wt/d	127 ± 14	113 ± 11.5	124 ± 13
% dry wt/d	25 ± 1.3	25 ± 1.1	26 ± 1.1
Fecal pH	6.9 ± 0.06	6.9 ± 0.07	6.9 ± 0.06
Fecal ammonia (µg/L)	316 ± 23	328 ± 24	336 ± 25
Calprotectin (µg/g)	31 ± 9.1	33 ± 7.8	26 ± 7.7

Healthy reference ranges: human ß-defensin-2 (<46.4 ng/mL), eosinophil cationic protein (2.45-14.12 ng/mL), radioallergosorbent test (<50.01-100 kU/L), faecal calprotectin (<50 µg/g).

Reference ranges for coeliac antibody assays: negative <20 U/ml; weak positive 20-30 U/ml; strong positive >30 U/ml.

DISCUSSION

Generally, NCGS is viewed as a defined illness, much like coeliac disease, where gluten is the cause and trigger for symptoms. In such a case, it would be anticipated that removal of gluten from the diet would lead to minimal symptoms and subsequent exposure to gluten would lead to specific triggering of symptoms. The results of the current study have not supported this concept. First, some of the patients were not minimally symptomatic despite apparent adherence to and previous considerable improvement on a GFD. Reduction of FODMAPs in their diets uniformly reduced gastrointestinal symptoms and fatigue in the run-in period, after which they were minimally symptomatic. Secondly, in two double-blind, randomized, placebo-controlled, crossover trials, specific and reproducible induction of symptoms with gluten could not be demonstrated.

Such findings must be reconciled with the results of our recent double-blind, randomized trial, in which gluten induced greater gastrointestinal symptoms and fatigue than did placebo in an identically-selected population of patients who fulfilled the criteria for NCGS[396]. Several key differences in study design may have potentially influenced the results. First, in contrast to the previous use of supplements with the habitual diet, food intake was carefully controlled. All food provided was low in FODMAPs and gluten-free to reduce 'background noise' and control for changes in participants' usual diet, particularly intake of other potential dietary triggers. FODMAPs were especially important since they are well-documented inducers of gastrointestinal symptoms[492] and some of the patients in the current study reported intolerance to one (38%) or multiple (27%) foods containing FODMAPs[49]. Symptoms uniformly improved following instruction on restricting FODMAPs in the run-in period. While the fibre intake might

have altered with this dietary change, education was given to alternative low FODMAP sources of fibre, and fibre alteration is not a reliable means of improving symptoms in IBS.

The restriction of all dairy products and food chemicals was also employed in the rechallenge trial in order to control other putative triggers of gut and other symptoms[492]. This ensured that known potential dietary confounders capable of inducing symptoms were minimised and that the only difference between the treatments was the nature of the protein intake. It is possible however, that provision of foods not normally consumed as part of some participants' diets may have led to negative associations of these foods with symptom induction and obscured their actual response to the challenges.

The third difference was the utilisation of a crossover design to reduce the influence of confounders and increase power. Adequate washout and run-in periods were employed (confirmed with checking of symptom diaries) to minimise carry-over and order effects[492,499]. Although there has been previous reserved criticism for the use of a crossover design within the IBS population[500], they have been used successfully in rechallenge dietary studies with IBS subjects[386,501]. However, in the current patient population, an order effect was apparent in both the 7-day and 3-day studies indicating that, in this patient group, a strong anticipatory symptomatic (i.e., nocebo) response was present independently of the nature of the challenge protein.

Fourthly, the duration of treatment was reduced from six to one week on the basis that symptoms were uniformly induced within the first week of the original study. It is unlikely that a longer time frame of challenge would capture any delayed responses to gluten as the three gluten

responders in the current study reached their highest symptom level at day 3. This also formed the rationale for the 3-day rechallenge study duration.

Fifthly, the high participant burden and rigorous demands of the 7-day trial included frequent visits to clinic for blood taking, faecal collection, wearing accelerometers, and completion of daily questionnaires, all whilst following a restrictive diet. This may have been perceived as stressful and may have contributed to the nocebo effect and, therefore, positive symptomatic responses across all treatment arms. This was at least partly addressed in the subsequent study by considerable simplification of the 3-day rechallenge, which was purposely designed to be of a short-duration, highly controlled and with less participant effort and application. Regardless, a nocebo response was again found.

Finally, pure lactose-free whey protein isolate was used as the placebo in the 7-day trial to balance overall protein levels. It was chosen for its rapid digestibility[502] and minimal effects it had on the study food's texture and flavour. The results from the 7-day trial suggested that whey protein itself may have triggered symptoms in some patients. However, the effects of whey protein independently of gluten were not reproduced in the 3-day rechallenge.

While such methodological criticisms can be waged against the current studies, most patients did not exacerbate their symptoms when exposed to gluten. Furthermore, multiple potential biomarkers that are associated with food-related gut disorders were performed as objective end-points. The serological pattern was mostly negative, but of note, there were a lower proportion of cases with positive IgG AGA compared to recent data on gluten sensitivity[503]. Concordant with symptomatic responses, no biomarker-specific changes were shown in the patients who had

gluten- or whey-specific symptoms induced, nor were there any trends amongst participants who had inconsistent or elevated biomarker results. Furthermore, given the apparently specific effect of gluten on fatigue when measured by a simple VAS in the previous study[396], the use of validated tools (D-FIS and accelerometry) for assessing fatigue also failed to show any gluten-specific effects.

A key feature of the present study was the care taken in selection of participants for the study. They were sought by advertising and underwent careful screening to ensure coeliac disease was not present. This included HLA-DQ assessment (non-coeliac haplotype in 47%) and assurance that those with an at-risk haplotype had duodenal biopsies that were performed whilst taking adequate gluten (assessed historically) with normal histopathology. Other studies of NCGS have often included patients with increased density of intraepithelial lymphocytes[504], increasing the risk that patients with latent coeliac disease are being included. None had evidence of wheat allergy (negative RAST). In addition, all patients were assessed for gliadin-specific T-cells using the ELISpot assay, performed using the methodology that identifies coeliac-specific responses[337]. Only one patient demonstrated a positive response, but follow-up testing was negative and her duodenal biopsy on gluten was normal. The other essential inclusion criterion was that the participants had well-controlled symptoms on a GFD pre-enrolment. When assessed by a VAS, eleven patients rated their gastrointestinal symptoms above 20mm for overall abdominal symptoms and 22% experienced clinically significant improvement (change >20mm) in overall symptoms. However, all patients indicated that they were markedly improved on the GFD and their symptoms were well controlled as per the entry criteria. This same feature was noted in the previous gluten-challenge study[396]. Thus, in order to participate in the study, the patients were carefully selected to fulfil current criteria for NCGS. It is likely then that these

criteria need modification so that the issue of whether gluten is indeed a trigger for gut and other symptoms in the broader IBS population can be addressed.

It is possible that the gluten used in the present study was different to that in the first (suppliers were different). The gluten content was similar, but the non-gluten-proteins were not characterised. For instance, α-amylase/trypsin inhibitors[5] may have been present in only the previously reported study. However, evidence points to inflammatory mechanisms by which they might induce symptoms[404] and no evidence of such a process was found in that study.

Alternatively, gluten might induce symptoms only in the presence of a moderate content of FODMAPs. Many gluten-containing cereals are high in fructans, which are a problem in patients with IBS[386] and their concomitant reduction with the introduction of the GFD might lead to improved gut symptoms, wrongly perceived to be due to reduction in gluten intake. Gluten is hypothesised to have direct effects on the brain leading to depression and other neurological maladies[505]. While fatigue did not change in the current study on exposure to gluten, it was a prominent effect in the initial study. A more focussed attention to anxiety and depression, rather than fatigue per se, might provide additional clues to why patients who partake a GFD feel better. Hence, one mechanism by which this interaction might work is that FODMAPs are predominant trigger of gut symptoms whereas gluten in the predominant trigger for a loss of wellness. This intriguing potential interaction deserves further investigation.

In conclusion, these consecutive double-blind, randomized, placebo-controlled, crossover rechallenge studies showed no evidence of specific or dose-dependent effects of gluten in patients with NCGS placed on a low FODMAP diet. A high nocebo response was found regardless of known background dietary triggers being controlled and reproducibility of symptom induction to a

specific protein was poor. These data suggest that NCGS, as currently defined, may not be a discrete entity or that this entity may be confounded by FODMAP restriction, and that, at least in this highly selected cohort, gluten may be not be a specific trigger of functional gut symptoms once dietary FODMAPs are reduced.

Monash University

Declaration for Thesis Chapter 5.3

Declaration by candidate

In the case of Chapter 5.1, the nature and extent of my contribution to the work was the following:

Nature of contribution
Dr Evan Newnham was involved in design of the questionnaire, patient recruitment, data analysis and preparation of the manuscript.

Extent of contribution: 40%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Dr Jessica Biesiekierski
Nature of contribution	Dr Biesiekierski was involved in design of the questionnaire, patient recruitment, data analysis and preparation of the manuscript.

Extent of contribution (%) for student co-authors only: 40%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature [redacted]

Date 16/2/14

Main Supervisor's Signature [redacted]

Date

5.3 Characterisation of adults with a self-diagnosis of non-coeliac gluten sensitivity

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INTRODUCTION

Coeliac disease is an autoimmune gastrointestinal disease estimated to affect 1% or more of Western populations[506]. It occurs when genetically suspectible patients (HLA-DQ2 and/or HLA-DQ8 haplotype) are exposed to dietary gluten, the major protein in wheat, rye, barley and related grains, activating a specific immune response, leading to small intestinal villous atrophy, intraepithelial lymphocytosis, and the development of gastrointestinal symptoms[17]. Diagnosis is achieved via serological screening tests followed by typical features on histopathology of endoscopic duodenal biopsies. Treatment is a lifelong, strict, gluten-free diet (GFD). Since adherence to the GFD normalises serological markers and leads to healing of the small intestine, disease investigation prior to removal of gluten from the diet is essential.

Many of the gastrointestinal symptoms seen in coeliac disease (such as diarrhoea, bloating, gut pain) can mimic irritable bowel syndrome (IBS), a disorder based on symptom patterns and duration, and characterised by a lack of biomarkers[507]. There is an emerging belief that gluten

might mediate IBS symptoms[97] and the term, non-coeliac gluten sensitivity (NCGS), defined as those without coeliac disease but whose gastrointestinal symptoms improve on a GFD, has been recently supported and defined by an expert group [222]. A randomized controlled parallel group trial in which patients fulfilling the definition of NCGS were rechallenged with carbohydrate-deplete gluten or placebo suggested gluten can specifically induce gastrointestinal symptoms and tiredness[396]. However, this gluten-specificity in triggering of symptoms was not detected in consecutive cross-over studies[408]. Related scientific evidence assessing the effects of gluten outside of coeliac disease have focussed on animal models or cancer cell lines[302,381,508]. Regardless of these inconclusive findings, the increased prescription of the GFD for gut and other symptoms continues may lead to missing the diagnosis of true coeliac disease for which the risks and associated complications, especially if left untreated, can include higher mortality, increased risk of malignancy, growth impairment in children, infertility, osteoporosis and autoimmune disease[214].

Compliance with the GFD is complex. It is generally inappropriate for patients to be on a life-long GFD except for those medically diagnosed with coeliac disease or dermatitis herpetiformis. It is very restrictive, and can be more expensive than that of a standard diet[132] and nutritionally inadequate for several nutrients[136]. It has been estimated in Australia, that for every person who has diagnosed coeliac disease, there are twenty others eating gluten-free food[490]. In the US lay press, 20% of the general population are reported to associate their symptoms with the ingestion of gluten [509]. There is a paucity of information about why this non-coeliac population choose to follow the GFD and whether they have had coeliac disease formally excluded. The present study analyzed data from respondents with self-characterized NCGS who expressed willingness to participate in a clinical trial and aimed to characterize the sub-group of people on a GFD who believed they fulfilled criteria for NCGS.

METHODS

From January 2010 to February 2011 in metropolitan Melbourne, Australia, flyers distributed through websites and local clinic rooms (including gastroenterology outpatient clinics and dietetic practices), and advertisements in a local newspaper sought adults who believed they had NCGS to participate in a clinical trial. The advertisements clearly stated the inclusion criteria, including living in Melbourne, had coeliac disease ruled out, have currently well-controlled symptoms, follow a GFD and aged 16 years or older, and that participation would involve consuming gluten, taking blood samples and collecting fecal samples. The clinical trial has already been published, and the current study cohort is derived from further analysis of information available from these volunteers and other survey respondents.

Respondents were asked to fill out a questionnaire comprising 23 items, divided into three dimensions of symptoms (e.g.: 'Describe your main symptoms', 'Do you currently feel in control of your symptoms?'), diet (e.g.: 'Do you follow a strict gluten free diet?', 'Dow long have you been following a gluten free diet?', 'Where did you find out about a gluten free diet?'), and coeliac disease investigation (e.g.: 'Have you had blood tests (or "coeliac antibodies") for diagnosis of coeliac disease?', 'Have you had the gene test for coeliac disease?', 'Have you had a gastroscopy (endoscopy) for diagnosis of coeliac disease?', 'If yes, were you consuming gluten in the lead up to the gastroscopy? How much gluten and for how long before the gastroscopy were you eating gluten? Were you specifically asked to consume gluten in the lead up to the gastroscopy?'). The questionnaire had not been previously validated, and all items were analyzed. Subjects eligible and willing to participate in the research study were evaluated in further detail, by keeping a 7-day food diary and filling in a verified flow chart[495,510] to give a subjective assessment of GFD adherence. This simple flow chart gives a numerical score of adherence to the GFD based on four short questions. These subjects were also asked about any

additional food intolerances by a registered nutritionist (JRB). The advertising, questionnaire and protocol were approved by the Eastern Health Research and Ethics Committee.

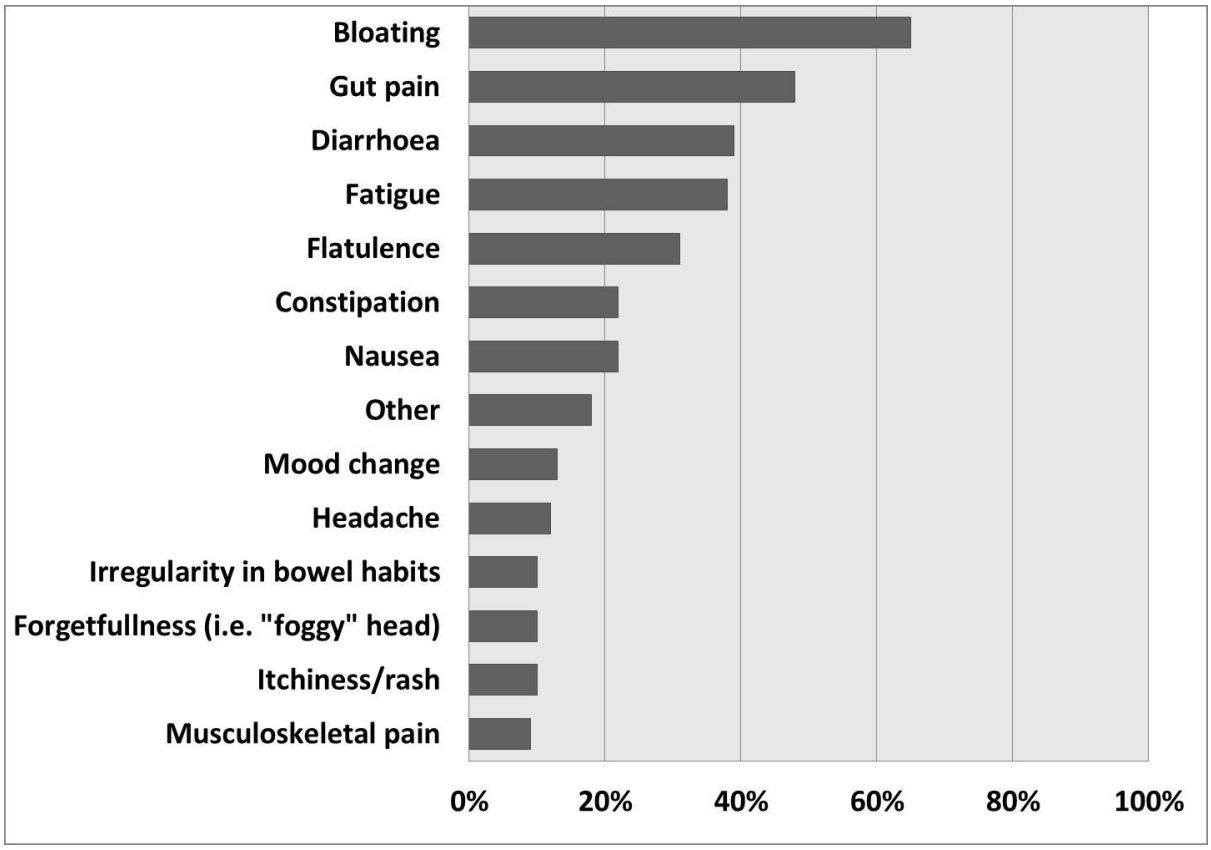
RESULTS

Of 248 respondents to advertising, 147 (59%) completed and returned the survey; these comprised the study cohort for the present analysis. The mean age of respondents was 43.5 (range 16 – 84) years and 130 (88%) were female. Forty subjects who met the inclusion criteria of the research study agreed to participate and were enrolled[396].

The range and frequency of symptoms described by respondents experienced after consuming gluten is shown in Figure 5.9. Gastrointestinal symptoms were, as anticipated from the advertising, most common. A variety of extra-intestinal symptoms were also commonly reported, especially fatigue (also described as tiredness or lethargy). In response to the question as to whether they currently felt in control of their symptoms, 22% of participants answered "no", 3% answered "sometimes", 16% answered "mostly" and 59% answered "yes". Just under a third (32%; n=47) of participants reported having had a hydrogen breath test for sugar malabsorption, 30 (64%) of whom reported positive results for fructose and 14 (30%) for lactose.

Participants were asked whether they believed they follow a strict GFD, answering "no" (22%), "sometimes" (3%), "mostly" (17%), or "yes" (58%). The median time for all respondents to be following a GFD was 36 (0.25 – 444) months. The reasons why participants initiated the GFD varied. It was self-initiated in 44%, and prescribed by alternative health professionals in 21%, dietitians in 23% or general practitioners in 12%.

Figure 5.9 Most common symptoms related to gluten intake reported by participants (n=147). "Other" refers to descriptions of flu-like symptoms, hot flushes, reflux, dry retching, congestion, mouth symptoms, belching, shivers/shudders, sore throat, sleeplessness, dizziness, poor balance, dry eyes, locomotion, hiccups and sweats.



Investigations performed for the diagnosis of coeliac disease are shown in Figure 5.10. No investigation whatsoever (HLA status, antibody testing or biopsy) had been performed in 15% of the respondents. For coeliac diagnosis, Australian guidelines recommend a gluten challenge as a prerequisite for duodenal biopsies of at least four weeks of 16 g gluten per day[511]. Of the 75 participants who had duodenal biopsies, 30% (n=22) had an inadequate gluten intake at the time of endoscopy, implying they had already removed gluten from their diet or did not implement an adequate gluten challenge. Only 40% (n=30) of participants who had duodenal biopsies were asked specifically to consume gluten. Despite this advice, one remained gluten-free and seven gluten-loaded for less than 4 weeks (consuming daily gluten for an average of 10 (1 – 17) days).

All biopsies had been performed in the previous eleven years (2000 to 2011), with only seven participants reporting having more than one biopsy.

Coeliac disease was inadequately excluded in 44 of the 65 (68%) participants who self-initiated the GFD, compared with 21 of the 30 (70%) initiated by alternative health practitioners, 12 of the 28 (43%) initiated by dietitians, and 14 of the 24 (58%) initiated by general practitioners (p=0.103; Chi-square). The only statistical significant difference in inter-group comparisons was for dietitians versus self-initiated (p=0.037; Fisher's exact).

Responses in the questionnaire were used to redefine who in this cohort with self-perceived NCGS actually met the defined criteria. Only 28% fulfilled the criteria of NCGS. The remaining had inadequate exclusion of coeliac disease (62%), and/or uncontrolled symptoms despite gluten restriction (24%), and/or was not following a GFD (27%).

Additional information was sought from the forty respondents who agreed to participate in the clinical trial. All were found to be adherent with the GFD for a median of 48 months prior to participation (see Table 5.7). Recorded information (including type and brand of food) from the 7-day food diaries confirmed strict GFD adherence. During the baseline interview, 65% of these participants described some other form of dietary intolerance, allergy or problem food (detailed in Table 5.7). Sixty percent described taking regular dietary supplementation, including calcium and/or vitamin D (28%), fish and/or omega-3 oil (28%), and multivitamins (25%). Other reported supplements included B-vitamins (10%), magnesium (10%), probiotics (8%), folate (5%) and biotin (5%). Less common supplements taken by 2% of participants included melatonin-5, glucosamine, protein, sage, iron, vitamin E, liver support (supplement powder

consisting of N acetyl cysteine, milk thistle, acetyl carnitine, alpha lipoic acid and vitamin E), zinc, St John's Wort and horsetail.

Figure 5.10 Investigations for coeliac disease in participants following a gluten-free diet (n=147)

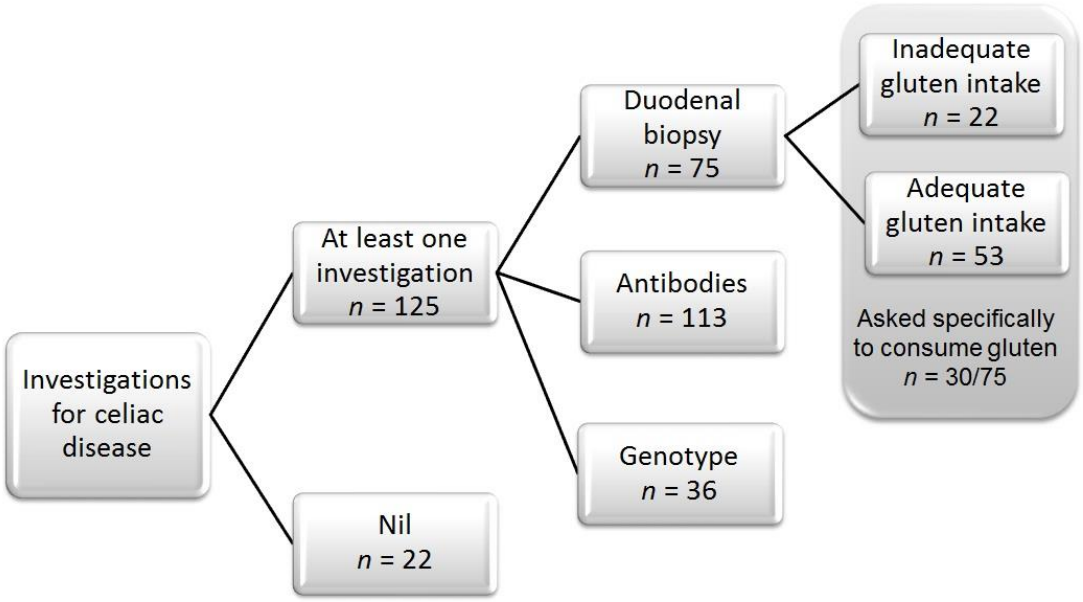


Table 5.7 Summary of baseline dietary habits of responding participants

Baseline Dietary Information		
Mean time spent following GFD (range)	48 (2-444 months)	
Subjective assessment of GFD adherence	Do not follow a strict GFD	0
	Follow a GFD but with errors that require correction	8%
	Follow a strict GFD	92%
Additional intolerances to gluten sensitivity	Nil	35%
	Single	38%
	Multiple	27%
Reported problem foods	FODMAP containing foods[a]	43%
	Dairy (including lactose, casein and whey)	17%
	Food chemicals (eg:, Amines, sulphites, benzoates)	8%
	Tomatoes	5%
	Other[b]	22%

a FODMAPs comprise fructose in excess of glucose, sorbitol, mannitol, fructans and galacto-oligosaccharides

b caffeine, corn, ginger, chilli, psyllium, capsicum, nuts, cinnamon, balsamic, gums, preservatives, spices

DISCUSSION

Although understanding of coeliac disease has considerably improved both clinically and pathologically during recent decades, the evidence behind NCGS remains incomplete. This survey has provided the first data to characterize adults with a self-diagnosis of NCGS, targeting those who use a GFD for relief of their gastrointestinal symtpoms.

There were several key findings in the survey. First, only 58% believed they were strictly gluten-free and, therefore, the remaining respondents could not be considered to be following a GFD. Of those who believed they were gluten-free, detailed assessment of the food intake of the sub-group that entered the clinical trial showed excellent adherence to the GFD. This was despite 44% of the whole cohort and 33% of the clinical trial participants having self-initiated the GFD without dietetic supervision or education. Perhaps this reflects the high standards of information widely available with regard to gluten content of foods.

Secondly, one in four participants who judged him or herself to be gluten-sensitive remained markedly symptomatic despite gluten avoidance. This suggested that either the symptoms were improved from a very high level while eating gluten or that gluten had little to do with the symptoms. One explanation is that, by markedly reducing gluten intake, a major source of other triggers of gastrointestinal symptoms is concomitantly eliminated. Other potential triggers in gluten-containing cereals include fermentable, poorly absorbed, short-chain carbohydrates (collectively termed FODMAPs; Fermentable Oligo- Di- and Mono-saccharides And Polyols) [492] and other wheat proteins[404]. Indeed, in the more detailed analysis of the 40 patients who entered the clinical trial, nearly one half had identified non-cereal foods that contain high levels of FODMAPs as additional triggers of symptoms. FODMAPs are found in a wide variety of

foods[406,480,494], including grains and cereals where wheat- and rye-derived products contain the highest FODMAP content, predominantly fructans. Cereal products with the lowest FODMAP content are mostly gluten-free, based on rice, oat, quinoa and corn ingredients[406]. It is likely, therefore, that 'gluten restriction' will automatically reduce a patient's dietary FODMAP intake.

Thirdly, almost two out of three respondents appeared not to have adequately excluded coeliac disease. The failure to exclude coeliac disease was not confined to those who self-initiated the diet or to alternative health practitioners, but also to general practitioners (although these were few in number). Dietitians seemed the best informed. The gold standard in the diagnosis of coeliac disease and best clinical practice remains duodenal biopsy, although new European Society for Pediatric Gastroenterology, Hepatology and Nutrition recommendations suggest this may not always be the case[28]. Falsely negative results can occur in association with gluten restriction prior to testing since gluten withdrawal is associated with improvement of duodenal histology and reduction in serum levels of coeliac-specific antibodies[214]. Hence, patients should not commence a GFD and, ideally, should be gluten-loaded prior to being tested for coeliac disease. There is yet to be consensus on the optimum dose of gluten needed and the length of time of such loading[169], although recent data show lower doses and shorter challenge duration may be efficacious[512]. However for the purpose of this study, the Australian Therapeutic Guidelines recommendations were used as a guide where an adequate challenge protocol to be the equivalent of four to six slices of wheat bread (16-24 g gluten) per day for at least four weeks[511]. More than one in four patients in the present study had inadequate gluten intake at the time of endoscopy. Forty percent of the patients who underwent endoscopic assessment were instructed to increase gluten intake prior. No data were collected on the patient's gluten intake at time of serological testing, nor was the timing or possible delay determined between serological/genetic testing and endoscopy. The only test independent of gluten intake is HLA

typing, but this can only exclude coeliac disease. Twenty-nine percent of the patients having any investigation of coeliac disease had such testing.

Fourthly, the survey results indicate that patients with self-perceived NCGS are highly heterogeneous in the levels and standards of health care they had received. A major observation was that the importance of a definitive diagnosis or exclusion of coeliac disease was poorly appreciated. Much of the confusion and controversy has arisen in part from a failure to distinguish clearly between the protein (gluten) and carbohydrate (fructan) components of wheat. Indeed, patients who believe they have NCGS are likely to benefit from lowering their dietary intake of FODMAPs [396]. Interestingly, we found a low number (27% of the 40 clinical trial participants) reported multiple food intolerances, compared with previous data showing 50% of IBS patients identified 2-5 foods and 31% identified 6-10 foods as causes of their intolerances[513]. Regular dietary and herbal supplement usage was more frequent (60%) than 16-45% reported for the general adult Australian population[514]. More recent data from the U.S show 55% are multiple supplement users [515].

There are several limitations to the study. The context of the advertising was recruitment for an international clinical trial and this may have attracted a biased sample of those with NCGS. Hence, the results can only apply to this cohort made up predominantly of women of mean age 43.5 years and their applicability to the NCGS population as a whole was not and cannot be currently assessed. In addition, just over one half of the respondents to the advertising completed the questionnaire, a response rate that indicates a risk of a sampling. However, the notion that a lower response rate means lower survey accuracy has been strongly challenged over recent

years[516]. The results were based on participant interpretation, and verification of aspects, such as the diagnostic test performed and their findings was limited to a subset that fulfilled the criteria.

Two relevant and important issues that arise from this survey deserve further discussion. First, the importance of diagnosing coeliac disease cannot be underplayed. Being definitively diagnosed with coeliac disease creates the opportunity for optimal outcomes and ensures ongoing monitoring for associated conditions, memberships to support groups, health fund assistance and in many European countries, a subsidy for the GFD. Coeliac disease demands serious adherence to the GFD, something not done in about half of the patients in the present study. Furthermore, the diagnosis has implications for family screening. The difficulty in excluding this diagnosis is considerable after the GFD has been instituted as outlined above. This message needs to be disseminated to patients and primary care health professionals, a proportion of each group failed in this respect in the present cohort.

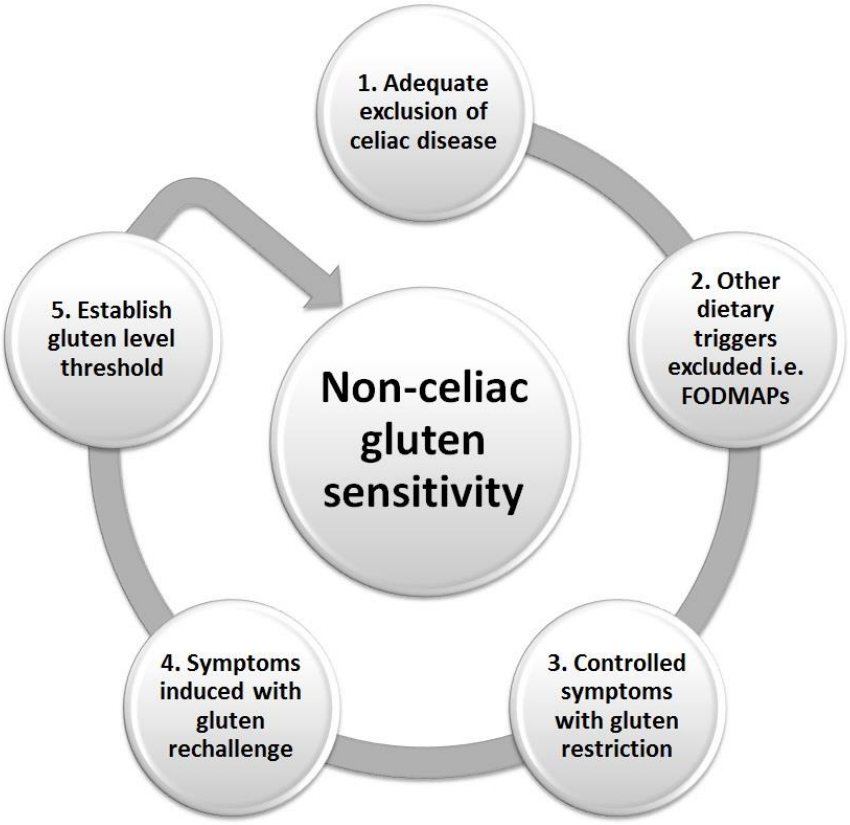
The second issue is that of the risks in committing to the GFD in the absence of coeliac disease. Apart from making diagnosis of coeliac disease difficult, the GFD is potentially nutritionally inadequate. A dietitian-taught GFD is characterized by attention to ensuring adequate intake of all nutrients. Even with excellent teaching, inadequacies of intake do occur and include fiber, thiamin, folate and calcium intakes[136]. It is likely that self-taught patients may not always understand the fundamentals to successfully identifying nutrient-dense gluten-free foods, which includes wholegrain foods (fortified where possible), legumes, fruits, vegetables, lean meat, fish and eggs. Compounding this is the finding of the present study that 65% of the clinical trial sub-group described avoidance of other foods that were perceived to be a problem. The health implications of following long-term restrictive diets require investigation. This area is especially

significant given the evidence for the important roles of fiber and prebiotics (such as fructans) in grain- and cereal-derived carbohydrates in relation to bowel health[517].

Much research is still needed to fulfill our understanding of NCGS, importantly the clinical phenotype to allow the accurate prevalence to be defined. However, for the meantime, a pathway for clinical application and diagnosis of NCGS has been suggested in Figure 5.11. After coeliac testing, other possible dietary triggers should be investigated (such as FODMAPs) and the amount of gluten tolerated should be established.

In conclusion, the practice of initiation of a GFD without adequate exclusion of coeliac disease is common. In one of four, symptoms are poorly controlled despite gluten avoidance, but most patients appear to be well versed in the GFD. Better definition and definitive characterization of this patient group is needed.

Figure 5.11 Suggested flow chart for defining non-coeliac gluten sensitivity: ***Step 1:*** Definitive exclusion of coeliac disease done by either absence of the coeliac-associated HLA-DQ genotype or negative coeliac serology and a normal duodenal biopsy on a gluten-rich diet. ***Step 2:*** After testing for coeliac disease, other possible dietary triggers should be investigated, importantly Fermentable Oligo- Di- and Mono-saccharides And Polyols (FODMAPs), by initiating and trialing the low FODMAP diet for 6 weeks. Skilled dietetic input is imperative. ***Step 3:*** If the patient experiences no or partial symptom improvement to the low FODMAP diet, it is then worth considering gluten. Patients should exclude dietary gluten for 4 weeks and record symptom response. ***Step 4:*** Provided there is marked improvement in symptoms with the GFD, blinded challenges (that is, monitored reintroduction of gluten) can be subsequently undertaken. ***Step 5:*** Following a positive challenge, the amount of gluten tolerated should be established by systematic re-challenges beginning with small amounts of gluten.



Chapter 6: Discussion and conclusion

This thesis has provided detailed analyses of the role of gluten in two highly topical conditions: coeliac disease and non-coeliac gluten sensitivity. Their common thread is that they are both treated with a gluten-free diet (GFD), but they provide contrasting, almost opposite challenges both to patients and the clinicians looking after them. Coeliac disease is unequivocally proven to be caused by the ingestion of specific gluten-derived peptides that result in well characterised biological and histological abnormalities in genetically susceptible individuals. Coeliac disease is common (affecting at least 1.5% of the population[10]) but is presenting with an increasingly mild clinical phenotype. This has created uncertainty in the mind of patients and clinicians as to whether it is worth the pain of adhering to a dietary treatment that is potentially associated with social isolation, increased cost and impaired quality of life. There is still debate as to how aggressively patients should be monitored, what treatment goals should be set and, indeed, whether asymptomatic patients need treatment[518]. Hindering rational arguments defining treatment goals is the relatively limited information on outcomes in CD. For example, the literature examining rates of healing of the small intestine has been dominated by retrospective analyses of populations where dietary compliance has been variable and where complete mucosal healing can be seen in as few as 8%[157]. Only recently have prospective studies been conducted, but even these have been limited in follow-up to 1 year[166].

On the other hand, it is anything but proven that gluten is the inducing protein in non-coeliac gluten sensitivity (NCGS). Questions remain over the very existence of NCGS, let alone how common it is. Outcome data are limited, but it is anticipated that those with NCGS do not have a poor prognosis as it is has been challenging demonstrating clinically significant injury to the intestine or systemically in such patients. The biggest challenge for clinicians is trying to prevent

people without CD from commencing a GFD because it is not necessarily nutritionally healthy and can create psychosocial problems. Previous studies in this area have either lacked a control group or investigators have not been blinded[381]. One major obstacle has been proving that gluten is indeed pathogenically associated with the development of those symptoms. Wheat is complex in structure - both the protein and carbohydrate components have the potential to cause gastrointestinal symptoms by distinct and separate mechanisms.

The work presented in this thesis has made considerable contribution to both of these arguments by presenting prospective studies in CD with up to 5 years of follow-up data, and by undertaking the first randomised, double blind, placebo controlled trials evaluating the effect of re-challenging subjects fulfilling criteria for NCGS with wheat protein devoid of potentially confounding carbohydrates.

Coeliac disease

For a condition that affects up to 1.5% of the population, it is important that solid prospective data are obtained, which will inform the many issues faced in the management of patients with CD. Population screening for CD continues to attract attention and has gained traction in countries such as Finland[519]. However, screening standards demand that the long-term disease outcomes be known and that treatment will prevent complications[520]. As with all population screening programs, the implications are particularly relevant to those who are asymptomatic. An increasing proportion of patients with CD are presenting with minimal or no gastrointestinal symptoms and this patient group can be most challenging to manage, particularly with regards to encouragement of dietary compliance. In clinical practice, patients in this group question what benefits there are to them as an individual. In a recent study of 40 asymptomatic adults with

untreated CD randomised to a GFD or gluten-containing diet, gastrointestinal symptoms, histology and coeliac antibodies improved in those randomised to a GFD but there were no differences in BMD or body composition indices[518]. Although symptoms might improve with treatment, even in those who are asymptomatic, health authorities will only embrace screening when costs can be reduced by outcomes such as reduction in mortality, reduced fracture risk or reduced incidence of malignancy. As it stands, controversy continues around these issues in CD[178,521,522]; for example, although intuitive that treatment might reduce the incidence of malignancies seen in CD, there is only scant evidence to support such a contention[142,179] and there are no prospective studies to guide us. One approach might be to target at risk populations, but even this method has been challenged by studies recently showing that neither gastrointestinal symptoms or body composition are reliable indicators in this regard[45,102]. As outlined below, the major studies presented in this thesis help clarify some, but not all of these important issues and provide a foundation upon which to guide future research.

The first major study in this thesis followed a cohort of patients with newly diagnosed CD prospectively for 5 years. At diagnosis, 1 year and 5 years participants underwent routine blood tests, detailed body composition analysis and endoscopy. A summary of the major findings is presented in Table 6.1. In this group of patients, it was shown that although mucosal response occurs in the vast majority (89%) at 5 years, half had mild ongoing mucosal inflammation (Marsh 1 or worse). In addition, only 37% had achieved mucosal remission at 1 year. This was despite excellent dietary adherence in the overwhelming majority of patients. Importantly, it was shown that neither coeliac antibodies nor gastrointestinal symptoms were an accurate marker of mucosal inflammation during follow-up. Male gender was associated with a greater chance of healing the small bowel at 1 (but not 5) year(s) but no other risk factors could be identified. Changes in body composition were also demonstrated. Skeletal muscle mass progressively

increased over the five years without concerning increases in fat mass. Indices of body fat tended to increase in those who have a normal or mildly elevated BMI rather than those with were already obese at diagnosis. Further, improvements in bone density were most pronounced in those with reduced BMD at diagnosis. In Chapter 3.2, genotype dose was described to have an impact upon the clinical phenotype at diagnosis and possibly on long-term outcomes such as mucosal and serological disease response.

Extra-intestinal complications of CD were also examined in this work. An elevated ALT was the most common liver function test abnormality at diagnosis (seen in 26%). Levels of ALT returned to normal in the majority, despite increases in weight and BMI, suggesting a treatment response in this cohort. Levels of GGT increased with measures of weight and fat whilst reduction in ALP over time was likely to be related to bone turnover. Although retrospective and short-term prospective studies have supported similar findings, the length of follow-up and comparison to detailed body composition and histological measures is unique. Furthermore, observations from enrolment into an unrelated clinical trial in CD resulted in an analysis of the incidence of leukopenia and neutropenia in the present cohort of patients. The finding of an increased incidence of neutropenia (noted in 19% at diagnosis) has not been previously described and there was not convincing evidence of a relationship to CD phenotype, genotype or response to treatment. Using serum collected at 5 years in this cohort, the application of point-of-care testing (PoCT) to the follow-up of CD was assessed by comparing titres of deamidated gliadin peptide antibodies to a qualitative PoCT kit of the same antibody (Simtomax®). Although inter-observer agreement in the results of PoCT was high, accuracy when compared to serum antibodies was poor.

Table 6.1 Summary of outcomes demonstrated in the prospective study presented in Chapter 3. Statistically significant values are shown in bold. Results from other prospective studies are referenced in brackets.

		Diagnosis	**1 year**	**5 Years**
Proportion with elevation of tTG (%)		96	**47** (70 [166])	**20**
Mucosal response (%)			**54** (75 [166])	**85** (64 [198])
Mucosal remission (%)			**37** (66 [166])	**50**
Change in weight (kg) from diagnosis			**+3** (+2.6 [518])	**+4.3**
Change in BMI (kg/m^2) from diagnosis			**+0.9** (0 to 1.0 [209,198,518])	**+1.5** (+2.3[198])
Change in total fat mass (kg) from diagnosis			**+4.2** (+1.1 [518])	+2.0
Change in skeletal muscle mass (kg) from diagnosis			**+0.2**	**+0.9**
Change in bone mass (grams) from baseline	Osteoporosis or osteopenia at baseline		**+65**	+77
	Normal BMD at baseline		-11	+34
Proportion with an elevated ALT (%)		26(10, 0.01 [230,231])	16	11
Proportion with neutropenia (%)		19	12	9

Cognitive improvement in CD has been anecdotally common. In a pilot study of a separate group of patients with newly diagnosed CD followed prospectively for 1 year, improvement in validated measures of cognition was noted that correlated with improvements seen in the small bowel mucosa. Specifically, measures of short-term memory, movement and processing speed were impaired at diagnosis and improved with treatment.

As outlined below, the results obtained in these two studies have described important clinical outcome data regarding the natural history of coeliac disease. When viewed together, these prospectively collected results serve to provide tangible outcomes for patients and clinicians. For patients, particularly the increasing proportion presenting with minimal or no gastrointestinal symptoms, the changes in body composition are especially relevant. It can be expected that skeletal muscle mass increases and that bone mineral density is likely to improve if reduced at diagnosis. Patients can also be reassured that compliance to treatment is highly likely to heal the small bowel and normalise coeliac antibodies. Although undesirable, weight gain is to be expected in those with a BMI less than 25 kg/m^2 but not in those who are overweight or obese. The pilot study of changes in cognition in CD has laid the foundation for further studies in an area that is perceived by patients to be of great importance. Even in those with average measures of cognition at diagnosis, encouragement for compliance to a GFD can be derived from the improved cognition noted in the cohort of patients analysed in Chapter 4. The changes in cognition have important implications for the broader community, and the results on their own suggest that we should be taking treatment of CD seriously.

For clinicians, the results provide insights into the expected timelines of response to treatment of both antibodies and histology. Importantly, the results imply care should be taken in interpreting coeliac antibodies as reflecting mucosal disease activity. Given antibodies were still elevated in 20% of this highly compliant population at 5 years, caution should also be exercised in attributing elevated antibodies to dietary indiscretion, particularly in view of the finding of mucosal response in most of this cohort. Population studies have shown ongoing mucosal inflammation increases risks of complications such as lymphoma and this supports the intuition that mucosal healing should be a treatment goal in CD[179]. Whilst histological follow-up may be the most accurate means of assessment in CD, it is impractical for an increasingly stretched healthcare system. Prospective studies will inform clinicians regarding histological response and large studies of this nature should enable identification of robust risk factors for mucosal response. However, it remains intuitive that mucosal remission be the desired endpoint in a condition defined by the mucosal pathology and there are documented consequences of even mild ongoing inflammation[415]. Other inflammatory bowel diseases have similarly adverse consequences in the absence of mucosal disease response[523]. Underpinning treatment of most inflammatory disease has been induction (often with corticosteroids) followed by maintenance therapy (usually an immunomodulator such as azathioprine, methotrexate or monoclonal antibodies). Although CD is by definition an inflammatory bowel disease, clinicians have thus far not embraced treating it as such. Our approach has been to commence maintenance therapy (i.e., a gluten free diet). As demonstrated in this thesis, such maintenance therapy can take a prolonged period to achieve histological and serological response. Driven by these concepts, we have completed enrolment for an investigator-initiated phase IIb randomised, double blind, placebo controlled trial evaluating the effect of induction therapy with oral effervescent budesonide on mucosal healing in patients with newly diagnosed CD[524]. Results from this trial are expected in the middle of 2015. Interest in therapeutics for CD has increased and, from the

patient perspective, alternatives to the gluten free diet are highly desirable[525]. The detailed understanding of CD has enabled specific therapies to be developed (reviewed in[526]). However it is unlikely that therapy will be indicated or funded for all with CD. As seen with other inflammatory bowel diseases clinicians will be asked to identify patients who will benefit the most from treatment. Prospective studies with well-defined patient groups including genotypic, serological and histological markers will further inform these treatment algorithms. Non-invasive markers such as i-FABP, antibodies to glycoprotein-2 and urinary volatile organic compound analysis[352,369,527] require validation in prospective studies to overcome the need for regular endoscopic assessment in future studies.

As demonstrated in the present work and recent studies of high risk populations[425], genotype dose has an impact upon disease presentation and possibly on long term outcomes such as mucosal and serological disease response. These observations are beginning to be adopted into diagnostic algorithms and might serve to identify high risk groups that warrant more stringent disease follow-up or groups that should receive therapy earlier in their disease course [10]. Genome wide association studies (GWAS) have thus far been exclusively applied to understanding at-risk groups, disease association or for investigating the pathogenesis of CD [528-530]. Whether any of these risk loci are associated with long-term disease outcomes (and therefore able to be utilised in risk-assessment algorithms) has not been assessed and should be included in future prospective analyses.

Based on the present work, clinicians can also be reassured that an elevated ALT will return to normal in most with CD but that this can take up to 5 years. Further, elevations in ALP at diagnosis are often bony in origin and also improve with treatment. If the findings regarding

neutropenia are validated, this might avoid unnecessary investigation for a secondary cause in patients with CD. Conversely, an incidentally discovered low neutrophil count during investigation for other illnesses might prompt diagnostic evaluation for CD. There was not a universal treatment response to neutropenia at diagnosis and thus the observation might point to an inherent phenomenon of CD unrelated to disease activity. Larger prospective studies are needed to validate such observations, elucidate the pathophysiology, and further dissect response to treatment of neutropenia. Measurement of changes in cognition might represent another non-invasive marker of disease response but more practical measures that reflect mucosal disease activity need to be validated. Patients with established CD similarly report cognitive effects upon inadvertent exposure to gluten (for example when eating outside of their home environment). To explore and validate this observation, a randomised, double blind, placebo controlled cross-over study has also been commenced at our institution. In this study, patients with CD treated for at least 2 years and with normal coeliac antibodies have been challenged with either gluten or placebo. Outcome measures include response to similar cognitive tests utilised in Chapter 4 as well as liver function tests and white blood cell indices. Abnormalities in liver function have been previously observed in response to gluten challenge[231], but not in a blinded fashion and not with defined low FODMAP gluten doses.

It is important to address the limitations of these prospective studies. Due to funding constraints for the 5 year follow-up study in Chapter 3, only 54 participants were included for repeat analysis at 1 year. At 5 years, 46 patients from the baseline cohort of 99 agreed to participate in follow-up assessments and data was only available for 26 patients at all 3 time points. There were also methodological limitations. The utilisation of differing assessments of gastrointestinal symptoms at each assessment adversely affected the ability to compare changes over time. The validation of clinical scores arising during the conduct of the present study should overcome this

obstacle in future studies[103]. Adequate numbers of biopsies were obtained at each time point, but assessment of histological outcomes was based on Marsh scores alone. Marsh scores are a useful adjunct to day to day clinical practice, but villous height to crypt depth ratio provides a more accurate representation of changes over time and, from the statistical view point, is a continuous variable that facilitates more robust analyses in the context of clinical trials[90,131]. Furthermore, each time point provided only a snapshot analysis over a comparatively long time period. For example, dietary compliance was not formally assessed between years 1 and 5 and, as explored in more detail below, there is likely to be responder bias when using tools such as dietitian interview and food diaries.

Although novel in design, the study of cognitive outcomes after treatment of patients with newly diagnosed CD would have been strengthened by a control population. The very nature of the psychological tests in this study involved intensive and lengthy study visits that introduced challenges in recruitment. The finding of change in comparatively simple tests such as the Subtle Cognitive Impairment Test (SCIT), trail making tests and Rey-Osterrieth Complex Figure (ROCF) should guide future studies and has been adopted into studies planned at our own institution, as discussed below. In particular, the SCIT is attractive in this regard in that it can be conducted efficiently and repeated without being subject to practise effect.

A significant weakness of all clinical studies in CD to date has been the absence of an objective measure of dietary compliance. As validated in the studies presented in Chapters 3 and 4, coeliac antibodies, intestinal histology and symptoms are all unreliable tool for assessment of adherence. In all studies presented in this thesis, formal assessment was subjective being reliant on completion of food diaries and dietitian interview. More robust and validated tools for

assessment of compliance have been subsequently developed but they remain subjective[149]. There is a pressing need for objective measures. The measurement of intact gluten peptides in the faeces has presented one realistic alternative that requires validation[155] whilst newer technologies such as measurement of the metabolome (in serum or urine) [531] or measurement of volatile compounds in the urine[527] also have potential. Techniques that allow real time assessment of gluten peptides in food may have a role in avoiding inadvertent gluten exposure but require further development[532].

Despite the above limitations, the studies describe the largest cohorts with the longest prospective follow-up yet reported. The breadth of indices measured and the excellent adherence rates enable the provision of data on the 'best case scenario'. The high incidence of CD compared to other inflammatory bowel disease and availability of validated outcome scores should facilitate the conduct of large-scale prospective studies. Clinicians require clarity in identifying those patients likely to have a more severe disease course in order that such patients be targeted for more intensive surveillance and potential therapies. Further, the utility of novel non-invasive markers can only be accurately assessed in sufficiently powered studies. Participants in these studies need to be clearly defined and characterised at diagnosis and have histological follow-up at predetermined time intervals. Contemporaneous measurement of coeliac antibodies, routine blood tests, genotype (both HLA and non-HLA genes) and non-invasive markers of intestinal healing is paramount if our understanding of the true natural history of CD is to be progressed.

Non-coeliac gluten sensitivity

In contrast to CD, NCGS is about wheat protein (not necessarily gluten) inducing gut (and extraintestinal symptoms). It is somewhat surprising that there is no proof that wheat protein is involved at all, since all re-challenge studies attempting to determine the existence of NCGS have used bread or other wheat flour-based products to induce symptoms. Wheat is a complex mix of protein, carbohydrates, fat, vitamins and minerals. Of these components, both protein (for example in CD, Baker's asthma and WDEIA) and carbohydrates (for example in irritable bowel syndrome) have evidence for causality in other human diseases. The two double-blind, randomised, placebo controlled studies outlined in this thesis in Chapter 5 have for the first time separated the potential effects of the carbohydrate component of wheat from the protein in NCGS. The first of these rechallenge trials in NCGS involved the administration of low FODMAP bread and muffins to 34 individuals with NCGS[396]. Subjects were randomised to either gluten (bread 'spiked' with 16g gluten) or placebo. Clear, consistent and statistically significant differences were noted in the group exposed to gluten. Striking differences were seen in the self-reported levels of fatigue, perhaps suggesting a more systemic process. All patients developed exacerbation of symptoms in response to gluten and did so within the first week of re-challenge in contrast to the placebo group where symptom induction occurred more slowly and the level of symptoms reached was less severe. In exploring a biological explanation for the observations, no differences were seen in intestinal permeability, coeliac antibodies, faecal lactoferrin or highly sensitive CRP.

The gold-standard way of determining food intolerance or allergy is a double-blind, placebo controlled rechallenge trial. The pilot study was not. Although randomised and placebo controlled, it did not control for a number of potentially important background variables and did not find a plausible biologic explanation for the observations. The study food provided in both

arms was low FODMAP but the background diets of study participants were only known to be gluten free (i.e. the FODMAP content of their daily meals and snacks were not quantified). As well as FODMAPs there are a number of other potential dietary triggers for gastrointestinal symptoms that include salicylates. With these issues in mind, a subsequent randomised controlled trial was conducted and has been presented in Chapter 5[408]. In this study participants were randomised to high dose gluten, low dose or placebo in a randomised double blinded and placebo controlled cross-over design. All main meals were supplied to participants to control for the issues alluded to above and lists of low-FODMAP foods were supplied for snacks. In the context of controlling for the background diet, no differences were noted between groups in gastrointestinal symptoms or the biological and immune markers assessed. But a sub-analysis of the effect of gluten on mood in these patients during the 3-day re-challenge component (described in details in Chapter 3), has suggested that depressive symptoms were more common in those receiving gluten[533]. The mechanisms for this effect remain unclear but may relate to centrally acting serotonin or exorphins acting on opioid receptors[533].

A randomised study was published almost simultaneously to the above[286]. Using a different methodology, 45 gluten consuming individuals with diarrhoea predominant IBS (D-IBS) were randomised to a GFD or gluten containing diet (GCD) for 4 weeks with all food supplied from the investigating centre. Participants underwent assessments of gastrointestinal symptoms, intestinal permeability (via urine IP studies and mucosal tight junction gene expression from small bowel and rectal biopsies) and intestinal motility. In addition, an in vitro assessment of cytokine response to in vitro stimulation of PBMC with gluten was also performed. Stool frequency reduced in those randomised to a GFD whilst intestinal permeability was increased in the GCD compared to the GFD. Both stool frequency and IP were reduced by more in those with an HLADQ2/DQ8 haplotype and alterations in mucosal tight junction gene expression

were also noted in those possessing the coeliac genotype. Increased TNF-α was observed (but not IFN-γ) pointing towards a possible innate immune response in NCGS, a finding supported by other groups[405]. No overall differences were observed in colonic permeability, stimulated cytokine response or GI motility. Although the results might suggest a biological explanation for NCGS, there are important caveats to explain. Patients at enrolment were on a gluten containing diet, but quantification of gluten was not undertaken. Randomisation to a GFD in the intervention arm resulted in the exclusion of both wheat protein and carbohydrates. It is therefore difficult to draw firm conclusions regarding cause and effect. Although utilised in some studies in IBS, the fractional excretion of lactulose when assessing intestinal permeability study results is to be used with caution due to the effect of intestinal motility and bacterial fermentation on absorption of this compound.

In a large retrospective analysis of double-blinded challenge with wheat-containing capsules, investigators in an Italian study noted histological changes in the small bowel in a group of individuals with NCGS[404]. As well as increased intra-epithelial lymphocytes, infiltration of both the small bowel and colon with eosinophils was noted. After *in vitro* stimulation with wheat protein, an increase in basophil activation was also observed in participants, but this has not been replicated in other populations[534]. Contrary to the studies above, no placebo response was noted. The finding of increased intraepithelial lymphocytes has been seen by others after challenge with wheat protein *in vitro*[534] and bread *in vivo*[535]. Some studies have included participants with intraepithelial lymphocytes amongst the defined population with NCGS in the absence of wheat protein challenge[405].

There are several lessons for future research into clinical syndromes or symptoms that appear to relate to the ingestion of wheat. It is essential to take into account the multiple components of wheat when interpreting findings associated with its ingestion so that attribution of an effect to a specific part (e.g., to gluten) is not incorrectly made. This might involve using specific parts of the wheat (e.g. carbohydrate-depleted wheat protein) when challenging a subject[396,408]. It is similarly important that dietary interventions are accurately described. For example, during intervention studies, the administering of a GFD in a population with IBS[286] results in the exclusion of wheat protein as well as carbohydrates and other cereals and grains that are known to relieve symptoms in this patient group[387,536,537]. Conclusions are also difficult to interpret when the baseline cohort includes participants both on a gluten-free and gluten-containing diet[534]. The amount of wheat used is another less direct method of making an educated guess as to the culprit; for example, the amount of wheat contained within capsules used in some challenge protocols is too small to anticipate carbohydrate-mediated effects to be observed[538]. Likewise, the presence of inflammatory lesions due to its ingestion are less likely to be carbohydrate-associated.

The definition of the target population is essential. The survey study presented in Chapter 5.3 identified a clinical phenotype of NCGS that has subsequently been seen in a much larger population study[402]. As outlined in detail below, future studies similarly need to focus on the clinical features of participants whilst also clearly defining the absence of CD by using validated definitions. Much controversy about NCGS has revolved around different study populations who have responded to a GFD. For example, some are defined histologically and immunologically[396,408], some defined by symptom response to blinded placebo-controlled challenges with wheat[404,538] and some with a variety of symptom clusters from intestinal to extra-intestinal alone or in combination. Some investigators have described changes in patients with

NCGS that share much in common with those seen in CD (for example increased IFN-γ and expression of CD3 positive t-cells[405,535]. Older studies have included participants with a variety of histological and immunological features in which some might indeed have coeliac disease[381]. The current definitions and terminology used create confusion and changes to diagnostic criteria for CD as well as the invention of newer CD diagnostic tests such as tissue staining for IgA serve to further complicate this area[539]. The consensus definition of gluten sensitivity recently reached[11,222] is impractical since there is no available challenge that is purely gluten and the gluten-free diet leads to a concomitant reduction of all wheat components in addition to gluten. There is no problem with the term if strong evidence implicates gluten in the symptom genesis, but, as outlined above, this has been a general weakness of many studies as uncovered by the cross-over study outlined in Chapter 5.2[408]. The proposal that the term, NCGS, is replaced with 'non-coeliac wheat sensitivity' is helpful in taking attention away from gluten as the culprit[538], but would apply to perhaps 10% of the population since wheat induces symptoms, presumably via the ingestion of fructans in the majority of patients with IBS. Furthermore, it would in many cases inappropriately excuse a similar role for other cereals such as rye and barely.

The need for biomarkers is paramount, since the use of patient-reported outcomes (i.e. symptom induction) during rechallenges with wheat/gluten or placebo may be fraught with nocebo responses, although not all groups have experienced this. Circulating anti-whole-gliadin antibodies have been proposed, but their frequency in the non-wheat sensitive population is too high for it to be discriminatory[538] and may be related to increased exposure to gliadin secondary to increased epithelial permeability from the intestinal lesion itself. The report of the high positive predictive value of the basophil activation test for those with wheat hypersensitivity is promising in this way[488]. However, another group did not find this test useful at all in a similar population[540]. To supplement the investigation of biomarkers, longer-term, prospective data on

clinical outcomes following dietary interventions is essential to define the true value of that dietary intervention. To date, the literature is devoid of such information. Recent advances in confocal endomicroscopy might also allow real-time observation of mucosal response to food challenge and guide clinical management[541].

Study design is also important. Using the gold standard of a double-blinded, placebo controlled trial will ensure robust results and minimise confounders whilst crossover studies, such as the one presented in Chapter 5.2, allow for observation within and between individuals. Re-challenge studies where the background diet of participants is accurately defined allow for such a design. If antigen withdrawal methods are used (such as the study by Vazquez-Roque et al [286]) and blinding is to be maintained, then it is important that the non-wheat protein composition and palatability of each intervention arm is identical.

Whether sensitivity to wheat protein in patients with gastrointestinal symptoms without CD exists as a clinical entity has been challenged by the only published randomised, double-blind, placebo controlled study. Only with carefully-designed studies addressing many of these issues will clarity emerge from the current confusion that is present in both the clinical and general community. Currently, CD is the only common condition that has been unequivocally linked to gluten. Inaccurate attribution will be associated with suboptimal therapeutic advice and at least partly underlies the current gluten-free epidemic gripping the Western world.

Conclusion

In conclusion, this thesis has presented unique and original work in CD and NCGS. As outlined above, the research projects have advanced knowledge in the natural history of CD, provided insights into the impact of genotype on clinical phenotype and the response to treatment of abnormal liver function tests. Although the discovery of gluten-derived peptides as the causative antigen in CD revolutionised treatment, the traditional perception that removal of the causative antigen results in rapid dissolution of symptoms, histology and serology has been challenged, as has the reliance on currently available non-invasive markers of intestinal healing and compliance. Neutropenia has been described as a new association with CD and the anecdotal experience of impaired cognition and 'brain fog' in newly diagnosed patients has been validated in a pilot study. Although treatment paradigms in CD have been somewhat challenged by work in this thesis, it has not altered the wealth of evidence that gluten causes CD and that its dietary exclusion results in disease response. In contrast, any role for gluten in the generation of symptoms in patients with NCGS has been significantly challenged by the trial presented in Chapter 4.2, designed according to the accepted gold standard for establishing causality of food-derived antigens. The published studies in NCGS have provided a solid platform for future research into a highly topical condition of great public interest and the studies remain the only publications undertaking a randomised, double blind placebo controlled trial of gluten versus placebo.

References

1. van Overbeek FM, Uil-Dieterman IG, Mol IW, Köhler-Brands L, Heymans HS, Mulder CJ. The daily gluten intake in relatives of patients with coeliac disease compared with that of the general Dutch population. *Eur J Gastroenterol Hepatol*. 1997;9(11):1097–1099. doi:10.3109/00365521.2012.688217.

2. Kasarda DD. Can an increase in celiac disease be attributed to an increase in the gluten content of wheat as a consequence of wheat breeding? *J Agric Food Chem*. 2013.

3. Shewry PR, Halford NG, Belton PS, Tatham AS. The structure and properties of gluten: an elastic protein from wheat grain. *Philos Trans R Soc Lond, B, Biol Sci*. 2002;357(1418):133–142. doi:10.1098/rstb.2001.1024.

4. Pasha I, Saeed F, Sultan MT, Batool R, Aziz M, Ahmed W. WHEAT ALLERGY & INTOLERENCE; RECENT UPDATES AND PERSPECTIVES. *Crit Rev Food Sci Nutr*. 2013. doi:10.1080/10408398.2012.659818.

5. Junker Y, Zeissig S, Kim S-J, et al. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. *J Exp Med*. 2012;209(13):2395–2408. doi:10.1084/jem.20102660.

6. Murray JA, Dyke CV, Plevak MF, Dierkhising RA, Zinsmeister AR, Melton LJ III. Trends in the identification and clinical features of celiac disease in a North American community, 1950-2001. *Clinical Gastroenterology and Hepatology*. 2003;1(1):19–27. doi:10.1053/jcgh.2003.50004.

7. Rampertab SD, Pooran N, Brar P, Singh P, Green PHR. Trends in the Presentation of Celiac Disease. *The American Journal of Medicine*. 2006;119(4):355.e9–355.e14. doi:10.1016/j.amjmed.2005.08.044.

8. Rubio-Tapia A, Robert AK, Edward LK, et al. Increased Prevalence and Mortality in Undiagnosed Celiac Disease. *Gastroenterology*. 2009;137(1):88–93. doi:10.1053/j.gastro.2009.03.059.

9. Lohi S, Mustalahti K, Kaukinen K, et al. Increasing prevalence of coeliac disease over time. *Alimentary Pharmacology & Therapeutics*. 2007;26(9):1217–1225. doi:10.1111/j.1365-2036.2007.03502.x.

10. Anderson RP, Henry MJ, Taylor R, et al. A novel serogenetic approach determines the community prevalence of celiac disease and informs improved diagnostic pathways. *BMC Medicine*. 2013;11(1):1–1. doi:10.1186/1741-7015-11-188.

11. Ludvigsson JF, Leffler DA, Bai JC, et al. The Oslo definitions for coeliac disease and related terms. *Gut*. 2012. doi:10.1136/gutjnl-2011-301346.

12. Hernandez L, Green PH. Extraintestinal manifestations of celiac disease. *Curr Gastroenterol Rep*. 2006;8(5):383–389.

13. Tio M, Cox MR, Eslick GD. Meta-analysis: coeliac disease and the risk of all-cause

mortality, any malignancy and lymphoid malignancy. *Alimentary Pharmacology & Therapeutics*. 2012;35(5):540–551. doi:10.1111/j.1365-2036.2011.04972.x.

14. Ludvigsson JF, Montgomery SM, Ekbom A, Brandt L, Granath F. Small-intestinal histopathology and mortality risk in celiac disease. *JAMA*. 2009;302(11):1171–1178. doi:10.1001/jama.2009.1320.

15. Katz KD, Rashtak S, Lahr BD, et al. Screening for Celiac Disease in a North American Population: Sequential Serology and Gastrointestinal Symptoms. *Am J Gastroenterol*. 2011;106(7):1333–1339. doi:10.1038/ajg.2011.21.

16. Wong RCW, Wilson RJ, Steele RH, Radford-Smith G, Adelstein S. A comparison of 13 guinea pig and human anti-tissue transglutaminase antibody ELISA kits. *J Clin Pathol*. 2002;55(7):488–494.

17. Shan L, Molberg Ø, Parrot I, et al. Structural basis for gluten intolerance in celiac sprue. *Science*. 2002;297(5590):2275–2279. doi:10.1126/science.1074129.

18. Bethune MT, Ribka E, Khosla C, Sestak K. Transepithelial transport and enzymatic detoxification of gluten in gluten-sensitive rhesus macaques. *PLoS ONE*. 2008;3(3):e1857. doi:10.1371/journal.pone.0001857.

19. Mazumdar K, Alvarez X, Borda JT, et al. Visualization of transepithelial passage of the immunogenic 33-residue peptide from alpha-2 gliadin in gluten-sensitive macaques. *PLoS ONE*. 2010;5(4):e10228. doi:10.1371/journal.pone.0010228.

20. Lammers KM, Lu R, Brownley J, et al. Gliadin induces an increase in intestinal permeability and zonulin release by binding to the chemokine receptor CXCR3. *Gastroenterology*. 2008;135(1):194–204.e3. doi:10.1053/j.gastro.2008.03.023.

21. Monsuur AJ, de Bakker PIW, 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. doi:10.1038/ng1680.

22. Wolters VM, Verbeek WH, Zhernakova A, et al. The MYO9B gene is a strong risk factor for developing refractory celiac disease. *Clin Gastroenterol Hepatol*. 2007;5(12):1399–405– 1405 e1–2. doi:10.1016/j.cgh.2007.08.018.

23. van Elburg RM, Uil JJ, Mulder CJ, Heymans HS. Intestinal permeability in patients with coeliac disease and relatives of patients with coeliac disease. *Gut*. 1993;34(3):354–357.

24. 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(10):2333–2340. doi:10.1111/j.1572-0241.2006.00741.x.

25. Anderson RP, Degano P, Godkin AJ, Jewell DP, Hill AV. In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. *Nat Med*. 2000;6(3):337–342. doi:10.1038/73200.

26. Siegel M, Strnad P, Watts RE, et al. Extracellular transglutaminase 2 is catalytically inactive, but is transiently activated upon tissue injury. El-Shemy HA, ed. *PLoS ONE*. 2008;3(3):e1861. doi:10.1371/journal.pone.0001861.

27. Tye-Din JA, Stewart JA, Dromey JA, et al. Comprehensive, quantitative mapping of T cell epitopes in gluten in celiac disease. *Sci Transl Med*. 2010;2(41):41ra51. doi:10.1126/scitranslmed.3001012.

28. Husby S, Koletzko S, Korponay-Szabó IR, et al. European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. *Journal of Pediatric Gastroenterology and Nutrition*. 2012;54(1):136–160. doi:10.1097/MPG.0b013e31821a23d0.

29. Ludvigsson JF, Bai JC, Biagi F, et al. Diagnosis and management of adult coeliac disease: guidelines from the British Society of Gastroenterology. *Gut*. 2014. doi:10.1136/gutjnl-2013-306578.

30. Rostom A, Murray JA, Kagnoff MF. American Gastroenterological Association (AGA) Institute technical review on the diagnosis and management of celiac disease. *Endoscopy*. 2006;131(6):1981–2002. doi:10.1053/j.gastro.2006.10.004.

31. Norström F, Lindholm L, Sandström O, Nordyke K, Ivarsson A. Delay to celiac disease diagnosis and its implications for health-related quality of life. *BMC Gastroenterol*. 2011;11:118. doi:10.1186/1471-230X-11-118.

32. Rostom A, Dubé C, Cranney A, Saloojee N, Sy R. The diagnostic accuracy of serologic tests for celiac disease: a systematic review. *Gastroenterology*. 2005. doi:10.1053/j.gastro.2005.02.028.

33. James M, Scott B. Endomysial antibody in the diagnosis and management of coeliac disease. *Postgraduate medical journal*. 2000. doi:10.1136/pmj.76.898.466.

34. Dieterich W, Ehnis T, Bauer M, Donner P, Volta U. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nature medicine*. 1997. doi:10.1038/nm0797-797.

35. Sárdy M, Kárpáti S, Merkl B, Paulsson M, Smyth N. Epidermal transglutaminase (TGase 3) is the autoantigen of dermatitis herpetiformis. *J Exp Med*. 2002;195(6):747–757. doi:10.1084/jem.20011299.

36. Martin A, Giuliano A, Collaro D, et al. Possible involvement of transglutaminase-catalyzed reactions in the physiopathology of neurodegenerative diseases. *Amino Acids*. 2013;44(1):111–118. doi:10.1007/s00726-011-1081-1.

37. Cascella NG, Santora D, Gregory P, Kelly DL, Fasano A, Eaton WW. Increased Prevalence of Transglutaminase 6 Antibodies in Sera From Schizophrenia Patients. *Schizophrenia Bulletin*. 2013;39(4):867–871. doi:10.1093/schbul/sbs064.

38. Carroccio A, Vitale G, Di Prima L, et al. Comparison of anti-transglutaminase ELISAs and an anti-endomysial antibody assay in the diagnosis of celiac disease: a prospective study. *Clinical Chemistry*. 2002;48(9):1546–1550.

39. Gillett HR, Freeman HJ. Comparison of IgA endomysium antibody and IgA tissue transglutaminase antibody in celiac disease. *Can J Gastroenterol*. 2000;14(8):668–671.

40. Tonutti E, Visentini D, Picierno A, et al. Diagnostic efficacy of the ELISA test for the

detection of deamidated anti-gliadin peptide antibodies in the diagnosis and monitoring of celiac disease. *J Clin Lab Anal*. 2009;23(3):165–171. doi:10.1002/jcla.20313.

41. Kumar V, Jarzabek-Chorzelska M, Sulej J. Celiac disease and immunoglobulin A deficiency: how effective are the serological methods of diagnosis? *Clinical and Vaccine Immunology*. 2002. doi:10.1128/CDLI.9.6.1295-1300.2002.

42. Egner W, Shrimpton A, Sargur R, Patel D, Swallow K. ESPGHAN guidance on coeliac disease 2012: multiples of ULN for decision making do not harmonise assay performance across centres. *Journal of Pediatric Gastroenterology and Nutrition*. 2012;55(6):733–735. doi:10.1097/MPG.0b013e31826531f6.

43. Tosco A, Salvati VM, Auricchio R, et al. Natural history of potential celiac disease in children. *Clin Gastroenterol Hepatol*. 2011;9(4):320–5– quiz e36. doi:10.1016/j.cgh.2010.09.006.

44. Vermeersch P, Geboes K, Mariën G, Hoffman I, Hiele M, Bossuyt X. Serological diagnosis of celiac disease: comparative analysis of different strategies. *Clin Chim Acta*. 2012;413(21-22):1761–1767. doi:10.1016/j.cca.2012.06.024.

45. Rosén A, Sandström O, Carlsson A, et al. Usefulness of symptoms to screen for celiac disease. *PEDIATRICS*. 2014;133(2):211–218. doi:10.1542/peds.2012-3765.

46. Walker MM, Murray JA, Ronkainen J, et al. Detection of Celiac Disease and Lymphocytic Enteropathy by Parallel Serology and Histopathology in a Population-Based Study. *Gastroenterology*. 2010;139(1):112–119. doi:10.1053/j.gastro.2010.04.007.

47. Hadithi M, Blomberg von BME, Crusius JBA, et al. Accuracy of serologic tests and HLA-DQ typing for diagnosing celiac disease. *Ann Intern Med*. 2007;147(5):294–302.

48. Hopper AD, Cross SS, Hurlstone DP, et al. Pre-endoscopy serological testing for coeliac disease: evaluation of a clinical decision tool. *BMJ : British Medical Journal*. 2007;334(7596):729–729. doi:10.1136/bmj.39133.668681.BE.

49. Biesiekierski JR, Newnham ED, Shepherd SJ, Muir JG, Gibson PR. Characterization of Adults With a Self-Diagnosis of Nonceliac Gluten Sensitivity. *Nutr Clin Pract*. 2014;29(4):504–509. doi:10.1177/0884533614529163.

50. McGowan KE, Lyon ME, Butzner JD. Celiac disease and IgA deficiency: complications of serological testing approaches encountered in the clinic. *Clinical Chemistry*. 2008;54(7):1203–1209. doi:10.1373/clinchem.2008.103606.

51. Volta U, Granito A, Parisi C, et al. Deamidated gliadin peptide antibodies as a routine test for celiac disease: a prospective analysis. *J Clin Gastroenterol*. 2010;44(3):186–190. doi:10.1097/MCG.0b013e3181c378f6.

52. Sugai E, Moreno ML, Hwang HJ, et al. Celiac disease serology in patients with different pretest probabilities: is biopsy avoidable? *World Journal of Gastroenterology : WJG*. 2010;16(25):3144–3152.

53. Bienvenu F, Besson Duvanel C, Seignovert C, Rouzaire P, Lachaux A, Bienvenu J. Evaluation of a point-of-care test based on deamidated gliadin peptides for celiac disease

screening in a large pediatric population. *Eur J Gastroenterol Hepatol*. 2012;24(12):1418–1423. doi:10.1097/MEG.0b013e3283582d95.

54. Benkebil F, Combescure C, Anghel SI, Besson Duvanel C, Schäppi MG. Diagnostic accuracy of a new point-of-care screening assay for celiac disease. *WJG*. 2013;19(31):5111–5117. doi:10.3748/wjg.v19.i31.5111.

55. Sorell L, Garrote JA, Acevedo B, Arranz E. One-step immunochromatographic assay for screening of coeliac disease. *Lancet*. 2002;359(9310):945–946. doi:10.1016/S0140-6736(02)08046-7.

56. Baldas V, Tommasini A, Trevisiol C, Berti I, Fasano A. Development of a novel rapid non-invasive screening test for coeliac disease. *Gut*. 2000. doi:10.1136/gut.47.5.628.

57. Garrote, Sorell, Alfonso, et al. A novel visual immunoassay for coeliac disease screening. *European Journal of Clinical Investigation*. 1999;29(8):697–699. doi:10.1046/j.1365-2362.1999.00518.x.

58. Korponay-Szabó IR, Szabados K, Pusztai J, et al. Population screening for coeliac disease in primary care by district nurses using a rapid antibody test: diagnostic accuracy and feasibility study. *BMJ*. 2007;335(7632):1244–1247. doi:10.1136/bmj.39405.472975.80.

59. Korponay-Szabó IR, Raivio T, Laurila K, et al. Coeliac disease case finding and diet monitoring by point-of-care testing. *Alimentary Pharmacology & Therapeutics*. 2005;22(8):729–737. doi:10.1111/j.1365-2036.2005.02663.x.

60. Raivio T, Korponay-Szabo I, Collin P, et al. Performance of a new rapid whole blood coeliac test in adult patients with low prevalence of endomysial antibodies. *Digestive and Liver Disease*. 2007;39(12):1057–1063. doi:10.1016/j.dld.2007.09.005.

61. Ferre-López S, Ribes-Koninckx C, Genzor C, et al. Immunochromatographic sticks for tissue transglutaminase and antigliadin antibody screening in celiac disease. *Clinical Gastroenterology and Hepatology*. 2004;2(6):480–484.

62. Nemec G, Ventura A, Stefano M, et al. Looking for celiac disease: diagnostic accuracy of two rapid commercial assays. *Am J Gastroenterol*. 2006;101(7):1597–1600. doi:10.1111/j.1572-0241.2006.00597.x.

63. Mooney PD, Kurien M, Evans KE, et al. Point-of-care testing for celiac disease has a low sensitivity in endoscopy. *Gastrointest Endosc*. 2014. doi:10.1016/j.gie.2014.02.009.

64. Centre for Clinical Practice at NICE (UK). Coeliac Disease: Recognition and Assessment of Coeliac Disease. 2009.

65. Raivio T, Kaukinen K, Nemes E, et al. Self transglutaminase-based rapid coeliac disease antibody detection by a lateral flow method. *Alimentary Pharmacology & Therapeutics*. 2006;24(1):147–154. doi:10.1111/j.1365-2036.2006.02957.x.

66. Zanchi C, Ventura A, Martelossi S, Di Leo G, Di Toro N, Not T. Rapid anti-transglutaminase assay and patient interview for monitoring dietary compliance in celiac disease. *Scand J Gastroenterol*. 2013;48(6):764–766.

doi:10.3109/00365521.2013.786129.

67. Popp A, Jinga M, Jurcut C, et al. Fingertip rapid point-of-care test in adult case-finding in coeliac disease. *BMC Gastroenterol*. 2013;13(1):115. doi:10.1186/1471-230X-13-115.

68. Meijer JWR, Wahab PJ, Mulder CJJ. Small intestinal biopsies in celiac disease: duodenal or jejunal? *Virchows Arch*. 2003;442(2):124–128. doi:10.1007/s00428-002-0709-7.

69. Bardella MT, Minoli G, Radaelli F, Quatrini M, Bianchi PA, Conte D. Reevaluation of duodenal endoscopic markers in the diagnosis of celiac disease. *Gastrointest Endosc*. 2000;51(6):714–716. doi:10.1067/mge.2000.104653.

70. Dickey W, Hughes D. Disappointing sensitivity of endoscopic markers for villous atrophy in a high-risk population: implications for celiac disease diagnosis during routine endoscopy. *Am J Gastroenterol*. 2001.

71. Lecleire S, Di Fiore F, Antonietti M, Savoye G. Endoscopic markers of villous atrophy are not useful for the detection of celiac disease in patients with dyspeptic symptoms. *Endoscopy*. 2006;38(7):696–701. doi:10.1055/s-2006-925373.

72. Ravelli A, Villanacci V, Monfredini C. How patchy is patchy villous atrophy?: distribution pattern of histological lesions in the duodenum of children with celiac disease. *Am J Gast*. 2010. doi:10.1038/ajg.2010.153.

73. Kiesslich R, Mergener K, Naumann C, et al. Value of chromoendoscopy and magnification endoscopy in the evaluation of duodenal abnormalities: a prospective, randomized comparison. *Endoscopy*. 2003;35(7):559–563. doi:10.1055/s-2003-40240.

74. Iovino P, Pascariello A, Russo I, Galloro G, Pellegrini L, Ciacci C. Difficult diagnosis of celiac disease: diagnostic accuracy and utility of chromo-zoom endoscopy. *Gastrointest Endosc*. 2013;77(2):233–240. doi:10.1016/j.gie.2012.09.036.

75. Ravelli AM, Tobanelli P, Minelli L, Villanacci V, Cestari R. Endoscopic features of celiac disease in children. *Gastrointest Endosc*. 2001;54(6):736–742. doi:10.1067/mge.2001.119217.

76. Johnston AJ, Kurien M, Avgerinos A, Mooney PD, Sanders DS. Is there a role for chromoendoscopy in the diagnosis of coeliac disease? *J Gastrointestin Liver Dis*. 2014;23(1):103–104.

77. De Luca L, Ricciardiello L, Rocchi MBL, et al. Narrow band imaging with magnification endoscopy for celiac disease: results from a prospective, single-center study. *Diagn Ther Endosc*. 2013;2013:580526. doi:10.1155/2013/580526.

78. Leong RWL, Nguyen NQ, Meredith CG, et al. In vivo confocal endomicroscopy in the diagnosis and evaluation of celiac disease. *Gastroenterology*. 2008;135(6):1870–1876. doi:10.1053/j.gastro.2008.08.054.

79. Levinson-Castiel R, Hartman C, Morgenstern S, et al. The role of duodenal bulb biopsy in the diagnosis of celiac disease in children. *J Clin Gastroenterol*. 2011;45(1):26–29. doi:10.1097/MCG.0b013e3181e04d26.

80. Hopper AD, Cross SS, Sanders DS. Patchy villous atrophy in adult patients with suspected gluten-sensitive enteropathy: is a multiple duodenal biopsy strategy appropriate? *Endoscopy*. 2008;40(3):219–224. doi:10.1055/s-2007-995361.

81. Weir DC, Glickman JN, Roiff T, Valim C, Leichtner AM. Variability of histopathological changes in childhood celiac disease. *Am J Gastroenterol*. 2010;105(1):207–212. doi:10.1038/ajg.2009.557.

82. Ravelli A, Bolognini S, Gambarotti M, Villanacci V. Variability of histologic lesions in relation to biopsy site in gluten-sensitive enteropathy. *Am J Gastroenterol*. 2005;100(1):177–185. doi:10.1111/j.1572-0241.2005.40669.x.

83. Gonzalez S, Gupta A, Cheng J, et al. Prospective study of the role of duodenal bulb biopsies in the diagnosis of celiac disease. *YMGE*. 2011;72(4):758–765. doi:10.1016/j.gie.2010.06.026.

84. Mangiavillano B, Masci E, Parma B, et al. Bulb biopsies for the diagnosis of celiac disease in pediatric patients. *Gastrointest Endosc*. 2010;72(3):564–568. doi:10.1016/j.gie.2010.05.021.

85. Vogelsang H, Hanel S, Steiner B, Oberhuber G. Diagnostic duodenal bulb biopsy in celiac disease. *Endoscopy*. 2001;33(4):336–340. doi:10.1055/s-2001-13702.

86. Bonamico M, Thanasi E, Mariani P. Duodenal bulb biopsies in celiac disease: a multicenter study. *Journal of Pediatric Gastroenterology and Nutrition*. 2008. doi:10.1097/MPG.0b013e3181677d6e.

87. Bonamico M, Mariani P, Thanasi E, et al. Patchy villous atrophy of the duodenum in childhood celiac disease. *Journal of Pediatric Gastroenterology and Nutrition*. 2004;38(2):204–207.

88. 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.

89. Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. *Eur J Gastroenterol Hepatol*. 1999;11(10):1185–1194.

90. Lähdeaho M-L, Kaukinen K, Laurila K, et al. Glutenase ALV003 attenuates gluten-induced mucosal injury in patients with celiac disease. *Gastroenterology*. 2014;146(7):1649–1658. doi:10.1053/j.gastro.2014.02.031.

91. Scott BB, Losowsky MS. Patchiness and duodenal-jejunal variation of the mucosal abnormality in coeliac disease and dermatitis herpetiformis. *Gut*. 1976;17(12):984–992. doi:10.1136/gut.17.12.984.

92. Kurien M, Evans KE, Hopper AD, Hale MF, Cross SS, Sanders DS. Duodenal bulb biopsies for diagnosing adult celiac disease: is there an optimal biopsy site? *Gastrointest Endosc*. 2012;75(6):1190–1196. doi:10.1016/j.gie.2012.02.025.

93. Murray JA, Rubio-Tapia A, Van Dyke CT, et al. Mucosal atrophy in celiac disease: extent

of involvement, correlation with clinical presentation, and response to treatment. *Clinical Gastroenterology and Hepatology*. 2008;6(2):186–193.

94. Sanders DS, Carter MJ, Hurlstone DP, Pearce A. Association of adult coeliac disease with irritable bowel syndrome: a case-control study in patients fulfilling ROME II criteria referred to secondary care. *Lancet*. 2001;358(9292):1504–1508. doi:10.1016/S0140-6736(01)06581-3.

95. Ford AC, Chey WD, Talley NJ, Malhotra A, Spiegel BMR, Moayyedi P. Yield of Diagnostic Tests for Celiac Disease in Individuals With Symptoms Suggestive of Irritable Bowel Syndrome: Systematic Review and Meta-analysis. *Arch Intern Med*. 2009;169(7):651–658. doi:10.1001/archinternmed.2009.22.

96. Cash BD, Schoenfeld P, Chey WD. The utility of diagnostic tests in irritable bowel syndrome patients: a systematic review. *Am J Gastroenterol*. 2002;97(11):2812–2819. doi:10.1111/j.1572-0241.2002.07027.x.

97. Cash BD, Rubenstein JH, Young PE, Gentry A. The prevalence of abnormal celiac antibodies and celiac disease in patients with suspected irritable bowel syndrome: a prospective multi-center US study. *Gastroenterology*. 2011. doi:10.1053/j.gastro.2011.06.084.

98. Brar P, Kwon GY, Egbuna II, et al. Lack of correlation of degree of villous atrophy with severity of clinical presentation of coeliac disease. *Digestive and Liver Disease*. 2007;39(1):26–9– discussion 30–2. doi:10.1016/j.dld.2006.07.014.

99. Godfrey JD, Brantner TL, Brinjikji W, et al. Morbidity and mortality among older individuals with undiagnosed celiac disease. *Gastroenterology*. 2010;139(3):763–769. doi:10.1053/j.gastro.2010.05.041.

100. Rubio-Tapia A, Rahim MW, See JA, Lahr BD, Wu T-T, Murray JA. Mucosal recovery and mortality in adults with celiac disease after treatment with a gluten-free diet. *Am J Gastroenterol*. 2010;105(6):1412–1420. doi:10.1038/ajg.2010.10.

101. Campanella J, Biagi F, Bianchi PI, Zanellati G, Marchese A, Corazza GR. Clinical response to gluten withdrawal is not an indicator of coeliac disease. *Scand J Gastroenterol*. 2008;43(11):1311–1314. doi:10.1080/00365520802200036.

102. van der Pals M, Myléus A, Norström F, et al. Body mass index is not a reliable tool in predicting celiac disease in children. *BMC Pediatrics*. 2014;14(1):165. doi:10.1186/1471-2431-14-165.

103. Leffler DA, Dennis M, Edwards George J, et al. A validated disease-specific symptom index for adults with celiac disease. *Clin Gastroenterol Hepatol*. 2009;7(12):1328–34, 1334.e1–3. doi:10.1016/j.cgh.2009.07.031.

104. Ploski R, Ek J, Thorsby E, Sollid LM. On the HLA-DQ(α1*0501, β1*0201)-associated susceptibility in celiac disease: A possible gene dosage effect of DQB1*0201. *Tissue Antigens*. 1993;41(4):173–177. doi:10.1111/j.1399-0039.1993.tb01998.x.

105. Greco L, Romino R, Coto I, et al. The first large population based twin study of coeliac

disease. *Gut*. 2002;50(5):624–628.

106. 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):562–567. doi:10.1111/j.0001-2815.2004.00237.x.

107. Gad Abraham JAT-DOGBAKJZMI. Accurate and Robust Genomic Prediction of Celiac Disease Using Statistical Learning. Akey JM, ed. *PLoS Genetics*. 2014;10(2):e1004137. doi:10.1371/journal.pgen.1004137.

108. Yuan J, Gao J, Li X, et al. The Tip of the "Celiac Iceberg" in China: A Systematic Review and Meta-Analysis. Paul F, ed. *PLoS ONE*. 2013;8(12):e81151. doi:10.1371/journal.pone.0081151.

109. Cummins AG, Roberts-Thomson IC. Prevalence of celiac disease in the Asia-Pacific region. *J Gastroenterol Hepatol*. 2009;24(8):1347–1351. doi:10.1111/j.1440-1746.2009.05932.x.

110. Wijmenga C, Gutierrez-Achury J. Celiac Disease Genetics: Past, Present and Future Challenges. *Journal of Pediatric Gastroenterology and Nutrition*. 2014;59:S4–S7. doi:10.1097/01.mpg.0000450392.23156.10.

111. Koning F. Pathophysiology of Celiac Disease. *Journal of Pediatric Gastroenterology and Nutrition*. 2014;59:S1–S4. doi:10.1097/01.mpg.0000450391.46027.48.

112. Fernandez-Arquero M, Figueredo MA, Maluenda C, la Concha de EG. HLA-linked genes acting as additive susceptibility factors in celiac disease. *Human Immunology*. 1995;42(4):295–300. doi:10.1016/0198-8859(94)00108-3.

113. Murray JA, Moore SB, Van Dyke CT, et al. HLA DQ Gene Dosage and Risk and Severity of Celiac Disease. *Clinical Gastroenterology and Hepatology*. 2007;5(12):1406–1412. doi:10.1016/j.cgh.2007.08.013.

114. Megiorni F, Mora B, Bonamico M, et al. HLA-DQ and risk gradient for celiac disease. *Human Immunology*. 2009;70(1):55–59. doi:10.1016/j.humimm.2008.10.018.

115. Vader W, Stepniak D, Kooy Y, et al. The HLA-DQ2 gene dose effect in celiac disease is directly related to the magnitude and breadth of gluten-specific T cell responses.

116. Stepniak D, Koning F. Celiac disease--sandwiched between innate and adaptive immunity. *Human Immunology*. 2006;67(6):460–468. doi:10.1016/j.humimm.2006.03.011.

117. Congia M, Cucca F, Frau F, et al. A gene dosage effect of the DQA1∗0501/DQB1∗0201 allelic combination influences the clinical heterogeneity of celiac disease. *Human Immunology*. 1994;40(2):138–142. doi:10.1016/0198-8859(94)90059-0.

118. Zubillaga P, Vidales MC, Zubillaga I, Ormaechea V, García-Urkía N, Vitoria JC. HLA-DQA1 and HLA-DQB1 genetic markers and clinical presentation in celiac disease. *Journal of Pediatric Gastroenterology and Nutrition*. 2002;34(5):548–554. doi:10.1097/01.MCG.0000014970.88508.64.

119. Karinen H, Kärkkäinen P, Pihlajamäki J, et al. Gene dose effect of the DQB1*0201allele contributes to severity of coeliac disease. *Scand J Gastroenterol*. 2006;41(2):191–199. doi:10.1080/00365520500206277.

120. Jores R-D, Frau F, Cucca F, et al. HLA-DQB1*0201 homozygosis predisposes to severe intestinal damage in celiac disease. *Scand J Gastroenterol*. 2007;42(1):48–53. doi:10.1080/00365520600789859.

121. Greco L, Percopo S, Clot F, et al. Lack of correlation between genotype and phenotype in celiac disease. *Journal of Pediatric Gastroenterology and Nutrition*. 1998;26(3):286–290.

122. Vermeulen BAN, Hogen Esch CE, Yüksel Z, et al. Phenotypic variance in childhood coeliac disease and the HLA-DQ/DR dose effect. *Scand J Gastroenterol*. 2009;44(1):40–45. doi:10.1080/00365520802116422.

123. Al-Toma A, Goerres MS, Meijer JWR, Peña AS, Crusius JBA, Mulder CJJ. Human leukocyte antigen-DQ2 homozygosity and the development of refractory celiac disease and enteropathy-associated T-cell lymphoma. *Clinical Gastroenterology and Hepatology*. 2006;4(3):315–319. doi:10.1016/j.cgh.2005.12.011.

124. Ciacci C, Iovino P, Amoruso D, et al. Grown-up coeliac children: the effects of only a few years on a gluten-free diet in childhood. *Alimentary Pharmacology & Therapeutics*. 2005;21(4):421–429. doi:10.1111/j.1365-2036.2005.02345.x.

125. Cosnes J, Cellier C, Viola S, et al. Incidence of autoimmune diseases in celiac disease: protective effect of the gluten-free diet. *Clin Gastroenterol Hepatol*. 2008;6(7):753–758. doi:10.1016/j.cgh.2007.12.022.

126. Tontini GE, Rondonotti E, Saladino V, Saibeni S, de Franchis R, Vecchi M. Impact of gluten withdrawal on health-related quality of life in celiac subjects: an observational case-control study. *Digestion*. 2010;82(4):221–228. doi:10.1159/000265549.

127. Shmerling DH, Franckx J. Childhood celiac disease: a long-term analysis of relapses in 91 patients. *Journal of Pediatric Gastroenterology and Nutrition*. 1986;5(4):565–569.

128. Matysiak-Budnik T, Malamut G, de Serre NP-M, et al. Long-term follow-up of 61 coeliac patients diagnosed in childhood: evolution toward latency is possible on a normal diet. *Gut*. 2007;56(10):1379–1386. doi:10.1136/gut.2006.100511.

129. Hopman EGD, Blomberg von ME, Batstra MR, et al. Gluten tolerance in adult patients with celiac disease 20 years after diagnosis? *Eur J Gastroenterol Hepatol*. 2008;20(5):423–429. doi:10.1097/MEG.0b013e3282f4de6e.

130. Kurppa K, Koskinen O, Collin P, Mäki M, Reunala T, Kaukinen K. Changing phenotype of celiac disease after long-term gluten exposure. *Journal of Pediatric Gastroenterology and Nutrition*. 2008;47(4):500–503. doi:10.1097/MPG.0b013e31817d8120.

131. Leffler D, Schuppan D, Pallav K, Najarian R. Kinetics of the histological, serological and symptomatic responses to gluten challenge in adults with coeliac disease. *Gut*. 2012.

132. Lee AR, Ng DL, Zivin J, Green PHR. Economic burden of a gluten-free diet. *J Hum Nutr Diet*. 2007;20(5):423–430. doi:10.1111/j.1365-277X.2007.00763.x.

133. Singh J, Whelan K. Limited availability and higher cost of gluten-free foods. *Journal of Human Nutrition and Dietetics*. 2011;24(5):479–486. doi:10.1111/j.1365-277X.2011.01160.x.

134. Thompson T, Dennis M, Higgins LA, Lee AR, Sharrett MK. Gluten-free diet survey: are Americans with coeliac disease consuming recommended amounts of fibre, iron, calcium and grain foods? *J Hum Nutr Diet*. 2005;18(3):163–169. doi:10.1111/j.1365-277X.2005.00607.x.

135. Wild D, Robins GG, Burley VJ, Howdle PD. Evidence of high sugar intake, and low fibre and mineral intake, in the gluten-free diet. *Alimentary Pharmacology & Therapeutics*. 2010;32(4):573–581. doi:10.1111/j.1365-2036.2010.04386.x.

136. Shepherd SJ, Gibson PR. Nutritional inadequacies of the gluten-free diet in both recently-diagnosed and long-term patients with coeliac disease. *Journal of Human Nutrition and Dietetics*. 2013;26(4):349–358. doi:10.1111/jhn.12018.

137. De Palma G, Nadal I, Collado MC, Sanz Y. Effects of a gluten-free diet on gut microbiota and immune function in healthy adult human subjects. *Br J Nutr*. 2009;102(8):1154–1160. doi:10.1017/S0007114509371767.

138. Herman ML, Rubio-Tapia A, Lahr BD, Larson JJ, Van Dyke CT, Murray JA. Patients with celiac disease are not followed up adequately. *Clin Gastroenterol Hepatol*. 2012;10(8):893–899.e1. doi:10.1016/j.cgh.2012.05.007.

139. Sharkey LM, Corbett G, Currie E, Lee J, Sweeney N, Woodward JM. Optimising delivery of care in coeliac disease - comparison of the benefits of repeat biopsy and serological follow-up. *Alimentary Pharmacology & Therapeutics*. 2013;38(10):1278–1291. doi:10.1111/apt.12510.

140. Johnston SD, Peter Watson RG, McMillan SA, Evans AE, Love AHG. Serological markers for coeliac disease: changes with time and relationship to enteropathy. *Eur J Gastroenterol Hepatol*. 1998;10(3):259.

141. Catassi C, Rossini M, Ratsch IM, et al. Dose dependent effects of protracted ingestion of small amounts of gliadin in coeliac disease children: a clinical and jejunal morphometric study. *Gut*. 1993;34(11):1515–1519.

142. Holmes GK, Prior P, Lane MR, Pope D, Allan RN. Malignancy in coeliac disease--effect of a gluten free diet. *Gut*. 1989;30(3):333–338. doi:10.1136/gut.30.3.333.

143. Ciacci C, Cirillo M, Cavallaro R, Mazzacca G. Long-term follow-up of celiac adults on gluten-free diet: prevalence and correlates of intestinal damage. *Digestion*. 2002;66(3):178–185.

144. Mustalahti K, Lohiniemi S, Collin P, Vuolteenaho N, Laippala P, Mäki M. Gluten-free diet and quality of life in patients with screen-detected celiac disease. *Eff Clin Pract*. 2002;5(3):105–113.

145. Bardella MT, Fredella C, Prampolini L, Molteni N, Giunta AM, Bianchi PA. Body composition and dietary intakes in adult celiac disease patients consuming a strict gluten-free diet. *Am J Clin Nutr*. 2000;72(4):937–939.

146. Barera G, Mora S, Brambilla P, et al. Body composition in children with celiac disease and the effects of a gluten-free diet: a prospective case-control study. *Am J Clin Nutr*. 2000;72(1):71–75.

147. Stazi AV, Mantovani A. A risk factor for female fertility and pregnancy: celiac disease.[see comment]. *Gynecological Endocrinology*. 2000;14(6):454–463.

148. Hall NJ, Rubin G, Charnock A. Systematic review: adherence to a gluten-free diet in adult patients with coeliac disease. *Alimentary Pharmacology & Therapeutics*. 2009;30(4):315–330. doi:10.1111/j.1365-2036.2009.04053.x.

149. Leffler DA, Dennis M, George JBE, et al. A Simple Validated Gluten-Free Diet Adherence Survey for Adults With Celiac Disease. *Clinical Gastroenterology and Hepatology*. 2009;7(5):530–536.e2. doi:10.1016/j.cgh.2008.12.032.

150. Sainsbury K, Mullan B. Measuring beliefs about gluten free diet adherence in adult coeliac disease using the theory of planned behaviour. *Appetite*. 2011;56(2):476–483. doi:10.1016/j.appet.2011.01.026.

151. Sainsbury K, Mullan B, Sharpe L. Gluten free diet adherence in coeliac disease. The role of psychological symptoms in bridging the intention–behaviour gap. *Appetite*. 2013;61:52–58. doi:10.1016/j.appet.2012.11.001.

152. Sainsbury K, Mullan B, Sharpe L. A Randomized Controlled Trial of an Online Intervention to Improve Gluten-Free Diet Adherence in Celiac Disease. *Am J Gastroenterol*. 2013. doi:10.1038/ajg.2013.47.

153. Wylie C, Geldart S, Winwood P. Dietitian-led coeliac clinic: A successful change in working practice in modern healthcare. *Gastroenterol Today*. 2005.

154. Addolorato G, De Lorenzi G, Abenavoli L, Leggio L, Capristo E, Gasbarrini G. Psychological support counselling improves gluten-free diet compliance in coeliac patients with affective disorders. *Alimentary Pharmacology & Therapeutics*. 2004;20(7):777–782. doi:10.1111/j.1365-2036.2004.02193.x.

155. Comino I, Real A, Vivas S, et al. Monitoring of gluten-free diet compliance in celiac patients by assessment of gliadin 33-mer equivalent epitopes in feces. *American Journal of Clinical Nutrition*. 2012. doi:10.3945/ajcn.111.026708.

156. Pulido O, Zarkadas M, Dubois S, et al. Clinical features and symptom recovery on a gluten-free diet in Canadian adults with celiac disease. *Can J Gastroenterol*. 2013;27(8):449–453.

157. Lanzini A, Lanzarotto F, Villanacci V, et al. Complete recovery of intestinal mucosa occurs very rarely in adult coeliac patients despite adherence to gluten-free diet. *Alimentary Pharmacology & Therapeutics*. 2009;29(12):1299–1308. doi:10.1111/j.1365-2036.2009.03992.x.

158. Bruins M. The Clinical Response to Gluten Challenge: A Review of the Literature. *Nutrients*. 2013;5(11):4614–4641. doi:10.3390/nu5114614.

159. Vécsei E, Steinwendner S, Kogler H, et al. Follow-up of pediatric celiac disease: value of

antibodies in predicting mucosal healing, a prospective cohort study. *BMC Gastroenterol*. 2014;14(1):28. doi:10.1186/1471-230X-14-28.

160. Dickey W, Hughes DF, McMillan SA. Disappearance of endomysial antibodies in treated celiac disease does not indicate histological recovery. *Am J Gastroenterol*. 2000;95(3):712–714. doi:10.1111/j.1572-0241.2000.01838.x.

161. Spatola BN, Kaukinen K, Collin P, Mäki M, Kagnoff MF, Daugherty PS. Persistence of elevated deamidated gliadin peptide antibodies on a gluten-free diet indicates nonresponsive coeliac disease. *Alimentary Pharmacology & Therapeutics*. 2014;39(4):407–417. doi:10.1111/apt.12603.

162. Lee SK, Lo W, Memeo L, Rotterdam H, Green PHR. Duodenal histology in patients with celiac disease after treatment with a gluten-free diet. *Gastrointest Endosc*. 2003;57(2):187–191. doi:10.1067/mge.2003.54.

163. 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):387–391.

164. Troncone R, Mayer M, Spagnuolo F, Maiuri L, Greco L. Endomysial antibodies as unreliable markers for slight dietary transgressions in adolescents with celiac disease. *Journal of Pediatric Gastroenterology and Nutrition*. 1995;21(1):69–72.

165. Vahedi K, Mascart F, Mary J-Y, et al. Reliability of antitransglutaminase antibodies as predictors of gluten-free diet compliance in adult celiac disease. *Am J Gastroenterol*. 2003;98(5):1079–1087. doi:10.1111/j.1572-0241.2003.07284.x.

166. Galli G, Esposito G, Lahner E, et al. Histological recovery and gluten-free diet adherence: a prospective 1-year follow-up study of adult patients with coeliac disease. *Alimentary Pharmacology & Therapeutics*. 2014;40(6):639–647. doi:10.1111/apt.12893.

167. Kaukinen K, Sulkanen S, Mäki M, Collin P. IgA-class transglutaminase antibodies in evaluating the efficacy of gluten-free diet in coeliac disease. *Eur J Gastroenterol Hepatol*. 2002;14(3):311–315.

168. Bannister EG, Cameron DJ, Ng J, et al. Can Celiac Serology Alone be Used as a Marker of Duodenal Mucosal Recovery in Children with Celiac Disease on a Gluten-Free Diet? *Am J Gastroenterol*. 2014. doi:10.1038/ajg.2014.200.

169. Rubio-Tapia A, Hill ID, Kelly CP, Calderwood AH, Murray JA, American College of Gastroenterology. ACG clinical guidelines: diagnosis and management of celiac disease. *Am J Gastroenterol*. 2013;108(5):656–76– quiz 677. doi:10.1038/ajg.2013.79.

170. Schnitzler F, Fidder H, Ferrante M, et al. Mucosal healing predicts long-term outcome of maintenance therapy with infliximab in Crohn's disease. *Inflamm Bowel Dis*. 2009;15(9):1295–1301. doi:10.1002/ibd.20927.

171. Rutgeerts P, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Endoscopy*. 1990;99(4):956–963.

172. Ardizzone S, Bollani S, Manzionna G, Imbesi V. Comparison between methotrexate and

azathioprine in the treatment of chronic active Crohn's disease: a randomised, investigator-blind study. *Digestive and Liver Disease*. 2003. doi:10.1016/S1590-8658(03)00372-4.

173. D'Haens G, Geboes K, Rutgeerts P. Endoscopic and histologic healing of Crohn's (ileo-) colitis with azathioprine. *Gastrointest Endosc*. 1999;50(5):667–671. doi:10.1016/S0016-5107(99)80017-0.

174. Clark M, Colombel JF, Feagan BC, et al. American gastroenterological association consensus development conference on the use of biologics in the treatment of inflammatory bowel disease, June 21-23, 2006. In: Vol 133. 2007:312–339. doi:10.1053/j.gastro.2007.05.006.

175. Summers RW, Switz DM, Sessions JT, et al. National Cooperative Crohn's Disease Study: results of drug treatment. *Endoscopy*. 1979;77(4 Pt 2):847–869.

176. Modigliani R, Mary JY, Simon JF, et al. Clinical, biological, and endoscopic picture of attacks of Crohn“s disease. Evolution on prednisolone. Groupe d”Etude Thérapeutique des Affections Inflammatoires Digestives. *Endoscopy*. 1990;98(4):811–818. doi:10.1016/0016-5085(90)90002-I.

177. Kaukinen K, Peräaho M, Lindfors K, et al. Persistent small bowel mucosal villous atrophy without symptoms in coeliac disease. *Alimentary Pharmacology & Therapeutics*. 2007;25(10):1237–1245. doi:10.1111/j.1365-2036.2007.03311.x.

178. Lebwohl B, Granath F, Ekbom A, et al. Mucosal healing and mortality in coeliac disease. *Alimentary Pharmacology & Therapeutics*. 2013;37(3):332–339. doi:10.1111/apt.12164.

179. Lebwohl B, Granath F, Ekbom A, et al. Mucosal healing and risk for lymphoproliferative malignancy in celiac disease: a population-based cohort study. *Ann Intern Med*. 2013;159(3):169–175. doi:10.7326/0003-4819-159-3-201308060-00006.

180. Wahab PJ, Meijer JWR, Mulder CJJ. Histologic follow-up of people with celiac disease on a gluten-free diet: slow and incomplete recovery. *AJCP*. 2002;118(3):459–463. doi:10.1309/EVXT-851X-WHLC-RLX9.

181. Lebwohl B, Murray JA, Rubio-Tapia A, Green PHR, Ludvigsson JF. Predictors of persistent villous atrophy in coeliac disease: a population-based study. *Alimentary Pharmacology & Therapeutics*. 2014;39(5):488–495. doi:10.1111/apt.12621.

182. Kumar PJ, Walker-Smith J, Milla P, Harris G, Colyer J, Halliday R. The teenage coeliac: follow up study of 102 patients. *Archives of Disease in Childhood*. 1988;63(8):916–920.

183. Grefte JM, Bouman JG, Grond J, Jansen W, Kleibeuker JH. Slow and incomplete histological and functional recovery in adult gluten sensitive enteropathy. 1988;41(8):886–891.

184. Tursi A, Brandimarte G, Giorgetti G, et al. Endoscopic and histological findings in the duodenum of adults with celiac disease before and after changing to a gluten-free diet: a 2-year prospective study. *Endoscopy*. 2006;38(7):702–707. doi:10.1055/s-2006-925178.

185. Bardella MT, Velio P, Cesana BM, et al. Coeliac disease: a histological follow-up study. *Histopathology*. 2007;50(4):465–471. doi:10.1111/j.1365-2559.2007.02621.x.

186. Carroccio A, Ambrosiano G, Di Prima L, et al. Clinical symptoms in celiac patients on a gluten-free diet. *Scand J Gastroenterol*. 2008;43(11):1315–1321. doi:10.1080/00365520802200044.

187. Meyer D, Stavropolous S, Diamond B, Shane E, Green PHR. Osteoporosis in a north american adult population with celiac disease. *Am J Gastroenterol*. 2001;96(1):112–119. doi:10.1111/j.1572-0241.2001.03507.x.

188. Valdimarsson T, Löfman O, Toss G, Ström M. Reversal of osteopenia with diet in adult coeliac disease. *Gut*. 1996;38(3):322–327.

189. Sategna-Guidetti C, Grosso SB, Grosso S, et al. The effects of 1-year gluten withdrawal on bone mass, bone metabolism and nutritional status in newly-diagnosed adult coeliac disease patients. *Alimentary Pharmacology & Therapeutics*. 2000;14(1):35–43. doi:10.1046/j.1365-2036.2000.00671.x.

190. Larussa T. No evidence of circulating autoantibodies against osteoprotegerin in patients with celiac disease. *WJG*. 2012;18(14):1622. doi:10.3748/wjg.v18.i14.1622.

191. Mazure R, Vazquez H, Gonzalez D, et al. Bone mineral affection in asymptomatic adult patients with celiac disease. *Am J Gastroenterol*. 1994;89(12):2130–2134.

192. Duerksen DR, Leslie WD. Positive celiac disease serology and reduced bone mineral density in adult women. *Can J Gastroenterol*. 2010;24(2):103–107.

193. Heikkilä K, Heliövaara M, Impivaara O, et al. Coeliac Disease Autoimmunity and Hip Fracture Risk: Findings from a Prospective Cohort Study. *J Bone Miner Res*. 2014. doi:10.1002/jbmr.2380.

194. Casella S, Zanini B, Lanzarotto F, Villanacci V, Ricci C, Lanzini A. Celiac disease in elderly adults: clinical, serological, and histological characteristics and the effect of a gluten-free diet. *J Am Geriatr Soc*. 2012;60(6):1064–1069. doi:10.1111/j.1532-5415.2012.03997.x.

195. McFarlane XA, Bhalla AK, Robertson DA. Effect of a gluten free diet on osteopenia in adults with newly diagnosed coeliac disease. *Gut*. 1996;39(2):180–184.

196. Corazza GR, Di Sario A, 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(6):525–530.

197. Mora S, Barera G, Ricotti A, Weber G, Bianchi C, Chiumello G. Reversal of low bone density with a gluten-free diet in children and adolescents with celiac disease. *Am J Clin Nutr*. 1998;67(3):477–481.

198. Kemppainen T, Kröger H, Janatuinen E, et al. Bone recovery after a gluten-free diet: a 5-year follow-up study. *Bone*. 1999;25(3):355–360.

199. Mautalen C, Gonzalez D, Mazure R, et al. Effect of treatment on bone mass, mineral

metabolism, and body composition in untreated celiac disease patients. *Am J Gastroenterol*. 1997;92(2):313–318.

200. Molteni N, Bardella MT, Vezzoli G, Pozzoli E, Bianchi P. Intestinal calcium absorption as shown by stable strontium test in celiac disease before and after gluten-free diet. *Am J Gastroenterol*. 1995;90(11):2025–2028.

201. T Valdimarsson G Toss O Löfman. Three Years' Follow-up of Bone Density in Adult Coeliac Disease: Significance of Secondary Hyperparathyroidism. *Scand J Gastroenterol*. 2000;35(3):274–280. doi:10.1080/003655200750024146.

202. Fornari MC, Pedreira S, Niveloni S, et al. Pre- and Post-Treatment Serum Levels of Cytokines IL-1beta, IL-6, and IL-1 Receptor Antagonist in Celiac Disease. Are They Related to the Associated Osteopenia? *Am J Gastroenterol*. 1998;93(3):413–418. doi:10.1111/j.1572-0241.1998.00413.x.

203. Bai JC, Gonzalez D, Mautalen C, et al. Long-term effect of gluten restriction on bone mineral density of patients with coeliac disease. *Alimentary Pharmacology & Therapeutics*. 1997;11(1):157–164.

204. García-Manzanares Á, Tenias JM, Lucendo AJ. Bone mineral density directly correlates with duodenal Marsh stage in newly diagnosed adult celiac patients. *Scand J Gastroenterol*. 2012:1–10. doi:10.3109/00365521.2012.688217.

205. Bode S, Hassager C, Gudmand-Hoyer E, Christiansen C. Body composition and calcium metabolism in adult treated coeliac disease. *Gut*. 1991;32(11):1342–1345.

206. Smecuol E, Gonzalez D, Mautalen C, et al. Longitudinal study on the effect of treatment on body composition and anthropometry of celiac disease patients. *Am J Gastroenterol*. 1997;92(4):639–643.

207. Mazure RM, Vazquez H, Gonzalez D, et al. Early changes of body composition in asymptomatic celiac disease patients. *Am J Gastroenterol*. 1996;91(4):726–730.

208. Kabbani TA, Goldberg A, Kelly CP, et al. Body mass index and the risk of obesity in coeliac disease treated with the gluten-free diet. *Alimentary Pharmacology & Therapeutics*. 2012;35(6):723–729. doi:10.1111/j.1365-2036.2012.05001.x.

209. Capristo E, Addolorato G, Mingrone G, et al. Changes in body composition, substrate oxidation, and resting metabolic rate in adult celiac disease patients after a 1-y gluten-free diet treatment. *Am J Clin Nutr*. 2000;72(1):76–81.

210. Cheng J, Brar PS, Lee AR, Green PHR. Body mass index in celiac disease: beneficial effect of a gluten-free diet. *J Clin Gastroenterol*. 2010;44(4):267–271. doi:10.1097/MCG.0b013e3181b7ed58.

211. Peracchi M, Conte D, Terrani C, et al. Circulating ghrelin levels in celiac patients. *Am J Gastroenterol*. 2003;98(11):2474–2478. doi:10.1111/j.1572-0241.2003.07709.x.

212. Thomas HJ, Ahmad T, Rajaguru C, Barnardo M, Warren BF, Jewell DP. Contribution of histological, serological, and genetic factors to the clinical heterogeneity of adult-onset coeliac disease. *Scand J Gastroenterol*. 2009;44(9):1076–1083.

doi:10.1080/00365520903100473.

213. Biagi F, Bianchi PI, Vattiato C, et al. Influence of HLA-DQ2 and DQ8 on severity in celiac Disease. *J Clin Gastroenterol*. 2012;46(1):46–50. doi:10.1097/MCG.0b013e318221077e.

214. Haines ML, Anderson RP, Gibson PR. Systematic review: The evidence base for long-term management of coeliac disease. *Alimentary Pharmacology & Therapeutics*. 2008;28(9):1042–1066. doi:10.1111/j.1365-2036.2008.03820.x.

215. Zakzanis K. Distinct Neurocognitive Profiles in Multiple Sclerosis Subtypes. *Archives of Clinical Neuropsychology*. 2000;15(2):115–136. doi:10.1016/S0887-6177(98)00157-7.

216. Carbotte RM, Denburg SD, Denburg JA. Cognitive dysfunction in systemic lupus erythematosus is independent of active disease. *J Rheumatol*. 1995;22(5):863–867.

217. Attree EA, Dancey CP, Keeling D, Wilson C. Cognitive function in people with chronic illness: inflammatory bowel disease and irritable bowel syndrome. *Appl Neuropsychol*. 2003;10(2):96–104. doi:10.1207/S15324826AN1002_05.

218. van Langenberg DR, Gibson PR. Factors associated with physical and cognitive fatigue in patients with Crohn's disease: a cross-sectional and longitudinal study. *Inflamm Bowel Dis*. 2014;20(1):115–125. doi:10.1097/01.MIB.0000437614.91258.70.

219. Hu WT, Murray JA, Greenaway MC, Parisi JE, Josephs KA. Cognitive impairment and celiac disease. *Arch Neurol*. 2006;63(10):1440–1446. doi:10.1001/archneur.63.10.1440.

220. Currie S, Hadjivassiliou M, Clark MJ, et al. Should we be “nervous” about coeliac disease? Brain abnormalities in patients with coeliac disease referred for neurological opinion. *Journal of Neurology, Neurosurgery & Psychiatry*. 2012. doi:10.1136/jnnp-2012-303281.

221. Zelnik N, Pacht A, Obeid R, Lerner A. Range of Neurologic Disorders in Patients With Celiac Disease. *PEDIATRICS*. 2004;113(6):1672–1676.

222. Sapone A, Bai J, Ciacci C, et al. Spectrum of gluten-related disorders: consensus on new nomenclature and classification. *BMC Medicine*. 2012;10(1):13. doi:10.1186/1741-7015-10-13.

223. Hadjivassiliou M, Davies-Jones GAB, Sanders DS, Grünewald RA. Dietary treatment of gluten ataxia. *Journal of Neurology, Neurosurgery & Psychiatry*. 2003;74(9):1221–1224.

224. Hadjivassiliou M, Aeschlimann P, Sanders DS, et al. Transglutaminase 6 antibodies in the diagnosis of gluten ataxia. *Neurology*. 2013;80(19):1740–1745. doi:10.1212/WNL.0b013e3182919070.

225. Anderson J. Gluten and Brain Fog. *celiacdiseaseaboutcom*. Available at: http://celiacdisease.about.com/od/symptomsofceliacdisease/a/Celiac-Disease-Brain-Fog.htm. Accessed August 12, 2014.

226. Bürk K, Bösch S, Müller CA, Melms A, Zühlke C. Sporadic cerebellar ataxia associated with gluten sensitivity. *Brain*. 2001.

227. Lurie Y, Landau DA, Pfeffer J. Celiac disease diagnosed in the elderly. *J Clin Gastroenterol*. 2008.

228. Hagander B, Berg NO, Brandt L, Norden A, Sjolund K, Stenstam M. Hepatic injury in adult coeliac disease. *Lancet*. 1977;2(8032):270–272.

229. Bardella MT, Fraquelli M, Quatrini M, Molteni N, Bianchi P, Conte D. Prevalence of hypertransaminasemia in adult celiac patients and effect of gluten-free diet. *Hepatology*. 1995;22(3):833–836.

230. Alavi Moghaddam M, Rostami Nejad M, Shalmani HM, et al. The effects of gluten-free diet on hypertransaminasemia in patients with celiac disease. *Int J Prev Med*. 2013;4(6):700–704.

231. Korpimäki S, Kaukinen K, Collin P, et al. Gluten-Sensitive Hypertransaminasemia in Celiac Disease: An Infrequent and Often Subclinical Finding. *Am J Gastroenterol*. 2011;106(9):1689–1696. doi:10.1038/ajg.2011.134.

232. Volta U, Rodrigo L, Granito A, et al. Celiac disease in autoimmune cholestatic liver disorders. *Am J Gastroenterol*. 2002;97(10):2609–2613. doi:10.1111/j.1572-0241.2002.06031.x.

233. Bardella MT, Vecchi M, Conte D, et al. Chronic unexplained hypertransaminasemia may be caused by occult celiac disease. *Hepatology*. 1999;29(3):654–657. doi:10.1002/hep.510290318.

234. Soresi M, Amplo M, Agliastro R, Sesti R, Di Giovanni G. Screening for autoantibodies to tissue transglutaminase reveals a low prevalence of celiac disease in blood donors with cryptogenic hypertransaminasemia. *Digestion*. 2001.

235. Kaukinen K, Halme L, Collin P, et al. Celiac disease in patients with severe liver disease: Gluten-free diet may reverse hepatic failure. *Gastroenterology*. 2002;122(4):881–888.

236. Pazo M, Abdulkader I, Otero-Anton E. [Liver abnormalities in adult celiac disease. Clinicopathologic characterization and outcome after therapy]. *Gastroenterologia y Hepatologia*. 2005;29(7):383–389. doi:10.1157/13091450.

237. Kingham JG, Parker DR. The association between primary biliary cirrhosis and coeliac disease: a study of relative prevalences. *Gut*. 1998;42(1):120–122.

238. Mounajjed T, Oxentenko A, Shmidt E, Smyrk T. The liver in celiac disease: clinical manifestations, histologic features, and response to gluten-free diet in 30 patients. *Am J Clin Pathol*. 2011;136(1):128–137. doi:10.1309/AJCPDOMY5RI5TPMN.

239. Volta U, De Franceschi L, Molinaro N, et al. Frequency and significance of anti-gliadin and anti-endomysial antibodies in autoimmune hepatitis. *Dig Dis Sci*. 1998;43(10):2190–2195. doi:10.1023/A:1026650118759.

240. Selcuk H, Kanbay M, Murat K, Yilmaz U. Liver Dysfunction After a Gluten-Free Diet in a Patient with Celiac Disease: A New Link? *Dig Dis Sci*. 2006;51(1):213–214. doi:10.1007/s10620-006-3110-6.

241. Di Biase AR, Colecchia A, Scaioli E, et al. Autoimmune liver diseases in a paediatric population with coeliac disease - a 10-year single-centre experience. *Alimentary Pharmacology & Therapeutics*. 2010;31(2):253–260. doi:10.1111/j.1365-2036.2009.04186.x.

242. Hsieh MM, Everhart JE, Byrd-Holt DD. Prevalence of neutropenia in the US population: age, sex, smoking status, and ethnic differences. *Ann Intern Med*. 2007.

243. Fisgin T, Yarali N, Duru F, Usta B, Kara A. Hematologic manifestation of childhood celiac disease. *Acta Haematol*. 2004;111(4):211–214. doi:10.1159/000077568.

244. Brandt L, Stenstam M. Letter: Subnormal lymphocyte-counts in adult coeliac disease. *Lancet*. 1975;1(7913):978–979.

245. Altintas A, Pasa S, Cil T, Bayan K, Gokalp D, Ayyildiz O. Thyroid and celiac diseases autoantibodies in patients with adult chronic idiopathic thrombocytopenic purpura. *Platelets*. 2008;19(4):252–257. doi:10.1080/09537100801894651.

246. Olén O, MONTGOMERY SM, Elinder G, Ekbom A, Ludvigsson JF. Increased risk of immune thrombocytopenic purpura among inpatients with coeliac disease. *Scand J Gastroenterol*. 2008;43(4):416–422. doi:10.1080/00365520701814028.

247. Ludvigsson JF, Welander A, Lassila R, Ekbom A, MONTGOMERY SM. Risk of thromboembolism in 14,000 individuals with coeliac disease. *Br J Haematol*. 2007;139(1):121–127. doi:10.1111/j.1365-2141.2007.06766.x.

248. Kallel L, Matri S, Karoui S, Fekih M, Boubaker J, Filali A. Deep Venous Thrombosis Related to Protein S Deficiency Revealing Celiac Disease. *Am J Gast*. 2009;104(1):256–257. doi:10.1038/ajg.2008.48.

249. Beyan E, Pamukcuoglu M, Beyan C. Deep vein thrombosis associated with celiac disease. *Bratisl Lek Listy*. 2009.

250. Thomas HJ, Wotton CJ, Yeates D, Ahmad T, Jewell DP, Goldacre MJ. Pneumococcal infection in patients with coeliac disease. *Eur J Gastroenterol Hepatol*. 2008;20(7):624–628. doi:10.1097/MEG.0b013e3282f45764.

251. Ludvigsson JF, Olén O, Bell M, Ekbom A, MONTGOMERY SM. Coeliac disease and risk of sepsis. *Gut*. 2008;57(8):1074–1080. doi:10.1136/gut.2007.133868.

252. Ludvigsson JF, Wahlstrom J, Grunewald J, Ekbom A, MONTGOMERY SM. Coeliac disease and risk of tuberculosis: a population based cohort study.[see comment]. *Thorax*. 2007;62(1):23–28. doi:10.1136/thx.2006.059451.

253. Kruetzmann S, Rosado MM, Weber H. Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. *J Exp Med*. 2003.

254. Marsh GW, Stewart JS. Splenic function in adult coeliac disease. *Br J Haematol*. 1970;19(4):445–457.

255. Di Sabatino A, Rosado MM, Cazzola P, et al. Splenic hypofunction and the spectrum of

autoimmune and malignant complications in celiac disease. *Clinical Gastroenterology and Hepatology*. 2006;4(2):179–186.

256. Freeman HJ, Chiu BK. Small bowel malignant lymphoma complicating celiac sprue and the mesenteric lymph node cavitation syndrome. *Endoscopy*. 1986;90(6):2008–2012.

257. Brown IS, Smith J, Rosty C. Gastrointestinal pathology in celiac disease: a case series of 150 consecutive newly diagnosed patients. *Am J Clin Pathol*. 2012;138(1):42–49. doi:10.1309/AJCPE89ZPVJTSPWL.

258. Brigden M, Graydon C. Eosinophilia detected by automated blood cell counting in ambulatory North American outpatients. Incidence and clinical significance. *Arch Pathol Lab Med*. 1997;121(9):963–967.

259. Lémann M, Mary J-Y, Colombel JF, et al. A randomized, double-blind, controlled withdrawal trial in Crohn's disease patients in long-term remission on azathioprine. *Gastroenterology*. 2005;128(7):1812–1818.

260. Mary JY, Modigliani R. Development and validation of an endoscopic index of the severity for Crohn“s disease: a prospective multicentre study. Groupe d”Etudes Thérapeutiques des Affections Inflammatoires du Tube Digestif (GETAID). *Gut*. 1989;30(7):983–989.

261. Daperno M, D'Haens G, van Assche G, et al. Development and validation of a new, simplified endoscopic activity score for Crohn's disease: the SES-CD. *Gastrointest. Endoscop*. 2004;60(4):505–512. doi:10.1016/S0016-5107(04)01878-4.

262. Fagan EA, Dyck RF, Maton PN. Serum levels of C-reactive protein in Crohn's disease and ulcerative colitis. *European journal of clinical investigation*. 1982. doi:10.1111/j.1365-2362.1982.tb02244.x.

263. Solem CA, Loftus EV, Tremaine WJ, Harmsen WS, Zinsmeister AR, Sandborn WJ. Correlation of C-reactive protein with clinical, endoscopic, histologic, and radiographic activity in inflammatory bowel disease. *Inflamm Bowel Dis*. 2005;11(8):707–712. doi:10.1097/01.MIB.0000173271.18319.53/asset/3780110801_ftp.pdf.

264. Rutgeerts P, De Vos M, Van Gossum A. A positive response to infliximab in Crohn disease: association with a higher systemic inflammation before treatment but not with-308 TNF gene polymorphism. *Scand. J. Gastroenterol*. 2002; 37(7):818-24.

265. Consigny Y, Modigliani R, Colombel JF, et al. A simple biological score for predicting low risk of short-term relapse in Crohn's disease. *Inflamm Bowel Dis*. 2006;12(7):551–557. doi:10.1097/01.ibd.0000225334.60990.5b.

266. Langhorst J, Elsenbruch S, Koelzer J, Rueffer A, Michalsen A, Dobos GJ. Noninvasive markers in the assessment of intestinal inflammation in inflammatory bowel diseases: performance of fecal lactoferrin, calprotectin, and PMN-elastase, CRP, and clinical indices. *Am J Gastroenterol*. 2008;103(1):162–169. doi:10.1111/j.1572-0241.2007.01556.x.

267. Canani RB, Terrin G, Rapacciuolo L, et al. Faecal calprotectin as reliable non-invasive

marker to assess the severity of mucosal inflammation in children with inflammatory bowel disease. *Dig Liver Dis*. 2008;40(7):547–553. doi:10.1016/j.dld.2008.01.017.

268. Roseth AG, Aadland E, Grzyb K. Normalization of faecal calprotectin: a predictor of mucosal healing in patients with inflammatory bowel disease. *Scand J Gastroenterol*. 2004;39(10):1017–1020. doi:10.1080/00365520410007971.

269. D'Incà R, Dal Pont E, Di Leo V, et al. Can calprotectin predict relapse risk in inflammatory bowel disease? *Am J Gastroenterol*. 2008;103(8):2007–2014.

270. Tibble JA, Sigthorsson G, Bridger S, Fagerhol MK, Bjarnason I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. *Endoscopy*. 2000;119(1):15–22.

271. Ertekin V, Selimoğlu MA, Turgut A, Bakan N. Fecal calprotectin concentration in celiac disease. *J Clin Gastroenterol*. 2010;44(8):544–546. doi:10.1097/MCG.0b013e3181cadbc0.

272. Montalto M, Santoro L, Curigliano V, et al. Faecal calprotectin concentrations in untreated coeliac patients. *Scand J Gastroenterol*. 2007;42(8):957–961. doi:10.1080/00365520601173632.

273. Wang W, Uzzau S, Goldblum SE, Fasano A. Human zonulin, a potential modulator of intestinal tight junctions. *J Cell Sci*. 2000;113 Pt 24:4435–4440.

274. Bruewer M, Luegering A, Kucharzik T, et al. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. *J Immunol*. 2003;171(11):6164–6172. doi:10.4049/jimmunol.171.11.6164.

275. Travis S, Menzies I. Intestinal permeability: functional assessment and significance. *Clin Sci*. 1992;82(5):471–488.

276. Fihn BM, Sjoqvist A, Jodal M. Permeability of the rat small intestinal epithelium along the villus-crypt axis: effects of glucose transport. *Endoscopy*. 2000;119(4):1029–1036. doi:10.1053/gast.2000.18148.

277. Holmes JL, Van Itallie CM, Rasmussen JE, Anderson JM. Claudin profiling in the mouse during postnatal intestinal development and along the gastrointestinal tract reveals complex expression patterns. *Gene Expr Patterns*. 2006;6(6):581–588. doi:10.1016/j.modgep.2005.12.001.

278. Berglund JJ, Riegler M, Zolotarevsky Y, Wenzl E, Turner JR. Regulation of human jejunal transmucosal resistance and MLC phosphorylation by Na(+)-glucose cotransport. *Am J Physiol Gastrointest Liver Physiol*. 2001;281(6):G1487–93.

279. Arrieta MC, Bistritz L, Meddings JB. Alterations in intestinal permeability. *Gut*. 2006;55(10):1512–1520. doi:10.1136/gut.2005.085373.

280. Laker MF, Menzies IS. Increase in human intestinal permeability following ingestion of hypertonic solutions. *J Physiol (Lond)*. 1977;265(3):881–894.

281. Hamilton I, Cobden I, Rothwell J, Axon AT. Intestinal permeability in coeliac disease: the

response to gluten withdrawal and single-dose gluten challenge. *Gut*. 1982;23(3):202–210.

282. Cox MA, Lewis KO, Cooper BT. Measurement of Small Intestinal Permeability Markers, Lactulose, and Mannitol in Serum (Results in Celiac Disease). *Dig Dis Sci*. 1999. doi:10.1023/A:1026679123148.

283. Menzies IS. *Absorption of Intact Oligosaccharide in Health and Disease*. Biochemical Society Transactions; 1974. doi:10.1042/bst0021042.

284. Bjarnason I, Maxton D, Reynolds AP, Catt S, Peters TJ, Menzies IS. Comparison of four markers of intestinal permeability in control subjects and patients with coeliac disease. *Scand J Gastroenterol*. 1994;29(7):630–639. doi:10.3109/00365529409092484.

285. Bjarnason I, Macpherson A, Hollander D. Intestinal permeability: an overview. *Endoscopy*. 1995.

286. Vazquez-Roque MI, Camilleri M, Smyrk T, et al. A Controlled Trial of Gluten-Free Diet in Patients with Irritable Bowel Syndrome-Diarrhea: Effects on Bowel Frequency and Intestinal Function. *Gastroenterology*. 2013;144(5):903–911.e3. doi:10.1053/j.gastro.2013.01.049.

287. Cobden I, Hamilton I, Rothwell J, Axon AT. Cellobiose/mannitol test: physiological properties of probe molecules and influence of extraneous factors. *Clin Chim Acta*. 1985;148(1):53–62.

288. Zhou Q, Souba WW, Croce CM, Verne GN. MicroRNA-29a regulates intestinal membrane permeability in patients with irritable bowel syndrome. *Gut*. 2010;59(6):775–784. doi:10.1136/gut.2009.181834.

289. Wyatt J, Vogelsang H, Hubl W, Waldhoer T, Lochs H. Intestinal permeability and the prediction of relapse in Crohri's disease. *Lancet*. 1993;341(8858):1437–1439.

290. D'Incà R, Leo V, Corrao G, et al. Intestinal permeability test as a predictor of clinical course in Crohn's disease. *Am J Gastroenterol*. 1999;94(10):2956–2960. doi:10.1111/j.1572-0241.1999.01444.x.

291. Irvine EJ, Marshall JK. *Increased Intestinal Permeability Precedes the Onset of Crohn's Disease in a Subject with Familial Risk*. Gastroenterology; 2000.

292. Peeters M, Geypens B, Claus D, et al. Clustering of increased small intestinal permeability in families with Crohn's disease. *Endoscopy*. 1997;113(3):802–807.

293. Dunlop SP, Hebden J, Campbell E, et al. Abnormal intestinal permeability in subgroups of diarrhea-predominant irritable bowel syndromes. *Am J Gastroenterol*. 2006;101(6):1288–1294. doi:10.1111/j.1572-0241.2006.00672.x.

294. Mujagic Z, Ludidi S, Keszthelyi D, et al. Small intestinal permeability is increased in diarrhoea predominant IBS, while alterations in gastroduodenal permeability in all IBS subtypes are largely attributable to confounders. *Alimentary Pharmacology & Therapeutics*. 2014;40(3):288–297. doi:10.1111/apt.12829.

295. Hollander D, VADHEIM CM. Increased intestinal permeability in patients with Crohn's disease and their relativesA possible etiologic factor. *Ann Intern Med*. 1986. doi:10.7326/0003-4819-105-6-883.

296. Feld JJ, Meddings J, Heathcote EJ. Abnormal Intestinal Permeability in Primary Biliary Cirrhosis. *Dig Dis Sci*. 2006;51(9):1607–1613. doi:10.1007/s10620-006-9544-z.

297. de Magistris L, Familiari V, Pascotto A, et al. Alterations of the Intestinal Barrier in Patients With Autism Spectrum Disorders and in Their First-degree Relatives. *Journal of Pediatric Gastroenterology and Nutrition*. 2010;51(4):418–424. doi:10.1097/MPG.0b013e3181dcc4a5.

298. Wood NC, Hamilton I, Axon AT, et al. Abnormal intestinal permeability. An aetiological factor in chronic psychiatric disorders? *BJP*. 1987;150(6):853–856. doi:10.1192/bjp.150.6.853.

299. Maes M, Kubera M, Leunis J-C. The gut-brain barrier in major depression: intestinal mucosal dysfunction with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory pathophysiology of depression. *Neuro Endocrinol Lett*. 2008;29(1):117–124.

300. Sapone A, de Magistris L, Pietzak M, et al. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. *Diabetes*. 2006;55(5):1443–1449. doi:10.2337/db05-1593.

301. Schulzke JD, Bentzel CJ, Schulzke I, Riecken EO, Fromm M. Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. *Pediatr Res*. 1998;43(4 Pt 1):435–441.

302. Sander GR, Cummins AG, Henshall T, Powell BC. Rapid disruption of intestinal barrier function by gliadin involves altered expression of apical junctional proteins. *FEBS Lett*. 2005;579(21):4851–4855. doi:10.1016/j.febslet.2005.07.066.

303. Bjarnason I, Peters TJ, Veall N. A persistent defect in intestinal permeability in coeliac disease demonstrated by a 51Cr-labelled EDTA absorption test. *Lancet*. 1983;1(8320):323–325.

304. Wilson JP. Surface area of the small intestine in man. *Gut*. 1967;8(6):618–621.

305. Brockow K, Kneissl D, Valentini L, et al. Using a gluten oral food challenge protocol to improve diagnosis of wheat-dependent exercise-induced anaphylaxis. *Journal of Allergy and Clinical Immunology*. 2014. doi:10.1016/j.jaci.2014.08.024.

306. Schumann M, Richter JF, Wedell I, et al. Mechanisms of epithelial translocation of the {alpha}2-gliadin-33mer in coeliac sprue. *Gut*. 2008;57(6):747–754. doi:10.1136/gut.2007.136366.

307. Smecuol E, Bai JC, Vazquez H, et al. Gastrointestinal permeability in celiac disease. *Gastroenterology*. 1997;112(4):1129–1136.

308. Kelly CP, Green PHR, Murray JA, et al. Larazotide acetate in patients with coeliac disease undergoing a gluten challenge: a randomised placebo-controlled study. *Alimentary*

Pharmacology & Therapeutics. 2012;37(2):252–262. doi:10.1111/apt.12147.

309. van Elburg RM, Uil JJ, Kokke FT, et al. Repeatability of the sugar-absorption test, using lactulose and mannitol, for measuring intestinal permeability for sugars. *Journal of Pediatric Gastroenterology and Nutrition*. 1995;20(2):184–188.

310. Cobden I, Rothwell J, Axon AT. Intestinal permeability and screening tests for coeliac disease. *Gut*. 1980;21(6):512–518. doi:10.1136/gut.21.6.512.

311. Sequeira IR, Lentle RG, Kruger MC, Hurst RD. Standardising the Lactulose Mannitol Test of Gut Permeability to Minimise Error and Promote Comparability. Hogan SP, ed. *PLoS ONE*. 2014;9(6):e99256. doi:10.1371/journal.pone.0099256.

312. Strobel S, Brydon WG, Ferguson A. Cellobiose/mannitol sugar permeability test complements biopsy histopathology in clinical investigation of the jejunum. *Gut*. 1984;25(11):1241–1246.

313. Juby LD, Rothwell J, Axon AT. Cellobiose/mannitol sugar test--a sensitive tubeless test for coeliac disease: results on 1010 unselected patients. *Gut*. 1989;30(4):476–480.

314. Juby LD, Rothwell J, Axon AT. Lactulose/mannitol test: an ideal screen for celiac disease. *Gastroenterology*. 1989;96(1):79–85. doi:10.5555/uri:pii:0016508589907671.

315. Greco L, D'Adamo G, Truscelli A, Parrilli G, Mayer M, Budillon G. Intestinal permeability after single dose gluten challenge in coeliac disease. *Archives of Disease in Childhood*. 1991;66(7):870–872.

316. Fernández-Calle P, Codoceo R, Polanco I, Gómez-Cerezo J, Orsi M, Tenias JM. Is an intestinal permeability test a valid marker for slight dietary transgressions in adolescents with coeliac disease? *Gut*. 1993;34(6):774–777.

317. Vogelsang H, Wyatt J, Penner E, Lochs H. Screening for celiac disease in first-degree relatives of patients with celiac disease by lactulose/mannitol test. *Am J Gastroenterol*. 1995;90(10):1838–1842.

318. Smecuol E, Bai JC, Vazquez H, Kogan Z. Gastrointestinal permeability in celiac disease. *112*(4), 1129–1136. *Gastroenterology*. 1997;112(4):1129–1136.

319. Smecuol E, Vazquez H, Sugai E, et al. Sugar tests detect celiac disease among first-degree relatives. *Am J Gastroenterol*. 1999;94(12):3547–3552. doi:10.1111/j.1572-0241.1999.01645.x.

320. Cummins AG, Thompson FM, Butler RN, et al. Improvement in intestinal permeability precedes morphometric recovery of the small intestine in coeliac disease. *Clin Sci*. 2001;100(4):379–386.

321. Abazia C, Ferrara R, Corsaro MM, Barone G, Coccoli P, Parrilli G. Simultaneous gas-chromatographic measurement of rhamnose, lactulose and sucrose and their application in the testing gastrointestinal permeability. *Clin Chim Acta*. 2003;338(1-2):25–32.

322. Duerksen DR, Wilhelm-Boyles C, Parry DM. Intestinal permeability in long-term follow-up of patients with celiac disease on a gluten-free diet. *Dig Dis Sci*. 2005;50(4):785–790.

323. Vilela EG, de Abreu Ferrari M de L, de Gama Torres HO, et al. Intestinal permeability and antigliadin antibody test for monitoring adult patients with celiac disease. *Dig Dis Sci*. 2007;52(5):1304–1309. doi:10.1007/s10620-006-9511-8.

324. Di Sabatino A, Ciccocioppo R, Cupelli F, et al. Epithelium derived interleukin 15 regulates intraepithelial lymphocyte Th1 cytokine production, cytotoxicity, and survival in coeliac disease. *Gut*. 2006;55(4):469–477. doi:10.1136/gut.2005.068684.

325. O'Mahony L, McCarthy J, Kelly P, et al. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology*. 2005;128(3):541–551.

326. Lahat N, Shapiro S, Karban A. Cytokine profile in coeliac disease. *Scand J Immunol*. 1999. doi:10.1046/j.1365-3083.1999.00523.x.

327. Allen ML, Hoschtitzky JA, Peters MJ, et al. Interleukin-10 and its role in clinical immunoparalysis following pediatric cardiac surgery. *Crit Care Med*. 2006;34(10):2658–2665. doi:10.1097/01.CCM.0000240243.28129.36.

328. Heagy W, Nieman K, Hansen C, Cohen M, Danielson D, West MA. Lower Levels of Whole Blood LPS-Stimulated Cytokine Release Are Associated with Poorer Clinical Outcomes in Surgical ICU Patients. *Surgical Infections*. 2003;4(2):171–180. doi:10.1089/109629603766956960.

329. Liebregts T, Adam B, Bredack C, et al. Immune activation in patients with irritable bowel syndrome. *Endoscopy*. 2007;132(3):913–920. doi:10.1053/j.gastro.2007.01.046.

330. Manavalan JS, Hernandez L, Shah JG, et al. Serum cytokine elevations in celiac disease: association with disease presentation. *Human Immunology*. 2010;71(1):50–57. doi:10.1016/j.humimm.2009.09.351.

331. Lettesjo H, Hansson T, Bergqvist A, Gronlund J, Dannaeus A. Enhanced interleukin-18 levels in the peripheral blood of children with coeliac disease - Lettesjö - 2004 - Clinical & Experimental Immunology - Wiley Online Library. *Clinical & Experimental Immunology*. 2005;139(1):138–143. doi:10.1111/j.1365-2249.2005.02661.x.

332. Romaldini CC, Barbieri D, Okay TS, Raiz R Jr, Cançado EL. Serum Soluble Interleukin-2 Receptor, Interleukin-6, and Tumor Necrosis Factor-[alpha] Levels in Children with Celiac Disease: Response to Treatment. *Journal of Pediatric Gastroenterology and Nutrition*. 2002;35(4):513–517.

333. Mizrachi A, Broide E, Buchs A, et al. Lack of correlation between disease activity and decreased stimulated secretion of IL-10 in lymphocytes from patients with celiac disease. *Scand J Gastroenterol*. 2002;37(8):924–930.

334. Tack GJ, van Wanrooij RL, Blomberg Von BM, et al. Serum parameters in the spectrum of coeliac disease: beyond standard antibody testing - a cohort study. *BMC Gastroenterol*. 2012;12(1):159. doi:10.1186/1471-230X-12-159.

335. Zhu S, Qian Y. IL-17/IL-17 receptor system in autoimmune disease: mechanisms and therapeutic potential. *Clin Sci*. 2012;122(11):487–511. doi:10.1042/CS20110496.

336. Hansson T, Dannaeus A, Klareskog L. Cytokine-producing cells in peripheral blood of children with coeliac disease secrete cytokines with a type 1 profile. *Clinical & Experimental Immunology*. 1999;116(2):246–250. doi:10.1046/j.1365-2249.1999.00882.x.

337. Anderson RP, van Heel DA, Tye-Din JA, et al. T cells in peripheral blood after gluten challenge in coeliac disease. *Gut*. 2005;54(9):1217–1223. doi:10.1136/gut.2004.059998.

338. Nilsen EM, Jahnsen FL, Lundin KE, et al. Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology*. 1998;115(3):551–563.

339. Fina D, Sarra M, Caruso R, et al. Interleukin 21 contributes to the mucosal T helper cell type 1 response in coeliac disease. *Gut*. 2008;57(7):887–892.

340. Gray GM. *Carbohydrate Digestion and Absorption*. *Role of the Small Intestine*. The New England journal of medicine; 1975:1225–1230. doi:10.1056/NEJM197506052922308.

341. Clarke JM, Pelton NC, Bajka BH. Use of the 13C-sucrose breath test to assess chemotherapy-induced small intestinal mucositis in the rat. *Cancer Biol Ther*. 2006;5(1):34–38. doi:10.4161/cbt.5.1.2235.

342. Tooley KL, Saxon BR, Webster J. Clinical Study A Novel Non-Invasive Biomarker for Assessment of Small Intestinal Mucositis in Children with Cancer Undergoing Chemotherapy. *Cancer biology & ...*. 2006.

343. Windmueller HG, Spaeth AE. Source and fate of circulating citrulline. *Am J Physiol*. 1981;241(6):E473–80.

344. Crenn P, Coudray-Lucas C, Thuillier F, Cynober L, Messing B. Postabsorptive Plasma Citrulline Concentration Is a Marker of Absorptive Enterocyte Mass and Intestinal Failure in Humans. *Gastroenterology*. 2000;119(6):1496–1505. doi:10.1053/gast.2000.20227.

345. Crenn P, De Truchis P, Neveux N, Galpérine T, Cynober L, Melchior JC. Plasma citrulline is a biomarker of enterocyte mass and an indicator of parenteral nutrition in HIV-infected patients. *American Journal of Clinical Nutrition*. 2009;90(3):587–594. doi:10.3945/ajcn.2009.27448.

346. Crenn P, Vahedi K, Lavergne-Slove A, Cynober L, Matuchansky C, Messing B. Plasma citrulline: A marker of enterocyte mass in villous atrophy-associated small bowel disease. *Gastroenterology*. 2003;124(5):1210–1219. doi:10.1016/S0016-5085(03)00170-7.

347. Ioannou HP, Fotoulaki M, Pavlitou A, Efstratiou I, Augoustides-Savvopoulou P. Plasma citrulline levels in paediatric patients with celiac disease and the effect of a gluten-free diet. *Eur J Gastroenterol Hepatol*. 2011;23(3):245–249. doi:10.1097/MEG.0b013e3283438ad7.

348. Peters JHC, Wierdsma NJ, Teerlink T, van Leeuwen PAM, Mulder CJJ, van Bodegraven AA. Poor diagnostic accuracy of a single fasting plasma citrulline concentration to assess intestinal energy absorption capacity. *Am J Gastroenterol*. 2007;102(12):2814–2819. doi:10.1111/j.1572-0241.2007.01513.x.

349. Peters J, Wierdsma NJ, Teerlink T, Van Leeuwen P, Mulder C, van Bodegraven AA. The citrulline generation test: proposal for a new enterocyte function test. *Alimentary Pharmacology & Therapeutics*. 2008;27(12):1300–1310.

350. 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):339–343. doi:10.1053/gast.1996.v110.pm8566578.

351. Derikx JPM, 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. doi:10.1371/journal.pone.0003954.

352. Adriaanse MPM, Tack GJ, Passos VL, et al. Serum I-FABP as marker for enterocyte damage in coeliac disease and its relation to villous atrophy and circulating autoantibodies. *Alimentary Pharmacology & Therapeutics*. 2013;37(4):482–490. doi:10.1111/apt.12194.

353. 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. doi:10.1097/MCG.0b013e31819194b0.

354. Vreugdenhil AC, Wolters VM, Adriaanse MP, et al. Additional value of serum I-FABP levels for evaluating celiac disease activity in children. *Scand J Gastroenterol*. 2011:1435–1441. doi:10.3109/00365521.2011.627447.

355. Fiehn O. Metabolomics – the link between genotypes and phenotypes. *Plant Mol Biol*. 2002;48(1-2):155–171. doi:10.1023/A:1013713905833.

356. Laurin P, Fälth-Magnusson K, Sundqvist T. Increase in nitric oxide urinary products during gluten challenge in children with coeliac disease. *Scand J Gastroenterol*. 2003;38(1):55–60.

357. Reimund JM, Duclos B, Koehl C, Lehr L, Ezenfis J, Baumann R. Nitric oxide end products in patients hospitalized for diarrhoea. *Eur J Gastroenterol Hepatol*. 1999;11(9):1013–1018. doi:10.1097/00042737-199909000-00011.

358. Bertini I, Calabrò A, De Carli V, et al. The Metabonomic Signature of Celiac Disease. *J Proteome Res*. 2009;8(1):170–177. doi:10.1021/pr800548z.

359. de Vos WM, de Vos EAJ. Role of the intestinal microbiome in health and disease: from correlation to causation. *Nutrition Reviews*. 2012;70(s1):S45–S56. doi:10.1111/j.1753-4887.2012.00505.x.

360. Bernini P, Bertini I, Calabrò A, et al. Are Patients with Potential Celiac Disease Really Potential? The Answer of Metabonomics. *J Proteome Res*. 2011;10(2):714–721. doi:10.1021/pr100896s.

361. Bernini P, Bertini I, Luchinat C, et al. Individual Human Phenotypes in Metabolic Space and Time. *J Proteome Res*. 2009;8(9):4264–4271. doi:10.1021/pr900344m.

362. Di Cagno R, De Angelis M, De Pasquale I, et al. Duodenal and faecal microbiota of celiac children: molecular, phenotype and metabolome characterization. *BMC Microbiol*.

2011;11(1):219. doi:10.1186/1471-2180-11-219.

363. Jara LJ, Vera-Lastra O, Miranda JM, Alcala M, Alvarez-Nemegyci J. Prolactin in human systemic lupus erythematosus. *Lupus*. 2001;10(10):748–756. doi:10.1191/096120301717164994.

364. Chikanza IC, Petrou P, Chrousos G, KINGSLEY G, PANAYI GS. Excessive and dysregulated secretion of prolactin in rheumatoid arthritis: immunopathogenetic and therapeutic implications. *Br J Rheumatol*. 1993;32(6):445–448. doi:10.1093/rheumatology/32.6.445.

365. Delvecchio M, Faienza MF, Lonero A, Rutigliano V, Francavilla R, Cavallo L. Prolactin May Be Increased in Newly Diagnosed Celiac Children and Adolescents and Decreases after 6 Months of Gluten-Free Diet. *Horm Res Paediatr*. 2014;0(0). doi:10.1159/000357064.

366. Capristo E, Farnetti S, Mingrone G, et al. Reduced plasma ghrelin concentration in celiac disease after gluten-free diet treatment. *Scand J Gastroenterol*. 2005;40(4):430–436. doi:10.1080/00365520510012028.

367. Lanzini A, Magni P, Petroni ML, et al. Circulating ghrelin level is increased in coeliac disease as in functional dyspepsia and reverts to normal during gluten-free diet. *Alimentary Pharmacology & Therapeutics*. 2006;23(7):907–913. doi:10.1111/j.1365-2036.2006.02852.x.

368. Bogdanos DP, Roggenbuck D, Reinhold D, et al. Pancreatic-specific autoantibodies to glycoprotein 2 mirror disease location and behaviour in younger patients with Crohn's disease. *BMC Gastroenterol*. 2012;12(1):102. doi:10.1186/1471-230X-12-102.

369. Gross S, Bakker SF, van Bodegraven AA, et al. Increased IgA glycoprotein-2 specific antibody titres in refractory celiac disease. *J Gastrointestin Liver Dis*. 2014;23(2):127–133.

370. Sander I, Merget R, Degens PO, Goldscheid N, Brüning T, Raulf Heimsoth M. Comparison of wheat and rye flour skin prick test solutions for diagnosis of baker's asthma. *Allergy*. 2004;59(1):95–98. doi:10.1046/j.1398-9995.2003.00349.x.

371. De Zotti R, Bovenzi M, Negro C, et al. Specific inhalation challenge with wheat flour in workers with suspected baker's asthma. *Int Arch Occup Environ Health*. 1999;72(5):335–337. doi:10.1007/s004200050384.

372. Salcedo G, Quirce S, Diaz-Perales A. Wheat allergens associated with Baker's asthma. *J Investig Allergol Clin Immunol*. 2011.

373. Uvackova L, Skultety L, Bekesova S, McClain S, Hajduch M. The MS(E)-proteomic analysis of gliadins and glutenins in wheat grain identifies and quantifies proteins associated with celiac disease and baker's asthma. *Journal of Proteomics*. 2013;93:65–73. doi:10.1016/j.jprot.2012.12.011.

374. Inomata N. Wheat allergy. *Curr Opin Allergy Clin Immunol*. 2009;9(3):238–243. doi:10.1097/ACI.0b013e32832aa5bc.

375. Matricardi PM, Bockelbrink A, Beyer K, et al. Primary versus secondary immunoglobulin E sensitization to soy and wheat in the Multi-Centre Allergy Study cohort. *Clinical & Experimental Allergy*. 2008;38(3):493–500. doi:10.1111/j.1365-2222.2007.02912.x.

376. Zuidmeer L, Goldhahn K, Rona RJ, et al. The prevalence of plant food allergies: A systematic review. *Journal of Allergy and Clinical Immunology*. 2008;121(5):1210–1218.e4. doi:10.1016/j.jaci.2008.02.019.

377. Battais F, Courcoux P, Popineau Y, Kanny G, Moneret-Vautrin DA, Denery-Papini S. Food allergy to wheat: differences in immunoglobulin E-binding proteins as a function of age or symptoms. *Journal of Cereal Science*. 2005;42(1):109–117. doi:10.1016/j.jcs.2005.01.004.

378. Varjonen E, Vainio E, Kalimo K. Antigliadin IgE--indicator of wheat allergy in atopic dermatitis. *Allergy*. 2000;55(4):386–391.

379. Battais F, Mothes T, Moneret-Vautrin DA, et al. Identification of IgE-binding epitopes on gliadins for patients with food allergy to wheat. *Allergy*. 2005;60(6):815–821. doi:10.1111/j.1398-9995.2005.00795.x.

380. Pastorello EA, Farioli L, Conti A, et al. Wheat IgE-mediated food allergy in European patients: alpha-amylase inhibitors, lipid transfer proteins and low-molecular-weight glutenins. Allergenic molecules recognized by double-blind, placebo-controlled food challenge. *Int Arch Allergy Immunol*. 2007;144(1):10–22. doi:10.1159/000102609.

381. Wahnschaffe U, Schulzke J-D, Zeitz M, Ullrich R. Predictors of clinical response to gluten-free diet in patients diagnosed with diarrhea-predominant irritable bowel syndrome. *Clin Gastroenterol Hepatol*. 2007;5(7):844–50; quiz 769. doi:10.1016/j.cgh.2007.03.021.

382. Wahnschaffe U, Stockmann M, Daum S. Intestinal Antibodies against Gliadin, Tissue-Transglutaminase, β-Lactoglobulin, and Ovalbumin in Patients with Irritable Bowel Syndrome. *Annals of the New …*. 1998.

383. Fan X, Sellin JH. Review article: Small intestinal bacterial overgrowth, bile acid malabsorption and gluten intolerance as possible causes of chronic watery diarrhoea. *Alimentary Pharmacology & Therapeutics*. 2009;29(10):1069–1077. doi:10.1111/j.1365-2036.2009.03970.x.

384. Jones VA, Shorthouse M, McLaughlan P. Food intolerance: a major factor in the pathogenesis of irritable bowel syndrome. *Lancet*. 1982. doi:10.1016/S0140-6736(82)92782-9.

385. Rumessen JJ, Gudmand-Hoyer E. Fructans of chicory: intestinal transport and fermentation of different chain lengths and relation to fructose and sorbitol malabsorption. *Am J Clin Nutr*. 1998;68(2):357–364.

386. Shepherd SJ, Parker FC, Muir JG, Gibson PR. Dietary triggers of abdominal symptoms in patients with irritable bowel syndrome: randomized placebo-controlled evidence. *Clin Gastroenterol Hepatol*. 2008;6(7):765–771. doi:10.1016/j.cgh.2008.02.058.

387. Halmos EP, Power VA, Shepherd SJ, Gibson PR, Muir JG. A diet low in FODMAPs reduces symptoms of irritable bowel syndrome. *Gastroenterology*. 2014;146(1):67–75.e5. doi:10.1053/j.gastro.2013.09.046.

388. Sollid LM. Molecular basis of celiac disease. *Annu Rev Immunol*. 2000;18:53–81. doi:10.1146/annurev.immunol.18.1.53.

389. Sollid LM. Coeliac disease: dissecting a complex inflammatory disorder. *Nature Reviews Immunology*. 2002;2(9):647–655. doi:doi:10.1038/nri885.

390. Cooper BT, Holmes GK, FPERGUSON R, Thompson R, Cooke WT. Proceedings: Chronic diarrhoea and gluten sensitivity. *Gut*. 1976;17(5):398.

391. Ellis A, Linaker BD. Non-coeliac gluten sensitivity? *Lancet*. 1978;1(8078):1358–1359.

392. Elli L, Dolfini E, Bardella MT. Gliadin cytotoxicity and in vitro cell cultures. *Toxicol Lett*. 2003;146(1):1–8.

393. Giovannini C, Matarrese P, Scazzocchio B, et al. Wheat gliadin induces apoptosis of intestinal cells via an autocrine mechanism involving Fas-Fas ligand pathway. *FEBS Lett*. 2003;540(1-3):117–124.

394. Giovannini C, Sanchez M, Straface E, Scazzocchio B, Silano M, De Vincenzi M. Induction of apoptosis in caco-2 cells by wheat gliadin peptides. *Toxicology*. 2000;145(1):63–71.

395. Rivabene R, Mancini E, De Vincenzi M. In vitro cytotoxic effect of wheat gliadin-derived peptides on the Caco-2 intestinal cell line is associated with intracellular oxidative imbalance: implications for coeliac disease. *Biochim Biophys Acta*. 1999;1453(1):152–160.

396. Biesiekierski JR, Newnham ED, Irving PM, et al. Gluten causes gastrointestinal symptoms in subjects without celiac disease: a double-blind randomized placebo-controlled trial. *Am J Gastroenterol*. 2011;106(3):508–14; quiz 515. doi:10.1038/ajg.2010.487.

397. Drago S, Asmar El R, Di Pierro M, et al. Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand J Gastroenterol*. 2006;41(4):408–419. doi:10.1080/00365520500235334.

398. Verdu EF, Huang X, Natividad J, et al. Gliadin-dependent neuromuscular and epithelial secretory responses in gluten-sensitive HLA-DQ8 transgenic mice. *Am J Physiol Gastrointest Liver Physiol*. 2008;294(1):G217–25. doi:10.1152/ajpgi.00225.2007.

399. Anderson IH, Levine AS, Levitt MD. Incomplete absorption of the carbohydrate in all-purpose wheat flour. *N Engl J Med*. 1981;304(15):891–892. doi:10.1056/NEJM198104093041507.

400. Tanpowpong P, Ingham TR, Lampshire PK, et al. Coeliac disease and gluten avoidance in New Zealand children. *Archives of Disease in Childhood*. 2011;97(1):12–16. doi:10.1136/archdischild-2011-300248.

401. Rubio-Tapia A, Ludvigsson JF, Brantner TL, Murray JA, Everhart JE. The Prevalence of Celiac Disease in the United States. *Am J Gastroenterol*. 2012. doi:10.1038/ajg.2012.219.

402. Golley S, Corsini N, Topping D, Morell M, Mohr P. Motivations for avoiding wheat consumption in Australia: results from a population survey. *Public Health Nutr*. 2014:1–10. doi:10.1017/S1368980014000652.

403. Tavakkoli A, Lewis SK, Tennyson CA, Lebwohl B, Green PHR. Characteristics of Patients Who Avoid Wheat and/or Gluten in the Absence of Celiac Disease. *Dig Dis Sci*. 2014;59(6):1255–1261. doi:10.1007/s10620-013-2981-6.

404. Carroccio A, Mansueto P, Iacono G, et al. Non-Celiac Wheat Sensitivity Diagnosed by Double-Blind Placebo-Controlled Challenge: Exploring a New Clinical Entity. *Am J Gastroenterol*. 2012:–. doi:10.1038/ajg.2012.236.

405. Sapone A, Lammers KM, Casolaro V, et al. Divergence of gut permeability and mucosal immune gene expression in two gluten-associated conditions: celiac disease and gluten sensitivity. *BMC Medicine*. 2011;9:23. doi:10.1186/1741-7015-9-23.

406. Biesiekierski JR, Rosella O, Rose R, et al. Quantification of fructans, galacto-oligosacharides and other short-chain carbohydrates in processed grains and cereals. *Journal of Human Nutrition and Dietetics*. 2011;24(2):154–176. doi:10.1111/j.1365-277X.2010.01139.x.

407. Gibson PR, Muir JG. Not all effects of a gluten-free diet are due to removal of gluten. *Gastroenterology*. 2013;145(3):693. doi:10.1053/j.gastro.2013.06.056.

408. Biesiekierski JR, Peters SL, Newnham ED, Rosella O, Muir JG, Gibson PR. No effects of gluten in patients with self-reported non-celiac gluten sensitivity after dietary reduction of fermentable, poorly absorbed, short-chain carbohydrates. *Gastroenterology*. 2013;145(2):320–8.e1–3. doi:10.1053/j.gastro.2013.04.051.

409. Gibson PR, Shepherd SJ, Din JT. For celiac disease, diagnosis is not enough. *Clinical Gastroenterology and Hepatology*. 2012.

410. Herman ML, Rubio-Tapia A, Lahr BD, Larson JJ, Van Dyke CT, Murray JA. Patients with celiac disease are not followed up adequately. *Clinical Gastroenterology and Hepatology*. 2012;10(8):893–899. e1.

411. Leibbrand R, Cuntz U, Hiller W. Assessment of functional gastrointestinal disorders using the gastro-questionnaire. 2002;9(2):155–172. doi:10.1207/S15327558IJBM0902_06.

412. Memeo L, Jhang J, Hibshoosh H, Green PH, Rotterdam H, Bhagat G. Duodenal intraepithelial lymphocytosis with normal villous architecture: common occurrence in H. pylori gastritis. *Mod Pathol*. 2005;18(8):1134–1144. doi:10.1038/modpathol.3800404.

413. Kim J, Heshka S, Gallagher D, et al. Intermuscular adipose tissue-free skeletal muscle mass: estimation by dual-energy X-ray absorptiometry in adults. *J Appl Physiol*. 2004;97(2):655–660. doi:10.1152/japplphysiol.00260.2004.

414. Neurath MF, Travis SPL. Mucosal healing in inflammatory bowel diseases: a systematic review. *Gut*. 2012;61(11):1619–1635. doi:10.1136/gutjnl-2012-302830.

415. Kurppa K, Collin P, Sievãnen H, Huhtala H, Mäki M, Kaukinen K. Gastrointestinal symptoms, quality of life and bone mineral density in mild enteropathic coeliac disease: a prospective clinical trial. *Scand J Gastroenterol*. 2010;45(3):305–314. doi:10.3109/00365520903555879.

416. Kumar PJ, O'Donoghue DP, Stenson K, Dawson AM. Reintroduction of gluten in adults and children with treated coeliac disease. *Gut*. 1979;20(9):743–749.

417. Murray JA, Watson T, Clearman B, Mitros F. Effect of a gluten-free diet on gastrointestinal symptoms in celiac disease. *Am J Clin Nutr*. 2004;79(4):669–673.

418. DIPPER CR, MAITRA S, THOMAS R, et al. Anti-tissue transglutaminase antibodies in the follow-up of adult coeliac disease. *Alimentary Pharmacology & Therapeutics*. 2009;30(3):236–244. doi:10.1111/j.1365-2036.2009.04039.x.

419. van Langenberg DR, Gatta Della P, Hill B, Zacharewicz E, Gibson PR, Russell AP. Delving into disability in Crohn“s disease: dysregulation of molecular pathways may explain skeletal muscle loss in Crohn”s disease. *J Crohns Colitis*. 2014;8(7):626–634. doi:10.1016/j.crohns.2013.11.024.

420. Valentini L, Schaper L, Buning C, et al. Malnutrition and impaired muscle strength in patients with Crohn's disease and ulcerative colitis in remission. *Nutrition*. 2008;24(7-8):694–702. doi:10.1016/j.nut.2008.03.018.

421. Szulc P, Beck TJ, Marchand F, Delmas PD. Low skeletal muscle mass is associated with poor structural parameters of bone and impaired balance in elderly men--the MINOS study. *J Bone Miner Res*. 2005;20(5):721–729. doi:10.1359/JBMR.041230.

422. Hjelle AM, Apalset E, Mielnik P, Bollerslev J, Lundin KEA, Tell GS. Celiac disease and risk of fracture in adults-a review. *Osteoporos Int*. 2014. doi:10.1007/s00198-014-2683-8.

423. Rubio-Tapia A, Murray JA. Classification and management of refractory coeliac disease. *Gut*. 2010;59(4):547–557. doi:10.1136/gut.2009.195131.

424. Woodward J. The management of refractory coeliac disease. *Ther Adv Chronic Dis*. 2013;4(2):77–90. doi:10.1177/2040622312473174.

425. Liu E, Lee H-S, Aronsson CA, et al. Risk of Pediatric Celiac Disease According to HLA Haplotype and Country. *N Engl J Med*. 2014;371(1):42–49. doi:10.1056/NEJMoa1313977.

426. Zanini B, Baschè R, Ferraresi A, et al. Factors That Contribute to Hypertransaminasemia in Patients With Celiac Disease or Functional Gastrointestinal Syndromes. *Clinical Gastroenterology and Hepatology*. 2014;12(5):804–810.e2. doi:10.1016/j.cgh.2013.10.033.

427. Sainsbury A, Sanders DS, Ford AC. Meta-analysis: coeliac disease and hypertransaminasaemia. *Alimentary Pharmacology & Therapeutics*. 2011. doi:10.1111/j.1365-2036.2011.04685.x.

428. Hadjivassiliou M, Grünewald RA, Davies-Jones GAB. Gluten sensitivity as a neurological illness. *Journal of Neurology, Neurosurgery & Psychiatry*. 2002;72(5):560–

563.

429. Ventura A, Magazzu G, Greco L. Duration of exposure to gluten and risk for autoimmune disorders in patients with celiac disease. SIGEP Study Group for Autoimmune Disorders in Celiac Disease. *Gastroenterology*. 1999;117(2):297–303.

430. Heikkilä K, Pearce J, Mäki M, Kaukinen K. Coeliac disease and bone fractures: a systematic review and meta-analysis. *The Journal of Clinical Endocrinology & Metabolism*. 2014:jc20141858. doi:10.1210/jc.2014-1858.

431. Lucendo AJ, Arias Á, Tenias JM. Systematic review: the association between eosinophilic oesophagitis and coeliac disease. *Alimentary Pharmacology & Therapeutics*. 2014:n/a–n/a. doi:10.1111/apt.12859.

432. Di Sabatino A, Bertrandi E, Casadei Maldini M, Pennese F, Proietti F, Corazza GR. Phenotyping of peripheral blood lymphocytes in adult coeliac disease. *Immunology*. 1998;95(4):572–576.

433. O'Donoghue DP, Lancaster-Smith M, Laviniere P, Kumar PJ. T cell depletion in untreated adult coeliac disease. *Gut*. 1976;17(5):328–331.

434. Bullen AW, Losowsky MS. Lymphocyte subpopulations in adult coeliac disease. *Gut*. 1978;19(10):892–897.

435. Moran CJ, Kolman OK, Russell GJ, Brown IS, Mino-Kenudson M. Neutrophilic infiltration in gluten-sensitive enteropathy is neither uncommon nor insignificant: assessment of duodenal biopsies from 267 pediatric and adult patients. *Am J Surg Pathol*. 2012;36(9):1339–1345. doi:10.1097/PAS.0b013e318254f413.

436. Pietro Capone ARNINCRT. Fecal calprotectin in coeliac disease. *World Journal of Gastroenterology : WJG*. 2014;20(2):611–612. doi:10.3748/wjg.v20.i2.611.

437. Martínez-Baños D, Crispín JC, Lazo-Langner A, Sánchez-Guerrero J. Moderate and severe neutropenia in patients with systemic lupus erythematosus. *Rheumatology (Oxford)*. 2006;45(8):994–998. doi:10.1093/rheumatology/kel016.

438. Balint GP, Balint PV. Felty's syndrome. *Best Pract Res Clin Rheumatol*. 2004;18(5):631–645. doi:10.1016/j.berh.2004.05.002.

439. Haines ML ARGP. Systematic review: the evidence base for long-term management of coeliac disease. *Alimentary Pharmacology & Therapeutics*. 2008;28(9):1042–1066. doi:10.1111/j.1365-2036.2008.03820.x.

440. Lebwohl B, Granath MF, Ekbom PA, et al. Mucosal Healing and Risk for Lymphoproliferative Malignancy in Celiac Disease. 2013:1–8.

441. Leffler DA, Edwards George JB, Dennis M, Cook EF, Schuppan D, Kelly CP. A prospective comparative study of five measures of gluten-free diet adherence in adults with coeliac disease. *Alimentary Pharmacology & Therapeutics*. 2007;26(9):1227–1235. doi:10.1111/j.1365-2036.2007.03501.x.

442. Kang JY, Kang AHY, Green A, Gwee KA, Ho KY. Systematic review: worldwide

variation in the frequency of coeliac disease and changes over time. *Alimentary Pharmacology & Therapeutics*. 2013;38(3):226–245. doi:10.1111/apt.12373.

443. Gibson PR, Shepherd SJ, Tye-Din JA. For celiac disease, diagnosis is not enough. *Clin Gastroenterol Hepatol*. 2012;10(8):900–901. doi:10.1016/j.cgh.2012.03.020.

444. Zelnik N, Pacht A, Obeid R, Lerner A. Range of neurologic disorders in patients with celiac disease. *PEDIATRICS*. 2004;113(6):1672–1676. doi:10.1542/peds.113.6.1672.

445. Siqueira Neto JI, Costa ACLV, Magalhães FG, Silva GS. Neurological manifestations of celiac disease. *Arq Neuro-Psiquiatr*. 2004;62(4):969–972. doi:10.1590/S0004-282X2004000600007.

446. Celiac Disease and Gluten Sensitivity. 2013. Available at: http://celiacdisease.about.com/od/symptomsofceliacdisease/a/Celiac-Disease-Brain-Fog.htm. Accessed October 14, 2014.

447. Lurie Y, Landau D-A, Pfeffer J, Oren R. Celiac disease diagnosed in the elderly. *J Clin Gastroenterol*. 2008;42(1):59–61. doi:10.1097/01.mcg.0000247995.12087.7b.

448. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. 1995;57(1):289–300. doi:10.2307/2346101.

449. Howell D. *Statistical Methods for Psychology*. 2012.

450. Friedman TW, Robinson SR, Yelland GW. Impaired perceptual judgment at low blood alcohol concentrations. *Alcohol*. 2011;45(7):711–718. doi:10.1016/j.alcohol.2010.10.007.

451. REITAN RM. The relation of the trail making test to organic brain damage. *J Consult Psychol*. 1955;19(5):393–394. doi:10.1037/h0044509.

452. *The Handbook of Rey-Osterrieth Complex Figure Usage: Clinical and Research Applications*. Lutz, FL, United States of America: Psychological assessment Resources, Inc.; 2003.

453. Strauss E, Spreen O. *A Compendium of Neuropsychological Tests: Administration, Norms, and Commentary*. Neurology; 1991:1856–1856. doi:10.1212/WNL.41.11.1856-a.

454. Rey A. *L'examen Clinique en Psychologie*. worldcat.org; 1964.

455. Klove H. *In: Forster FM, Editor. the Medical Clinics of North America*.

456. Spielberger CD. *State-Trait Personality Inventory (STPI) Mind Garden; Redwood City*. 1995.

457. Wechsler D. *Wechsler Test of Adult Reading*. 2001.

458. Friedman T, Hutchison C, Robinson S. Assessment of cognitive impairment. 2004;(10/…).

459. Lezak MD. *Neuropsychological Assessment*. 2004.

460. Balion C, Griffith LE, Strifler L, et al. Vitamin D, cognition, and dementia: a systematic review and meta-analysis. *Neurology*. 2012;79(13):1397–1405. doi:10.1212/WNL.0b013e31826c197f.

461. Murray-Kolb LE, Beard JL. Iron treatment normalizes cognitive functioning in young women. *Am J Clin Nutr*. 2007;85(3):778–787.

462. Ramos MI, Allen LH, Mungas DM, et al. Low folate status is associated with impaired cognitive function and dementia in the Sacramento Area Latino Study on Aging. *Am J Clin Nutr*. 2005;82(6):1346–1352.

463. Banks WA, Farr SA, Morley JE. Entry of blood-borne cytokines into the central nervous system: effects on cognitive processes. *Neuroimmunomodulation*. 2003. doi:10.1159/000071472.

464. Kapadia M, Sakic B. Autoimmune and inflammatory mechanisms of CNS damage. *Prog Neurobiol*. 2011;95(3):301–333. doi:10.1016/j.pneurobio.2011.08.008.

465. Larson SJ, Dunn AJ. Behavioral effects of cytokines. *Brain Behav Immun*. 2001;15(4):371–387. doi:10.1006/brbi.2001.0643.

466. Maier SF, Watkins LR. Cytokines for psychologists: implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. *Psychological review*. 1998. doi:10.1037/0033-295X.105.1.83.

467. O'Connor JJ, Coogan AN. Actions of the pro-inflammatory cytokine IL-1 beta on central synaptic transmission. *Experimental physiology*. 1999. doi:10.1111/j.1469-445X.1999.01892.x.

468. Turrin NP, Plata-Salamán CR. Cytokine–cytokine interactions and the brain. *Brain research bulletin*. 2000. doi:10.1016/S0361-9230(99)00203-8.

469. Wilson CJ, Finch CE, Cohen HJ. Cytokines and Cognition—The Case for A Head-to-Toe Inflammatory Paradigm. *J Am Geriatr Soc*. 2002;50(12):2041–2056. doi:10.1046/j.1532-5415.2002.50619.x.

470. Choi S, DiSilvio B, Fernstrom MH, Fernstrom JD. Meal ingestion, amino acids and brain neurotransmitters: effects of dietary protein source on serotonin and catecholamine synthesis rates. *Physiol Behav*. 2009;98(1-2):156–162. doi:10.1016/j.physbeh.2009.05.004.

471. Takahashi M, Fukunaga H, Kaneto H. Behavioral and pharmacological studies on gluten exorphin A5, a newly isolated bioactive food protein fragment, in mice. *The Japanese Journal of Pharmacology*. 2000. doi:10.1254/jjp.84.259.

472. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci*. 2012;13(10):701–712. doi:10.1038/nrn3346.

473. Diaz Heijtz R, Wang S, Anuar F, et al. Normal gut microbiota modulates brain development and behavior. *PNAS*. 2011;108(7):3047–3052. doi:10.1073/pnas.1010529108.

474. Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology & Motility*. 2011;23(3):255–64– e119. doi:10.1111/j.1365-2982.2010.01620.x.

475. Bercik P, Denou E, Collins J, et al. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology*. 2011;141(2):599–609– 609.e1–3. doi:10.1053/j.gastro.2011.04.052.

476. Tillisch K, Labus J, Kilpatrick L, et al. Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology*. 2013;144(7):1394–401– 1401.e1–4. doi:10.1053/j.gastro.2013.02.043.

477. Henderson KN, Tye-Din JA, Reid HH, et al. A structural and immunological basis for the role of human leukocyte antigen DQ8 in celiac disease. *Immunity*. 2007;27(1):23–34. doi:10.1016/j.immuni.2007.05.015.

478. 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.

479. Green P, Jabri B. Coeliac disease. *Lancet*. 2003. doi:10.1016/S0140-6736(03)14027-5.

480. Muir JG, Rose R, Rosella O, et al. Measurement of short-chain carbohydrates in common Australian vegetables and fruits by high-performance liquid chromatography (HPLC). *J Agric Food Chem*. 2009;57(2):554–565. doi:10.1021/jf802700e.

481. Miki K, Butler R, Moore D, Davidson G. Rapid and simultaneous quantification of rhamnose, mannitol, and lactulose in urine by HPLC for estimating intestinal permeability in pediatric practice. *Clinical Chemistry*. 1996;42(1):71–75.

482. Verdu EF, Armstrong D, Murray JA. Between celiac disease and irritable bowel syndrome: the “no man's land” of gluten sensitivity. *Am J Gastroenterol*. 2009;104(6):1587–1594. doi:10.1038/ajg.2009.188.

483. Piche T, Huet P-M, Gelsi E, et al. Fatigue in irritable bowel syndrome: characterization and putative role of leptin. *Eur J Gastroenterol Hepatol*. 2007;19(3):237–243. doi:10.1097/01.meg.0000252627.50302.b4.

484. kindt S, van oudenhove L, broekaert D, et al. Immune dysfunction in patients with functional gastrointestinal disorders. *Neurogastroenterology & Motility*. 2009;21(4):389–398. doi:10.1111/j.1365-2982.2008.01220.x.

485. Poullis A, Foster R, Northfield TC, Mendall MA. Review article: faecal markers in the assessment of activity in inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics*. 2002;16(4):675–681.

486. Gibson PR, Shepherd SJ. Evidence-based dietary management of functional gastrointestinal symptoms: The FODMAP approach. *J Gastroenterol Hepatol*. 2010;25(2):252–258. doi:10.1111/j.1440-1746.2009.06149.x.

487. Barbara G, Stanghellini V, Brandi G, et al. Interactions between commensal bacteria and gut sensorimotor function in health and disease. *Am J Gastroenterol*. 2005;100(11):2560–

2568. doi:10.1111/j.1572-0241.2005.00230.x.

488. Carroccio A, Brusca I, Mansueto P, et al. A cytologic assay for diagnosis of food hypersensitivity in patients with irritable bowel syndrome. *Clin Gastroenterol Hepatol*. 2010;8(3):254–260. doi:10.1016/j.cgh.2009.11.010.

489. Hadjivassiliou M, Williamson CA, Woodroofe N. The immunology of gluten sensitivity: beyond the gut. *Trends Immunol*. 2004;25(11):578–582. doi:10.1016/j.it.2004.08.011.

490. Vinning G, McMahon G. *Gluten-Free Grains: a Demand-and-Supply Analysis of Prospects for the Australian Grains Industry*. Canberra, Australia: Rural Industries Research and Development Corporation, Australian Government; 2006.

491. Cooper BT, Holmes GK, Ferguson R, Thompson RA, Allan RN, Cooke WT. "Gluten-sensitive diarrhea without evidence of celiac disease". *Endoscopy*. 1981;81(1):192–194.

492. Gibson PR, Shepherd SJ. Food choice as a key management strategy for functional gastrointestinal symptoms. *Am J Gastroenterol*. 2012;107(5):657–66– quiz 667. doi:10.1038/ajg.2012.49.

493. Swain A, Soutter VL, Loblay RH. *RPAH Elimination Diet Handbook*. Camperdown, NSW, Australia: Allergy Unit, Royal Prince Alfred Hospital; 2009.

494. Muir JG, Shepherd SJ, Rosella O, Rose R, Barrett JS, Gibson PR. Fructan and free fructose content of common Australian vegetables and fruit. *J Agric Food Chem*. 2007;55(16):6619–6627. doi:10.1021/jf070623x.

495. Biagi F, Andrealli A, Bianchi PI, Marchese A, Klersy C, Corazza GR. A gluten-free diet score to evaluate dietary compliance in patients with coeliac disease. *Br J Nutr*. 2009;102(6):882–887. doi:10.1017/S0007114509301579.

496. Fisk JD, Doble SE. Construction and validation of a fatigue impact scale for daily administration (D-FIS). *Qual Life Res*. 2002;11(3):263–272.

497. Trost SG, McIver KL, Pate RR. Conducting accelerometer-based activity assessments in field-based research. *Med Sci Sports Exerc*. 2005;37(11 Suppl):S531–43.

498. Gordon-Larsen P, McMurray RG, Popkin BM. Determinants of adolescent physical activity and inactivity patterns. *PEDIATRICS*. 2000;105(6):E83.

499. Welch RW, Antoine J-M, Berta J-L, et al. Guidelines for the design, conduct and reporting of human intervention studies to evaluate the health benefits of foods. *Br J Nutr*. 2011;106 Suppl 2(S2):S3–15. doi:10.1017/S0007114511003606.

500. Klein KB. Controlled treatment trials in the irritable bowel syndrome: a critique. *Endoscopy*. 1988;95(1):232–241.

501. Ong DK, Mitchell SB, Barrett JS, et al. Manipulation of dietary short chain carbohydrates alters the pattern of gas production and genesis of symptoms in irritable bowel syndrome. *J Gastroenterol Hepatol*. 2010;25(8):1366–1373. doi:10.1111/j.1440-1746.2010.06370.x.

502. Boirie Y, Dangin M, Gachon P, Vasson MP, Maubois JL, Beaufrere B. Slow and fast

dietary proteins differently modulate postprandial protein accretion. *Proc Natl Acad Sci USA*. 1997;94(26):14930–14935.

503. Volta U, Tovoli F, Cicola R, et al. Serological tests in gluten sensitivity (nonceliac gluten intolerance). *J Clin Gastroenterol*. 2012;46(8):680–685. doi:10.1097/MCG.0b013e3182372541.

504. Wahnschaffe U, Ullrich R, Riecken EO, Schulzke JD. Celiac disease-like abnormalities in a subgroup of patients with irritable bowel syndrome. *Gastroenterology*. 2001;121(6):1329–1338.

505. Ford RPK. The gluten syndrome: a neurological disease. *Med Hypotheses*. 2009;73(3):438–440. doi:10.1016/j.mehy.2009.03.037.

506. Dubé C, Rostom A, Sy R, Cranney A, Saloojee N. The prevalence of celiac disease in average-risk and at-risk Western European populations: a systematic review. *Gastroenterology*. 2005. doi:10.1053/j.gastro.2005.02.014.

507. Drossman DA. The functional gastrointestinal disorders and the Rome III process. *Gastroenterology*. 2006;130(5):1377–1390. doi:10.1053/j.gastro.2006.03.008.

508. Giovannini C, Sanchez M, Straface E, Scazzocchio B. Induction of apoptosis in caco-2 cells by wheat gliadin peptides. *Toxicology*. 2000. doi:10.1016/S0300-483X(99)00223-1.

509. Painter K. **Gluten-free diets gaining in popularity**. *httpusatodayusatodaycomnewshealthpainter---glutenNhtm*. Available at: http://usatoday30.usatoday.com/news/health/painter/2008-08-17-gluten_N.htm? Accessed October 14, 2014.

510. Biagi F, Bianchi PI, Marchese A, et al. A score that verifies adherence to a gluten-free diet: a cross-sectional, multicentre validation in real clinical life. *Br J Nutr*. 2012:1–5. doi:10.1017/S0007114511007367.

511. Group GE. *Therapeutic Guidelines: Gastrointestinal*. *Version 5*. *Melbourne: Therapeutic Guidelines Limited*. 2011.

512. Leffler D, Schuppan D, Pallav K, et al. Kinetics of the histological, serological and symptomatic responses to gluten challenge in adults with coeliac disease. *Gut*. 2013;62(7):996–1004. doi:10.1136/gutjnl-2012-302196.

513. Nanda R, James R, Smith H, Dudley CR, Jewell DP. Food intolerance and the irritable bowel syndrome. *Gut*. 1989. doi:10.1136/gut.30.8.1099.

514. Worsley A, Crawford D. Australian dietary supplementation practices. Health and dietary supplements. *Med J Aust*. 1984;140(10):579–583.

515. Radimer K, Bindewald B, Hughes J, Ervin B, Swanson C, Picciano MF. Dietary supplement use by US adults: data from the National Health and Nutrition Examination Survey, 1999-2000. *Am J Epidemiol*. 2004;160(4):339–349. doi:10.1093/aje/kwh207.

516. Singer E. Introduction Nonresponse Bias in Household Surveys. *Public Opinion Quarterly*. 2006. doi:10.1093/poq/nfl034.

517. Gibson GR, Probert HM, Loo JV, Rastall RA, Roberfroid MB. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr Res Rev*. 2004;17(2):259–275. doi:10.1079/NRR200479.

518. Kurppa K, Paavola A, Collin P, et al. Benefits of a Gluten-Free Diet for Asymptomatic Patients With Serologic Markers of Celiac Disease. *Gastroenterology*. 2014;147(3):610–617.e1. doi:10.1053/j.gastro.2014.05.003.

519. Mäki M, Mustalahti K, Kokkonen J, et al. Prevalence of Celiac disease among children in Finland. *N Engl J Med*. 2003;348(25):2517–2524. doi:10.1056/NEJMoa021687.

520. Wilson JMG, Jungner G. Principles and practice of screening for disease. *World Health Organisation*. 1968.

521. Elli L, Contiero P, Tagliabue G, Tomba C, Bardella MT. Risk of intestinal lymphoma in undiagnosed coeliac disease: Results from a registered population with different coeliac disease prevalence. *Dig Liver Dis*. 2012. doi:10.1016/j.dld.2012.04.020.

522. Askling J, Linet M, Gridley G, Halstensen TS, Ekström K. Cancer incidence in a population-based cohort of individuals hospitalized with celiac disease or dermatitis herpetiformis. *Gastroenterology*. 2002. doi:10.1053/gast.2002.36585.

523. Frøslie KF, Jahnsen J, Moum BA, Vatn MH, IBSEN Group. Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. *Gastroenterology*. 2007;133(2):412–422. doi:10.1053/j.gastro.2007.05.051.

524. Newnham ED, Shepherd SJ, Gibson PR. A phase II, randomised, double-blind, placebo-controlled study to evaluate the efficacy and safety of oral effervescent budesonide in the induction of mucosal healing in newly diagnosed coeliac disease: Australia and New Zealand Clinical Trials Registry: ACTRN12609000738224. *wwwanzctrorgau*. Available at: https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=308403. Accessed November 21, 2014.

525. Tennyson CA, Simpson S, Lebwohl B, Lewis S, Green PHR. Interest in medical therapy for celiac disease. *Therapeutic Advances in Gastroenterology*. 2013;6(5):358–364. doi:10.1177/1756283X13492580.

526. Anita Bakshi SSMLBDBD. Emerging Therapeutic Options for Celiac Disease: Potential Alternatives to a Gluten-Free Diet. *Gastroenterology & Hepatology*. 2012;8(9):582.

527. Arasaradnam RP, Westenbrink E, McFarlane MJ, et al. Differentiating coeliac disease from irritable bowel syndrome by urinary volatile organic compound analysis - a pilot study. *PLoS ONE*. 2014;9(10):e107312. doi:10.1371/journal.pone.0107312.

528. Garner C, Ahn R, Ding YC, et al. Genome-Wide Association Study of Celiac Disease in North America Confirms FRMD4B as New Celiac Locus. Sestak K, ed. *PLoS ONE*. 2014;9(7):e101428. doi:10.1371/journal.pone.0101428.

529. Ahn R, Ding YC, Murray J, et al. Association Analysis of the Extended MHC Region in Celiac Disease Implicates Multiple Independent Susceptibility Loci. Goldberg AC, ed. *PLoS ONE*. 2012;7(5):e36926. doi:10.1371/journal.pone.0036926.t002.

530. Zhernakova A, Stahl EA, Trynka G, et al. Meta-analysis of genome-wide association studies in celiac disease and rheumatoid arthritis identifies fourteen non-HLA shared loci. *PLoS Genetics*. 2011;7(2):e1002004. doi:10.1371/journal.pgen.1002004.

531. Ryan D, Newnham ED, Prenzler PD, Gibson PR. Metabolomics as a tool for diagnosis and monitoring in coeliac disease. *Metabolomics*. 2014. doi:10.1007/s11306-014-0752-9.

532. Amaya González S, de los Santos Álvarez N, Lobo Castañón MJ, Miranda Ordieres AJ, Tuñón Blanco P. Amperometric Quantification of Gluten in Food Samples Using an ELISA Competitive Assay and Flow Injection Analysis. *Electroanalysis*. 2011;23(1):108–114. doi:10.1002/elan.201000583.

533. Peters SL, Biesiekierski JR, Yelland GW, Muir JG, Gibson PR. Randomised clinical trial: gluten may cause depression in subjects with non-coeliac gluten sensitivity - an exploratory clinical study. *Alimentary Pharmacology & Therapeutics*. 2014;39(10):1104–1112. doi:10.1111/apt.12730.

534. Bucci C, Zingone F, Russo I, et al. Gliadin does not induce mucosal inflammation or basophil activation in patients with nonceliac gluten sensitivity. *Clin Gastroenterol Hepatol*. 2013;11(10):1294–1299.e1. doi:10.1016/j.cgh.2013.04.022.

535. Brottveit M, Beitnes A-CR, Tollefsen S, et al. Mucosal Cytokine Response After Short-Term Gluten Challenge in Celiac Disease and Non-Celiac Gluten Sensitivity. *Am J Gastroenterol*. 2013;108(5):842–850. doi:10.1038/ajg.2013.91.

536. de Roest RH, Dobbs BR, Chapman BA, et al. The low FODMAP diet improves gastrointestinal symptoms in patients with irritable bowel syndrome: a prospective study. *Int J Clin Pract*. 2013;67(9):895–903. doi:10.1111/ijcp.12128.

537. Staudacher HM, Whelan K, Irving PM, Lomer MCE. Comparison of symptom response following advice for a diet low in fermentable carbohydrates (FODMAPs) versus standard dietary advice in patients with irritable bowel syndrome. *Journal of Human Nutrition and Dietetics*. 2011;24(5):487–495. doi:10.1111/j.1365-277X.2011.01162.x.

538. Carroccio A, Mansueto P, D'Alcamo A, Iacono G. Non-Celiac Wheat Sensitivity as an Allergic Condition: Personal Experience and Narrative Review. *Am J Gastroenterol*. 2013:–. doi:10.1038/ajg.2013.353.

539. Villanacci V, Not T, Sblattero D, et al. Mucosal tissue transglutaminase expression in celiac disease. *J Cell Mol Med*. 2009;13(2):334–340. doi:10.1111/j.1582-4934.2008.00325.x.

540. Bucci C, Zingone F, Russo I, Morra I, Tortora R. Gliadin Does Not Induce Mucosal Inflammation or Basophil Activation in Patients with Non-Celiac Gluten Sensitivity. *Clinical Gastroenterology and Hepatology*. 2013.

541. Fritscher-Ravens A, Schuppan D, Ellrichmann M, et al. Confocal Endomicroscopy Reveals Food-Associated Changes in the Intestinal Mucosa of Patients With Irritable Bowel Syndrome. *Gastroenterology*. 2014;147(5):1–13. doi:10.1053/j.gastro.2014.07.046.