Understanding gluten sensitivity: The role of gluten and dietary carbohydrates in the genesis of gastrointestinal symptoms in individuals who do not have coeliac disease

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List of Abbreviations

ADHD	Attention deficit hyperactivity disorder
ANZCTR	Australia and New Zealand Clinical Trials Register
ANOVA	Analysis of variance
APCs	Antigen-presenting cells
BCAA	Branched-chain amino acid
BMI	Body mass index
CHD	Coronary heart disease
CT	Chymotrypsin
D-FIS	Daily Fatigue Impact Scale
DMSO	Dimethyl sulfoxide
DP	Degree of polymerisation
EATL	Enteropathy-associated T-cell lymphoma
ECP	Eosinophil cationic protein
EER	Estimated energy requirement
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
ELSD	Evaporating light scattering detector
ENS	Enteric nervous system
ESPGAN	European Society for Paediatric Gastroenterology and Nutrition
ESPGHAN	European Society for Paediatric Gastroenterology, Hepatology and Nutrition

FCS	Foetal calf serum
FFQ	Food frequency questionnaire
FGID	Functional gastrointestinal disorder
FODMAP	Fermentable oligo-, di-, mono-saccharides and polyols
FOS	Fructo-oligosaccharide
FSANZ	Food Standards Australia New Zealand
g	gram
GEE	Generalized estimating equation
GFD	Gluten-free diet
GI	Gastrointestinal
GOS	Galacto-oligosaccharide
GS	Glutenin subunits
HBD-2	Human ß-defensin-2
HLA	Human leukocyte antigen
HMW	High molecular weight
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High performance liquid chromatography
hsCRP	Highly-sensitive C-reactive protein
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IEL	Intraepithelial lymphocyte
IFN-γ	Interferon gamma
IgE	Immunoglobulin E

kD	kilodalton
LMW	Low molecular weight
LSA	Linseed, sunflower seed and almond mix
SCC	Short-chain carbohydrate
S	Sulphur
RS	Resistant starch
NSP	Non-starch polysaccharide
L/R	Ratio of lactulose:rhamnose
MHC	Major histocompatibility complex
min	minute
MJ	mega joule
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSG	Monosodium glutamate
NCGS	Non-coeliac gluten sensitivity
NHMRC	National Health and Medical Research Council
NS	Not significant
NSP	Non-starch polysaccharide
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDCAAS	Protein digestibility-corrected amino acid score
PHA	Phytohemagglutinin
PVDF	Polyvinylidene fluoride

RAST	Radioallergosorbent test
RP-HPLC	Reverse-phase high performance liquid chromatography
RPAH	Royal Prince Alfred Hospital
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SCC	Short-chain carbohydrate
SCFA	Short chain fatty acid
SCIT	Subtle Cognitive Impairment Test
SE-HPLC	Size-exclusion high performance liquid chromatography
SEM	Standard error of the mean
SFU	Spot forming unit
STPI	State-Trait Personality Inventory
tet tox	Tetanus toxoid
TNF-α	Tumor necrosis factor-alpha
TRP	Transient receptor potential
tTG	Transglutaminase
VAS	Visual analogue scale
WDEIA	Wheat dependent exercise induced anaphylaxis
µm	Micro meter

Abstract

Despite worldwide increased prescription of a gluten-free diet (GFD) for gastrointestinal (GI) and other symptoms in individuals who do not have coeliac disease, there is minimal evidence that suggests gluten is a trigger. It is not known whether it is the removal of protein (gluten) or the carbohydrate (fructan) component in wheat responsible, given fructans are capable of provoking GI symptoms themselves. A series of studies were undertaken to provide an evidence-base for this so-called non-coeliac gluten sensitivity (NCGS) and make sense of the increasing avoidance of gluten- and wheat-containing products.

The objectives of this thesis were: to evaluate the content of poorly absorbed, short-chain carbohydrates (termed FODMAPs) in a variety of grains and processed cereal products; to characterise patients who believed they have NCGS; and, to evaluate the effects of gluten in patients with irritable bowel syndrome (IBS) in who coeliac disease was excluded (histology or gene typing) and who were symptomatically controlled on a GFD in three randomised, placebo-controlled, double-blind trials on GI symptoms, fatigue and markers of potential mechanism.

Carbohydrate analysis of 55 commonly consumed grain and cereal products found fructans to be the most common FODMAP present, further developing the FODMAP composition tables and expanding our understanding of natural food sources of prebiotics (galacto- and fructo-oligosaccharides). Many high FODMAP-containing products were gluten-containing, and conversely many low FODMAP products were gluten-free, which may help explain the symptom improvement that individuals with IBS experience whilst following a GFD.

A survey of 132 people who believe they had NCGS found the practice of initiation of a GFD without adequate exclusion of coeliac disease is common and nearly 25% believed that they were gluten-sensitive despite having uncontrolled symptoms.

The results from the initial double-blind, randomised, placebo-controlled trial provided for the first time, high-quality evidence that gluten itself may trigger GI symptoms and fatigue in individuals who do not have coeliac disease (termed NCGS). A second trial used a cross-over design, controlling for other dietary triggers of symptoms, FODMAPs, and investigated more sensitive markers of possible immune mechanisms. Very limited evidence of gluten specificity was observed, but FODMAP restriction uniformly reduced residual symptoms. A strong nocebo response and an order effect were found. No clues to the mechanism were elucidated in either trial. The third trial invited previous participants to complete a rechallenge and did not show any protein specific changes in GI symptoms. Gluten did, however influence current feelings of depression, but had no influence on depression as a personality trait. A very high nocebo response was again found regardless of all background dietary triggers being controlled and reproducibility of symptom induction to gluten was poor. Either the patients did not have NCGS as self-reported or the trial design precluded its recognition because of a high nocebo effect.

Clarification of the phenotype of NCGS patients, the mechanism(s) by which gluten induces symptoms and clinical significance is required. Standardised guidelines into the design and conduct of IBS dietary studies are needed. Patients who believe they have NCGS are likely to benefit from lowering their dietary intake of FODMAPs.

Declaration

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 contains no material previously published or written by another person, except where due reference is made in the text of the thesis. All experimental work was performed by myself except where due acknowledgement is given.

The length of this thesis does not exceed 100,000 words.

Jessica Rose Biesiekierski

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List of Publications

The following papers were published during my PhD tenure (2008-2012) and relate to the individual studies or concepts discussed in this thesis:

- Ong DK, Mitchell SB, Barrett JS, Shepherd SJ, Irving PM, **Biesiekierski JR**, Smith S, Gibson PR, Muir JG. 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):1366-73. *(25 citations as at 12/08/2012)*

- **Biesiekierski JR**, Newnham ED, Irving PM, Barrett JS, Haines M, Doecke JD, Shepherd SJ, Muir JG, Gibson PR. Gluten causes gastrointestinal symptoms in subjects without coeliac disease: a double blind randomised placebo controlled trial. Am J Gastroenterol 2011; 106[1]:508-14. *(63 citations as at 12/08/2012)*

- **Biesiekierski JR**, Rosella O, Rose R, Liels K, Barrett JS, Shepherd SJ, Gibson PR, Muir JG. Fructan and short-chain carbohydrate content of common Australian processed grains and cereals. J Hum Nutr Diet 2011; 24:154-176. *(8 citations as at 12/08/2012)*

- **Biesiekierski JR**, Newnham ED, Gibson PR. Response to Bernardo et al. Am J Gastroenterol 2011; 106(12):2201-2. *(0 citations as at 12/08/2012)*

Please note that the final studies in this thesis (Chapter 7 and 8) have not yet been published due to contractual arrangement in the Australian Research Council Linkage.

Chapter 1 – Background

1.1 Wheat- and gluten-avoidance

Avoidance of wheat-containing products is a worldwide phenomenon. People are avoiding wheat and gluten for putative health benefits. Gluten has been linked to a wide range of conditions including various skin problems,[2] fatigue and migraine,[3] weight gain[4] and autism,[5] moreover wheat and gluten are most often blamed for gastrointestinal (GI) symptoms.[6] The common belief that wheat- and gluten-containing foods are responsible for such a wide range of health problems has led to the soaring demand for specialised wheat- and gluten-free products. In Australia this market is growing at between 15–20% per annum.[7] In the United States, the wheat- and gluten-free market has been estimated to be worth $1.75 billion and is growing by 23% per annum.[8] An Australian Government report suggested that one million Australians purchase 'gluten- and wheat-free' products[7] and in the USA, this figure is suggested to be as high as 8% of the population.[8]

The most common types of functional gastrointestinal disorders (FGID) are irritable bowel syndrome (IBS) and functional bloating.[9] IBS affects approximately 9% of the Australian population[10] and is characterised by abdominal pain, bloating, wind, distension and altered bowel habit but with no abnormal pathology.[11] IBS presents as a major challenge for clinicians, because of multiple contributing factors and conflicting evidence for management strategies and suitable pharmaceutical therapies.

The role of dietary components in inducing gastrointestinal (GI) symptoms is a complex area. The major goals of this thesis were to gain a greater understanding about 'wheat- and gluten-intolerance' and to investigate the components of wheat that are the most likely 'triggers' of GI symptoms – namely protein (gluten) and poorly absorbed short chain carbohydrate (FODMAP).

1.2 Components of wheat – gluten

1.2.1 Structure of gluten proteins

Gluten is the main storage protein contained within the germ of wheat grains.[12] The wheat kernel contains 8-15% of protein from which 10-15% is albumin/globulin and 85-90% is gluten (see Figure 1.1). Gluten is a complex mixture of hundreds of related but distinct proteins, mainly gliadin and glutenin. Different wheat varieties vary in their protein content and in their composition and distribution of the gluten proteins.[12-14] Collectively, the gliadin and glutenin proteins are referred to as prolamins, which represent seed proteins insoluble in water, but extractable in aqueous ethanol, and are characterised by high levels of glutamine residues (38%) and proline residues (20%).[15,16]

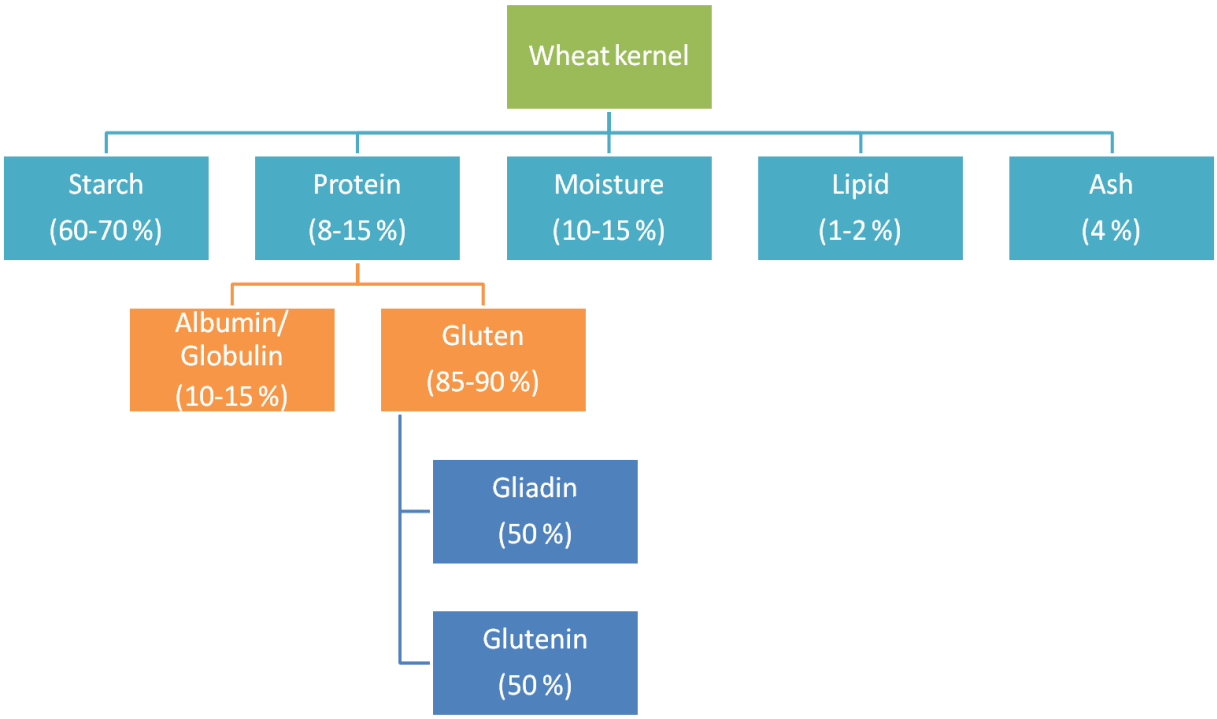

Figure 1.1 Approximate breakdown of wheat components

Gluten protein networks are among the most complex due to their different components and sizes, and the variability caused by genotype, growing conditions and technological processes.[12] Wheat gluten can be classified into three groups, each with a number of subgroups (as shown in Figure 1.2) dependent on key structural differences.[17]

- sulphur-rich (S-rich) with a molecular weight (MW) of ~50 kD
- sulphur-poor (S-poor) with a MW of ~50 kD
- high molecular weight (HMW) with a MW of ~100 kD

The individual gluten proteins are associated by strong covalent and non-covalent forces, and together with the structures and interactions of these proteins contribute to the unique properties of gluten.[18] Gliadin contains peptide sequences (known as epitopes) that are highly resistant to gastric, pancreatic and intestinal proteolytic digestion in the GI tract, escaping degradation in the human gut.[19-21] This is because many proteases are unable to cleave the peptide bonds located amino- or carboxy-terminally to proline.[19]

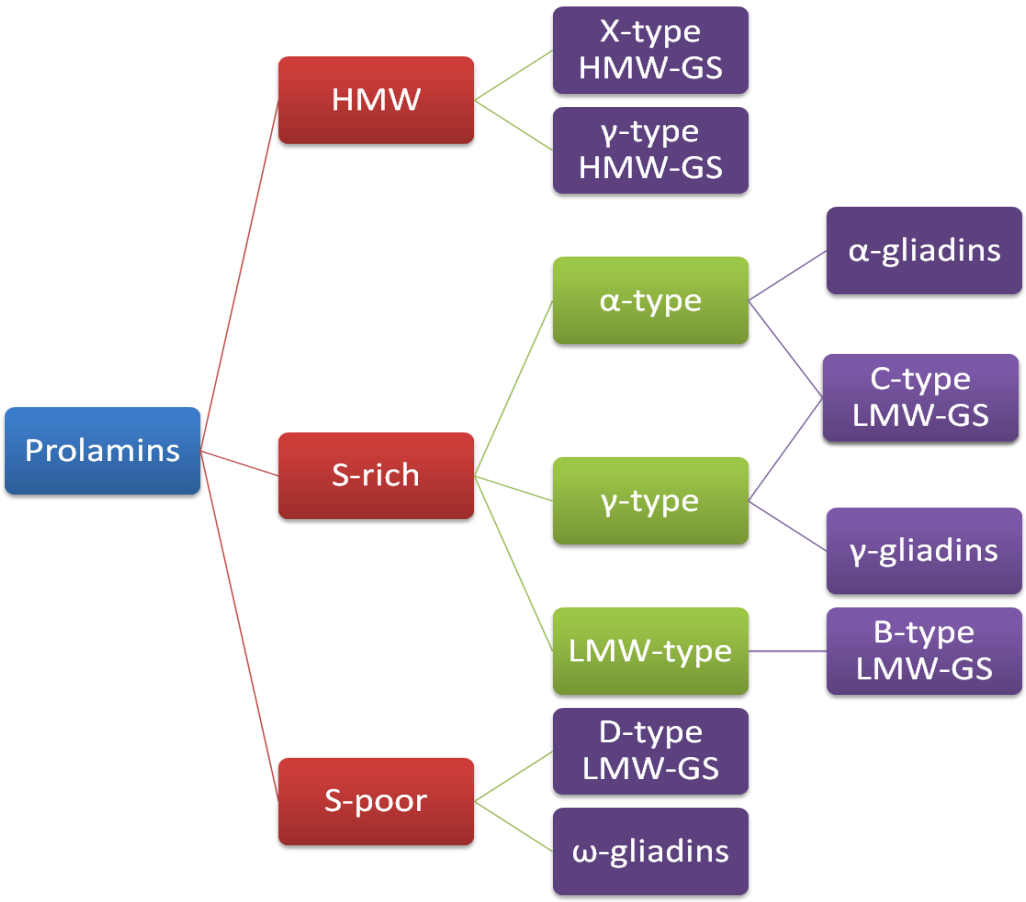

Figure 1.2 Breakdown of wheat gluten prolamins (HMW, high molecular weight; LMW, low molecular weight; GS, glutenin subunits; S, sulphur)[17]

1.2.2 Gluten properties and sources

The gluten matrix and its resulting functions are essential to determining the dough quality of bread and other baked products and are, therefore, present in many flours, pastas, cakes, pastries, biscuits and cereals. The gliadin and glutenin components each have different functions crucial in determining the 'viscoelastic' (entrapment of carbon dioxide released during bread leavening) properties of dough and quality of the end product. Purified hydrated gliadins contribute to the viscosity and extensibility of the dough, where as hydrated glutenins are cohesive and contribute to dough strength and elasticity.[12] Much work has focused on improving dough strength, for example increasing the HMW subunit gene copy number for increased glutenin elasticity.[18,22,23]

Gluten is heat-stable and has the capacity to act as a binding and extending agent, and is commonly used as an additive in processed foods for improved texture, moisture retention and flavour. Thus, less obvious sources containing gluten-derived ingredients are found in batter, seasonings, stuffings, or fillers and coatings used in medications or confectionary.

Similar proteins to the gliadin found in wheat, exist as secalin in rye, hordein in barley and avenins in oats, and are collectively referred to as 'gluten'. Derivatives of these grains such as triticale and malt and other ancient wheat varieties such as spelt and kamut also contain gluten. The gluten found in all of these grains has been identified as the component capable of triggering the immune-mediated disorder, coeliac disease.

1.2.3 Dietary gluten intakes

Wheat is an important staple food because of its high nutritional characteristics, technological properties and long shelf life. Wheat is a good source of several nutrients and is a fermentable substrate for the human colonic microflora, which conveys substantial benefits to the host. These include subsequent expansion of bacterial populations, bifidobacteria and lactobacilli (believed to mediate a range of responses),[24-26] and also produces a number of important by-products such as short-chain fatty acids (SCFA). Wheat can form the basis of all daily meals and is eaten in large amounts worldwide. The National Health and Medical Research Council of Australia (NHMRC) report cereals to be one of the most important food groups,[27] where the average daily gluten intake in a Western diet is thought to be around 10-20 g/day.[28] While wheat-containing bread is one of our major sources of gluten (each slice of bread contains approximately 4 g of gluten), there is some evidence that exposure to gluten may be increasing with changes in cereal technology. Modern baking practices have shortened bread leavening, increased the use of chemical/yeast leavening agents, and also increased inputs of nitrogen fertilizer and agrochemicals for higher yields of protein content required for bread making.[18,20,29]

1.3 Components of wheat – carbohydrates

Wheat protein, in particular, gluten has been blamed for various problems, of which GI complaints are perhaps one of the most common.[30] Other components of wheat have been studied in detail, such as the carbohydrate or more specifically, the fructan component.[31-37] The findings of these studies highlight the importance of wheat consumption for health benefits, but also provide some understanding into the relationship between wheat and GI symptoms in patients with IBS.

Most foods including whole grain cereals, legumes, fruit and vegetables contain a wide range of carbohydrates essential for health. Carbohydrates are a diverse group, classified according to their chemical, physical and physiological properties.[38] Primary classification is based on their chemical form including degree of polymerisation (DP), the type of linkage/bonds and character of monomers.[39] The major classes of importance to human nutrition are the 'short-chain' carbohydrates; monosaccharides (DP = 1) including glucose, sucrose, fructose and polyols, the disaccharide (DP = 2) lactose, the oligosaccharides (DP 3-9) especially galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), and the 'long-chain' carbohydrates; polysaccharides (DP ≥10) including starch, non-starch polysaccharides (NSP) and inulin.[38-40]

Aside from the provision of energy, carbohydrates can affect satiety,[41] blood glucose and insulin responses, lipid metabolism,[42] may be immunomodulatory,[38] and may also influence calcium absorption.[43] In relation to the health of the GI tract, colonic function, bowel habit, transit, the metabolism and balance of microflora and large bowel epithelial cell health can also be affected.[31,38] Many of the physiological properties and health effects can be attributed to the primary chemical form of the carbohydrates and their physical properties,[38] including the location and extent of absorption and fermentation.

Dietary non-digestible carbohydrates (resistant starch, NSP, dietary fibres, non-digestible oligosaccharides of plant origin) escape digestion in the small intestine,[26] producing unique end-products (SCFAs acetate, propionate and butyrate) that may offer protection against colorectal

cancer.[44] Inulin and its FOS derivatives, together with various forms of GOS have attracted a great deal of research interest for their potential health benefits as prebiotic oligosaccharides.[45-49] They selectively promote the growth of beneficial bifidobacteria and lactobacilli in the human gut.[50] Wheat is one of the most common sources of inulin-type fructans[33] and has been studied extensively in well-designed human studies, for example daily intakes of 3.5-7 g per day of FOS and inulin as pure powders for a few weeks have been found to have prebiotic effects, significantly changing the composition of human faecal flora.[25]

Aside from the well-established health effects mentioned above, indigested or malabsorbed fructans result in subsequent delivery to the colon providing a substrate that is rapidly fermented,[33,40] with the potential to induce abdominal symptoms.[51,52] Other short-chain carbohydrates that can be poorly absorbed include the monosaccharide fructose, disaccharide lactose, the sugar polyols – sorbitol, mannitol and xylitol, and oligosaccharides especially GOS. Their poor absorption and rapid fermentation by bacteria in the small and proximal large intestine[9] can induce luminal distension via a combination of osmotic effects and gas production and trigger GI symptoms in people with visceral hypersensitivity or abnormal motility responses[53,54] (further discussed in this Chapter, Section 1.8).

1.4 Clinical problems – Irritable bowel syndrome

1.4.1 Background – irritable bowel syndrome

Patients with symptoms of IBS represent 20-50% of referrals to gastroenterologists, although there are many who do not seek medical care.[55,56] It is recognised as one of the most common functional GI disorders, affecting around 9% of the Australian population.[10] Globally, the range of the affected population is reported to be between 3-20% in North America, 1.1-21.6% in Europe and 2.3-8.5% in Asia.[57]

Gastrointestinal infection,[58-60] low-grade inflammation in the intestinal wall,[61] and food components that influence the composition of the microbiota[62] have all been proposed as potential causes of IBS. There has, however, been no single biological abnormality identified to explain the recurrence of symptoms in IBS. The physiological basis for the genesis of many functional gut symptoms is likely to be luminal distension. Evidence for this comes from barostat and gas infusion studies.[53,63] Luminal distension not only induces symptoms of pain, sensation of bloating and visible abdominal distension, it may also lead to secondary motility changes. An osmotic load and rapid gas production can further result in luminal distension, motility changes (such as delayed or accelerated small bowel and colonic transit) and potential laxative effects, leading to abdominal discomfort and disturbed bowel function[64] Patients with IBS experience an enhanced perception of visceral events throughout the GI tract,[56] are more likely to notice contractions[65] and gas,[66] and may have elevated somatic pain thresholds.[67,68]

1.4.2 Diagnosis of irritable bowel syndrome

In the early 1990's, the Rome Foundation introduced a standard for the classification and diagnosis of functional GI disorders, termed the Rome criteria.[69] The Rome III diagnostic criteria, developed by an expert international consensus, is the current standard for IBS and is outlined in Table 1.1.[70]

Generally, IBS is recognised when the occurrence of abdominal pain and discomfort are present concurrently with altered bowel habits that cannot be explained by any other physiological, biochemical or inflammatory cause.[6] Because IBS is multi-factorial in its aetiology and heterogeneous in its clinical presentation and pathogenesis, it is imperative that exclusion of other conditions and detectable organic causes with similar presentations is undertaken. Alarm or warning signs include worsening or major change of symptoms, family history of colon cancer or inflammatory bowel disease (IBD), raised coeliac antibodies, rectal bleeding, significant unexplained weight loss and new onset of symptoms.[56]

Table 1.1 Definition of IBS according to the Rome III diagnostic criteria[70]

Guidelines	Criteria (≥ 2 must be present)
Recurrent abdominal pain or discomfort* for at least 3 days per month in the last 3 months, associated with two or more of the listed symptom criteria	1) Improvement with defecation
	2) Onset associated with a change in frequency of stool
	3) Onset associated with a change in form (appearance) of stool

*'Discomfort' is an uncomfortable sensation not described as pain

The burden of IBS and its associated symptoms have a detrimental effect on health-related quality of life.[71-74] In addition to the diagnostic criteria characterising symptoms of abdominal pain and changes in bowel habit (ranging from diarrhoea to constipation), other common symptoms of IBS include excessive wind, abdominal bloating and distension.[75] Non-bowel symptoms including fatigue and lethargy, back pain and headaches are also commonly reported.[71,76]

Co-morbidities of anxiety and depression are commonly reported in IBS, but it is not known whether this is a consequence, part of the symptom array or a vulnerability factor.[77-79] Peripheral mechanisms have been suggested, involving signalling from visceral afferents to the brain, emotion regulation and modulation of GI function by stress and emotions.[78] Perceptual hypersensitivity to gut signals is thought to be common with IBS.[80] Alterations in brain responses to controlled gut distension and expectation of gut discomfort have also been reported.[81] This project will address the difficulty of assessing depression, fatigue and cognitive impairment by using some novel approaches.

1.5 Clinical problems – coeliac disease

1.5.1 Background – coeliac disease

Dietary gluten unequivocally causes coeliac disease, a common immune-mediated disease that affects 1% of Western populations.[82,83] Coeliac disease is the best studied of the spectrum of disorders related to gluten ingestion, being first described in 1888,[84] and then in 1950, when a Dutch paediatrician, Dicke, established the link between the feeding of wheat flour and coeliac disease.[85,86] The immune response triggered is specific to toxic peptides within the gliadin fraction of the gluten protein[87] and initiates an immune response causing mucosal inflammation, small intestinal villous atrophy[88] and increased gut permeability.[89]

The damage in the small intestine can cause common functional GI symptoms (diarrhoea, constipation, excessive wind, bloating) that may or may not relate to malabsorption. These can disrupt nutrient absorption from the intestine leading to nutritional implications.[90] Other non-GI presentations potentially secondary to the malabsorption were historically observed (listed in Table 1.2), although are seen less so today.[91] Although the predominate or 'classic' presentation of coeliac disease is GI manifestations and malabsorptive symptoms, a recent shift in clinical presentation has been towards milder symptoms, silent or atypical presentations.[83,91] Regardless, all coeliac disease patients are exposed to the risk of long-term complications including infertility and lymphoma[92] (further complications are listed in Table 1.3).

Table 1.2 Non-gastrointestinal symptoms secondary to malabsorption[90]

Non-gastrointestinal symptom:	Nutritional association:
Peripheral neuropathy	Vitamin B12 and B1 deficiency
Anaemia, chronic fatigue	Iron, vitamin B12 and folate deficiency
Reduced bone density **Bone or joint pain**	Vitamin D and calcium deficiency leading to osteoporosis and osteopenia
Muscle cramps	Magnesium and calcium deficiency
Night blindness	Vitamin A deficiency
Weight loss **Growth failure in children i.e., short stature**	Impaired absorption of most nutrients
Oedema	Protein and albumin loss
Weakness	Hypokalemia and electrolyte depletion
Bleeding and hematoma	Vitamin K deficiency

Table 1.3 Examples of long-term complications shown to have an increased risk in untreated coeliac disease

Health Problem	Presentation	Possible association	Reference
Neurological symptoms	Depression	GI symptoms	73
	Ataxia (damage to the cerebellum with positive gluten serological markers), epilepsy, migraines, peripheral neuropathy	Suggested that anti-tTG immunoglobulins compromise neuronal function	93-96
Dermatitis herpetiformis	Skin manifestation of coeliac disease, presenting with a blistering rash and pathognomonic cutaneous IgA deposits	Reaction between gluten and dermal elastin, although the pathogenesis remains unknown. Diagnosis is based on skin biopsy and serological evidence of coeliac-type autoimmunity	97
Reduced fertility, delayed puberty, early menopause, miscarriage		Unclear but implicated to malnutrition	98
Autoimmune disorders	Insulin dependent type-1 diabetes, thyroid disease, autoimmune liver disease	Combination of a similar genetic background (HLA alleles) and immunological alterations	90,99
Malignant diseases	Small bowel adenocarcinoma, oesophageal carcinoma, T-cell lymphomas such as non-Hodgkin lymphoma	Trends appear concomitantly; combination of a similar genetic background (HLA alleles) and immunological alterations	99,100
Other	Stomatitis, arthritis, dental enamel defects	Combination of a similar genetic background (HLA alleles) and immunological alterations	93-96,99

1.5.2 Physiological mechanisms of coeliac disease

The pathological process of coeliac disease is well understood where, in the small intestine, gliadin escapes degradation due to the rich proline content imparting resistance to digestive protease action.[19-21] Post-translational modification (deamidation) of the gliadin peptides by tissue transglutaminase (tTG) forms negatively charged amino acids that bind better to the disease associated human leukocyte antigen (HLA)-DQ2 or -DQ8 receptors on the cell surface of antigen-presenting cells (APCs).[101] Once bound, this complex is presented with high affinity to the major histocompatibility complex (MHC) Class II T-cells. The CD4+ T-cell activation leads to the secretion of pro-inflammatory cytokines dominated by IFN-γ and TNF-α,[102,103] inflammation and damaged intestinal villi.[101,104-106] In addition, it is believed intestinal tight junctions loosen, allowing increased entry of antigenic peptides and consequently further development of immune events.[107]

A variety of sequences from α-, γ-, and ω-gliadins, as well as from the glutenins have been identified to activate T-cells in coeliac disease, although several hundred gluten peptides are predicted to be immunogenic.[104,108-110] The most immunodominant T-cell epitope is from α-gliadin[87] and is also found in multiple copies in a longer peptide, 33 amino acids long – p57-73 (QLQPFPQPQLPYPQPQS).[111] T-cell cross-reactivity against gluten-, secalin-, and hordein-derived peptides have been confirmed. Additionally, within each grain there exists a distinct hierarchy of immunostimulatory gluten peptides.[112] Of all the peptides known to stimulate the inappropriate T-cell response in coeliac disease, a given patient may react to only a few.[113] Although there is a low rate of avenin-specific T-cell responses after oat challenges in certain coeliac disease patients[114-118] and reports of oats being tolerated by most,[119] there have also been reports of patients with coeliac disease who are oat sensitive and develop histologic damage.[120,121]

1.5.3 Genetics of coeliac disease

The immunogenicity of α-gliadin is under control of the MHC genes,[122] where the genetic susceptibility locus (HLA-DQ2 or -DQ8) is expressed in 99.4% of coeliac patients.[102,123] HLA molecules bind toxic α-gliadin short peptides for presentation to CD4+ T cells, triggering T-cell proliferation,[124] and leading to increased density of intraepithelial lymphocytes (IELs) and shortening epithelial height.[125]

The functional heterodimer HLA-DQ2 is encoded by the alleles *DQA1*05* and *DQB1*02*.[126] HLA-DQ2 is present in 90-95% of affected coeliac disease individuals and 20-30% of the general population.[127] Although a very small number of coeliac disease patients have been reported in whom only one DQ2 allele is present.[128] The HLA-DQ8 heterodimer is composed of the *DQA1*03* and *DQB1*03:02* alleles.[129] HLA-DQ8 is present in 10% of the general population and 5-10% of individuals with coeliac disease.[129,130] Linkage studies have identified other chromosomal non-HLA complex genes that may contribute, but the nature and effects are not well described.[91,105,131] The T-cell epitope hierarchy has been shown to be critically determined by the HLA-DQ2 or -DQ8 status.[112]

Given this genetic association, high-risk groups for coeliac disease include relatives of coeliac patients and those with co-existing autoimmune conditions, including insulin-dependent diabetes mellitus, Down's syndrome, multiple sclerosis, and Williams or Turner syndrome.[108]

1.5.4 Diagnosis of coeliac disease

The prevalence of coeliac disease appears to be increasing. The most likely contributor to this is an increase in clinical awareness,[132,133] an improved definition and improvements in diagnostic tests.[133-136] Despite this, coeliac disease remains undiagnosed in the majority of patients.[83,137] Data from epidemiological studies of large cohorts in North America and Europe, resulted in one review estimating that there are approximately 7-10 undiagnosed cases for each diagnosed coeliac patient.[90]

According to European Society for Paediatric Gastroenterology, Hepatology and Nutrition criteria published in 1990, which remain the most widely accepted, the diagnosis of coeliac disease is based on the demonstration of small intestinal mucosal damage.[138] New diagnostic criteria published by the ESPGHAN suggest this may not always be the case.[139] Particularly, in a subgroup of children with high serum transglutaminase 2 antibody values, coeliac disease could be established without histological confirmation. Early results have indicated that this approach may be able to be extended to the adult population.[140] Nevertheless, until studies confirm this, coeliac diagnosis is made using a combination of:

(i) Histological findings from small bowel biopsy (via gastroscopy) including villous atrophy, crypt hyperplasia and intra-epithelial lymphocytosis. Figure 1.3A shows a normal biopsy with numerous surface villi. In contrast, Figure 1.3B shows an individual with untreated coeliac disease and total villous atrophy with flattened abnormal surface epithelial.[91]

(ii) Raised coeliac disease-associated antibodies (serology), such as for deamidated gliadin peptide (DGP) IgA and IgG, endomysial IgA or transglutaminase IgA

(iii) Histological, serological or clinical improvement after adherence to a gluten-free diet (GFD)

(iv) Specific allelic variants in the two HLA genes: HLA-DQA and HLA-DQB

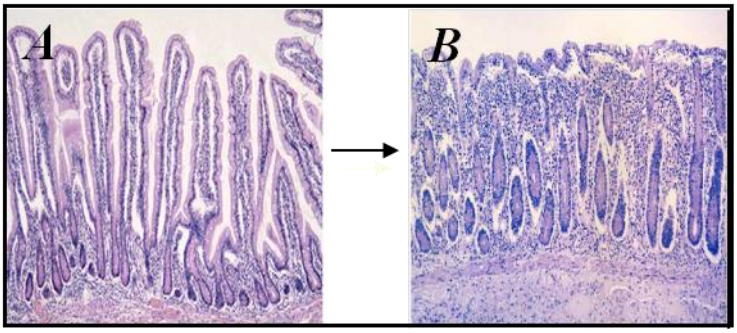

Figure 1.3 Small intestinal biopsy; A) Biopsy from subject showing healthy villous, B) Biopsy from coeliac disease patient showing atrophic villous[141]

Table 1.4 further outlines the main diagnostic approaches used to determine coeliac disease, of which there are several admissions needed. First, the expression of the HLA-class II haplotypes, DQ2 and DQ8 is necessary, but not sufficient to develop the disease.[90] The genotype is of important screening value, given it is the only test that seems capable of excluding coeliac disease for life.[128,142] Secondly, adequate dietary gluten intake is required prior to having the gastroscopy and serology. If gluten intake has already been removed or reduced, gluten challenges should be implemented and should comprise a daily intake of at least 10 g of gluten for a minimum four weeks.[90,91,143] In addition, the duodenal mucosa can be patchy, requiring a multiple-biopsy regime[144] and, although serological tests are predictive, they alone are not sufficient for diagnosis. Finally, a single serological test for coeliac disease is inadequate to exclude coeliac disease for life;[145] for example, in a cohort enrolled in 1974 and followed up in 1989, the seroprevalence of coeliac disease more than doubled from 0.2% to 0.5%.[134]

Marsh identified and classified a spectrum of consecutive stages of disease activity based on mucosal abnormalities, ranging from minimal change lesions - Marsh I and II (termed latent-coeliac disease), to villous atrophy - Marsh III (active coeliac disease),[131,141] and finally to the rare histological finding of flat atrophic mucosa - Marsh IV lesion, related to refractory coeliac disease and the development of enteropathy-associated T-cell lymphoma (EATL).[143]

Table 1.4 Features of tests for the diagnosis of coeliac disease

Test	Outline	Characteristics	Disadvantages	Advantages
HLA typing	Expression of alleles encoding HLA-DQ2 and -DQ8 genes investigated by polymerase chain reaction (PCR) sequence-specific oligonucleotide typing[146]	HLA-DQ2 and/or HLA-DQ8 positive	• Low specificity[146]	• Highly sensitive as >99% coeliac patients carry these alleles • Independent of disease activity or diet[146]
Serology	Antibody titres measured at diagnosis and then confirmed with disappearance after treatment[105] using ELISA assays or commercial indirect immunofluorescence test[146]	• Elevated antibodies to gliadin (IgA-AGA, IgG-AGA)[147] • deamidated gliadin peptides (DGP) antibodies (IgA and IgG) • Endomysial antibody positive (IgA anti-EMA) • Elevated concentrations of anti-tissue transglutaminase (TTG, a-tTG, TTA)	• Titres can show false negative and false positive results • Affected by diet, immunosuppressants and additional disorders[91] • Selective IgA deficiency incidence 1:400–500[148,149] (associated with 10–20-fold increased risk of coeliac disease)[150-153]	• DGP antibodies higher sensitivity and specificity than AGA counterparts[154] and similar to tTG • tTG antibodies have high (85-90%) specificity[141,147]
Intestinal biopsy	• Endoscopic biopsies of duodenum 1st to 3rd parts • Jejunal biopsy via capsule seldom performed now • Ideally after adequate gluten intake[155]	• Partial to total villous atrophy and decreased villous:crypt ratio, elongation of crypts (crypt hyperplasia)[155,156] • Increased intraepithelial lymphocyte (IELs) count >25/100 cells[146,157]	• Villous atrophy can be patchy[91] • 1st part duodenal biopsies may increase yield[158] • Partial villous atrophy can be undetected[155] • Invasive and expensive[159]	Remains gold standard for both coeliac disease diagnosis and in determining adequacy of mucosal remission on GFD

1.6 Other wheat-related illnesses – allergy

Clinical manifestations of reactions to foods can vary in degrees of severity and causation. The great challenge is to precisely characterise adverse reactions given the diversity of possible mechanisms at play and overlapping, non-specific symptoms. Differentiating food allergies (see definition below) from food intolerances is based on a combination of clinical, biological, genetic and histological data. Specific diagnosis of the three wheat-related conditions (wheat allergy, coeliac disease and the suggested entity of 'non-coeliac gluten sensitivity' further discussed in Section 1.7) can follow the algorithm shown in Figure 1.4.[160]

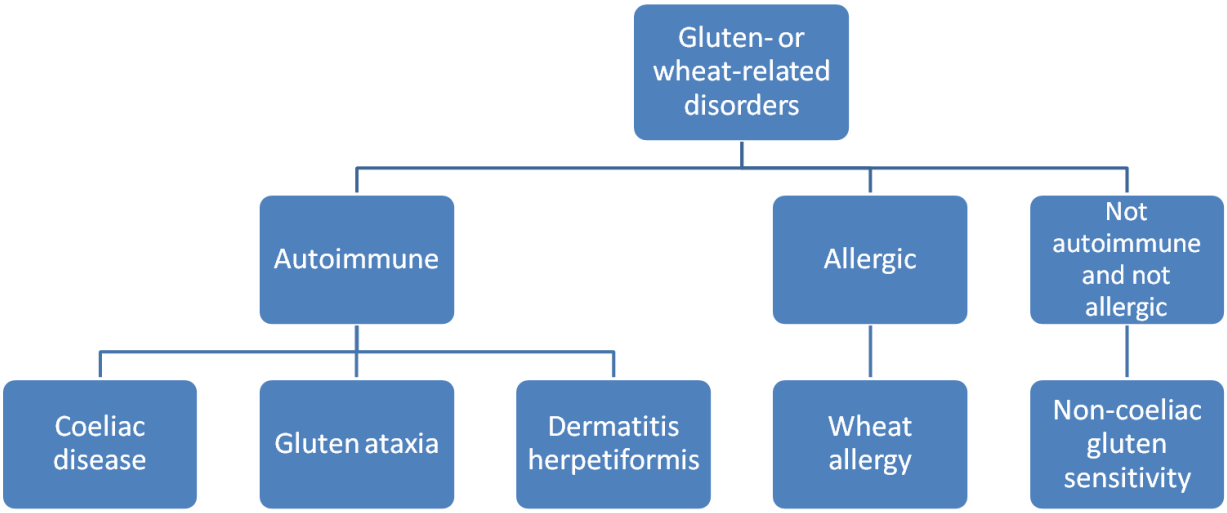

Figure 1.4 Classification of gluten- or wheat-related disorders[160]

A food allergy is defined as an "adverse immune response that occurs reproducibly on exposure to food",[161] where symptoms typically have a rapid onset within minutes to hours of ingestion and can involve many organ symptoms. There is no reliable data regarding the prevalence of food allergy in Australian adults. In children, wheat is listed as 1 of the 6 most commonly implicated allergens with skin allergies (alongside milk, soy, peanuts, eggs, and fish),[162-164] where wheat allergy has been confirmed by double-blind, placebo-controlled food challenges in subjects with atopic dermatitis.[162,165-167] Wheat allergy has been classified into classic food allergy affecting the skin, GI

tract or respiratory tract; wheat-dependent, exercise-induced anaphylaxis (WDEIA); occupational asthma (baker's asthma) and rhinitis; and contact urticaria.[160]

There has been little progress in understanding the mechanisms of food allergy, particularly those involved at the molecular level.[168] It is thought wheat allergy involves cross-linking of immunoglobulin E (IgE) by repeat sequences in gluten peptides, which triggers the release of chemical mediators including histamine from basophils and mast cells.[169] There is no direct evidence to suggest that classical IgE mediated type 1 allergic reactions to food antigens play a role in the symptoms of IBS.[170,171]

Traditional measures of type 1 IgE-mediated food allergy, such as serum specific IgE and RAST assays, and skin prick tests continue to be used for diagnosis of wheat allergy, regardless of their poor predictive value (less than 75%).[160] This is because there is serological cross-reactivity between cereal allergens and grass pollens, mixed commercial reagents used for skin prick tests[172,173] and their results are generally identical to healthy controls.[174] Given there are no laboratory tests that will confirm or exclude food allergy with certainty, diagnosis depends on carefully obtained clinical histories and the application of double-blind, placebo-controlled food challenges.[175]

1.7 Evidence for gluten as a trigger of symptoms in adults without coeliac disease

Evaluation of 'exclusion diets' has consistently shown wheat to be one of the most common factors inducing GI symptoms.[171] Dickerson and colleagues first identified that wheat may cause different kinds of chronic ill-health in individuals without coeliac disease in 1978.[176] Ellis and Linaker also described normal biopsies and normal lymphocyte counts existing in combination with rapid disappearance of symptoms when their patients withdrew gluten from the diet.[177] The frequency of patients presenting with IBS-type symptoms similar to coeliac disease who are reportedly responding well to a GFD, but have no other clinical or diagnostic biomarkers of coeliac disease, is

increasing and has recently been claimed to affect up to 15% of the population.[178] The growing gluten-free market suggested to be between 15–20% is a figure not confined to only coeliac patients.[7] Gluten has also been linked to a wide range of conditions including various skin problems,[2] fatigue and migraine,[3] weight gain,[4] and autism.[5] These gluten reactions appear to involve neither allergic nor immune mechanisms and, for patients with no features of coeliac disease, they are so-called 'non-coeliac gluten sensitive' (NCGS). While coeliac disease is a well-established entity, the evidence-base for NCGS is poor; for example, there have been no adequately-designed studies investigating whether gluten is directly responsible for generating intestinal symptoms and mucosal inflammation in individuals without coeliac disease.

To be somewhere between coeliac disease and IBS, has been described as the "no man's land of gluten sensitivity"[179] and has created speculation,[180] highlighting the need for definitive research to clearly identify gluten as a dietary trigger in patients without coeliac disease, and to importantly, allow a plausible explanation of the pathogenesis of NCGS.

The effect of gluten outside of coeliac disease has been limited to human in vitro cancer cell lines and uncontrolled clinical studies.[181-183] Gliadin has been shown to increase epithelial permeability and alter protein expression of components of the tight junction in Caco-2 (human colon adenocarcinoma) cells, used as a surrogate model for the human gut epithelium.[184] In this same line of cells, gliadin was shown to induce apoptosis[185,186] and increase oxidative stress.[187] Studies using animal models of gluten-sensitivity (not coeliac disease) have also directly investigated the role of gluten challenge in inducing gut dysfunction[185] and changes in neuromotor function and microbiota independently of inducing intestinal inflammation or injury.[188]

Functional GI disorders have already been shown to have an immune basis and there have been some studies on gluten exclusion diets in support of the reduction of gluten containing foods in improving symptoms in IBS patients.[181] Wahnschaffe and colleagues identified the gluten sensitivity concept particularly in individuals carrying the HLA-DQ2 allele and who did not have villous atrophy on duodenal biopsy, with varying pathological, immunological (positive IgA anti-

gliadin or anti-TTG antibodies and higher IELs)[146] and symptomatic improvement.[181] However, these coeliac-specific HLA antigens have not been found in other studies.[189] Sapone *et al* suggested innate immunity and the epithelial barrier function of the intestinal mucosa may have a pathogenic role in gluten sensitivity.[190] Immunological markers (including serum IgE, eosinophil counts, histamine release) have appeared normal in NCGS.[171] Although these studies provide a strong rationale for the clinical question of gluten as a symptom inducer in some IBS patients, these were mostly uncontrolled studies and have also reported subjects with intraepithelial lymphocytosis in the duodenum.

Although many people report that a GFD relieves their gut symptoms, it is not known whether it is the removal of protein (gluten) or of some other component in wheat (e.g., fructans) that is known to be capable of provoking GI symptoms themselves.[51] What is needed is a definitive experiment where the effect of gluten that is free from contamination from carbohydrates with the potential to induce symptoms is evaluated in patients with IBS where coeliac disease has been definitively excluded. This is the target of the present thesis.

1.8 Dietary managements for IBS

The functional disorder of IBS is characterised by chronically recurring abdominal pain or discomfort and altered bowel habits, and is one of the most common syndromes seen by gastroenterologists and primary care providers.[56] There are various treatment options for individuals with IBS, but convincing data for the use of pharmacologic and psychological treatments are limited and not supported by high quality, well-designed randomised, controlled trials. Currently available drugs are targeted to the management of individual symptoms, such as constipation (using osmotic laxatives, fibre or bulking agents), diarrhoea (using anti-diarrhoeal agents), and abdominal pain (using antispasmodic agents). Psychotherapy or cognitive behavioural therapy (a combination of cognitive and behavioural techniques) have also been studied as psychological treatments for IBS.[191]

A relationship between symptoms and food intake, has issues surrounding diagnostic tools, inaccurate patient observations and limited well-designed dietary trials resulting in questionable outcomes[192] Regardless, various dietary therapies including increasing dietary fibre intake, addition of probiotics, and complex exclusion diets – such as gluten-free, wheat-free, anti-candidal, carbohydrate-free – are often explored.[6,193,194] Evidence for efficacy of these is anecdotal at best. There is however, an increasing evidence base that certain food components can indeed contribute to symptoms through the effects of malabsorption of carbohydrates.[195,196]

1.8.1 FODMAPs

1.8.1.1 Background – FODMAPs

Poorly absorbed short-chain carbohydrates have been grouped together into a collective term – FODMAPs (Fermentable Oligo-, Di-, and Mono-saccharides And Polyols (FODMAPs),[75,196] due to similarities of effects in the intestine. They have been shown to induce GI symptoms in the majority of patients with IBS.[52,196-200] Evidence for the efficacy of the low FODMAP diet began with a retrospective study of patients with IBS and fructose malabsorption on a low-fructose/fructan diet[75] where 74% of patients reported symptomatic improvement. A randomised, placebo-controlled rechallenge trial in patients with IBS with fructose malabsorption was then undertaken.[196] All patients improved on a low-fructose/fructan diet, with induction of symptoms by rechallenge of fructose or fructans, further exacerbated by a combination of fructose and fructans.

In Australia, the low FODMAP diet is increasingly being accepted as the primary management strategy for IBS, recently adopted by the 2011 National Therapeutic Guidelines.[201] International studies have shown a low FODMAP diet to be more effective than standard dietary advice in IBS.[202] The low FODMAP approach is not only limited to the treatment of IBS where it has also been shown to improve gut symptoms in more than 50% of patients with IBD, who are experiencing ongoing gut symptoms despite having inactive disease.[203] In patients without a colon, the issue of frequent loose stool production was also reduced significantly.[204]

1.8.1.2 FODMAP mechanism of action

Visceral hypersensitivity is central to the pathophysiological mechanisms that underlie the majority of patients with IBS. For individuals with IBS, their enteric nervous system (ENS) is more likely to respond to normal sensory input, such as distension of the gut, by altering motility patterns and/or by sending messages to the brain that may be interpreted as bloating, discomfort and pain.[192] Dietary FODMAPs have three key physiological effects which contribute to luminal distension and can therefore exacerbate symptoms associated with IBS:[205,206]

(i) *Osmotic effect:* By virtue of the small size of FODMAP molecules and the fact that they remain in the intestinal lumen because of their poor absorption, the osmotic effect leads to more water being present in the lumen in order to maintain the osmolality within the narrow physiological range. In support of this, FODMAP intake has been shown to increase ileostomy output,[195] increase water delivery through the small bowel,[207] and increase severity of diarrhoea (in relation to chain length).[208]

(ii) *Rapid fermentation by bacteria*: FODMAPs are readily fermented by intestinal bacteria and the rate of fermentation for carbohydrates of short-chain length is more rapid than those of longer chain length. This was shown with the increase of daily breath hydrogen production on ingestion of a diet of high FODMAP-containing foods compared to low FODMAP-containing foods, despite similar fibre content.[200] The increased fermentation and associated gas production is likely to increase the distension induced, thereby exacerbating symptom severity.

(iii) *Alter motility:* The osmotic effect of FODMAPs has been shown to accelerate small intestinal and colonic transit.[208,209] In addition, products of their fermentation, SCFA, can promote motility.[210]

Individuals vary in their intestinal absorptive capacity, and thus only malabsorbed FODMAPs are likely to trigger symptoms in sensitive individuals. Fructose and lactose are common examples of FODMAPs that are variably absorbed across individuals, where malabsorption affects around 40% and 1-95% (depending on ethnicity) of the population, respectively.[211,212] Lactose is poorly absorbed mainly due to deficiency of the brush border disaccharidase, lactase. Free fructose and polyols are slowly absorbed in all, due to low capacity absorptive mechanisms, including GLUT5 facultative transporter for fructose[213] and passive diffusion for polyols. Fructans and GOS are poorly absorbed in all, due to the absence of small intestinal hydrolases to split fructose-fructose and galactose-galactose bonds. The degree of absorption can also vary depending on small intestinal transit time and/or small intestinal microbiota (bacterial overgrowth).[214,215] Other potential factors include alterations in the number, composition, function and location of the microbiota.[216]

1.8.1.3 FODMAP food composition

FODMAPs are found in a wide variety of foods and include lactose (in milk), excess fructose (in pears, apples), fructans and FOS (in artichoke, garlic, onions, wheat and rye), GOS (stachyose and raffinose in legumes), and sugar polyols (sorbitol and mannitol in stone fruits and artificial sweeteners) (further detailed in Table 1.5). Knowledge of food composition has been greatly expanded by the measurement of FODMAP content in Australian foods using established methodology of a combination of high-performance liquid chromatography and enzymatic assays.[217,218]

Table 1.5 Dietary FODMAPs and their sources[217,218]

Food component	Dietary form	Common sources
Fructose	Free monosaccharide constituent (fructose in excess of glucose)	Apple, pear, watermelon, honey, high fructose corn syrup, asparagus, artichoke
Lactose	Free disaccharide	Milk, yoghurt, ice cream, soft cheese
Fructans	Fructo-oligosaccharide (FOS) and inulin	Wheat, rye, barley, garlic, leek, onion, asparagus, artichoke, peach, persimmon, watermelon, pistachio, inulin
Polyols	Sorbitol, mannitol, xylitol maltitol, isomalt	Apple, pear, plum, apricot, nectarine, mushroom, cauliflower, reduced caloric sweetener
Galacto-oligosaccharides (GOS)	Raffinose, stachyose	Legumes, chickpeas, lentils

In clinical practice, the low FODMAP diet is usually recommended for 4-6 weeks, following which, rechallenge of any of the potentially well absorbed carbohydrates (that is, fructose, lactose, sorbitol and mannitol) can be undertaken. Tolerance to fructans and GOS can then be tested. The published tables of food composition currently available focuses on fruits and vegetables.[217,218] Continuing to expand the FODMAP food composition lists is important for improved nutritional adequacy, wider spread application and further development of the low FODMAP approach.

1.8.2 Other management strategies for IBS symptoms

Many approaches and studies have been undertaken to understand the role specific foods have in provoking symptoms of IBS. Elimination diets consisting of individual foods – a single fruit, meat or rice,[171] or by various exclusion diets[219-221] have all been used to identify food intolerance(s) in IBS patients. There is some clinical support for an approach involving elimination of food chemicals, which incorporates restriction of chemical substances including salicylates, benzoates, and other common food allergens,[192,222] listed in Table 1.6.

These problem chemicals and additives are suggested to be other sources of GI symptoms due to stimulation of hypersensitivity through food chemical ingestion.[223,224] However, evidence is scarce, particularly for the prevalence of food chemical sensitivities in GI conditions.

Table 1.6 Food chemicals and their sources restricted during the elimination diet for food chemical sensitivity[225]

Chemical	Sources
Salicylates (monohydroxybenzoates)	Widely found in fruits, vegetables, herbs, spices, nuts, tea, coffee
Glutamates	Food additives
Amines	Chocolate, canned/smoked fish, sauces, stock, nuts, seeds, vinegar, some fruit and vegetables
Monosodium gluatamate (MSG)	Strong cheeses, soy sauce, as flavour enhancers
Other	Preservatives, benzoates, propionate, sulphites, nitrites, sorbic acid, added antioxidants and colours

Most of the evidence for the use of the elimination diet for food chemical sensitivity is in conditions affecting the nose (rhinitis),[226] respiratory tract,[227,228] skin (eczema and urticaria),[226] and behaviour, such as attention deficit hyperactivity disorder (ADHD).[229] Additionally, in clinical practice, food chemicals have received some attention in the pathogenesis and management of headaches and anaphylactoid reactions.[192] Much research is still needed before we fully understand the mechanistic concept. However, it has been suggested that natural food chemicals potentially stimulate the ENS,[223] inducing their effects through stimulation of nerve endings in hypersensitive people.[230] They may be able to activate transient receptor potential (TRP) channels,[231,232] which appear to be central to visceral hypersensitivity, shown in an animal model.[233] Salicylates are currently the best studied food chemical, having been shown to trigger hives (urticaria), anaphylactoid reactions and asthma in susceptible individuals.[234,235] Salicylates may have a direct effect on mast cells producing cysteinyl leukotrienes,[236] which are pro-inflammatory, promote smooth muscle contraction, and increase vascular permeability.[237]

1.9 Dietary management for coeliac disease – the gluten-free diet

1.9.1 Definition of the gluten-free diet

The only available treatment for coeliac disease is life-long strict avoidance of gluten-containing foods.[110] In people with coeliac disease, 50 mg is generally considered to be the minimum quantity of gluten needed to induce damage to the lining of the small intestine.[238] Clinical effectiveness of the GFD as treatment for coeliac disease is well documented, including for clinical improvement of symptoms and nutrition, for disease remission and prevention of long-term complications.[239-242]

The Codex Alimentarius Standard (CODEX STAN 118 – 1979; adopted in 1979 and last revised in 2008) used in European countries, defines "gluten-free" as food having less than 20 ppm gluten[243] and recommend the enzyme-linked immunoassayR5 Mendez Method be used for measurement.[244] The R5 ELISA test kits do not detect the avenins in oats or malt products, which require separate assays.

In Australia, the GFD is completely devoid of detectable gluten. Food Standards Australia New Zealand (FSANZ; Standard 1.2.8) defines 'gluten-free' as having no detectable gluten (sensitive to <3 ppm) and must not contain oats or malt, using the most sensitive and specific testing method.[245] This approach permits inclusion of some gluten-free wheat-derived ingredients; for example, dextrose and glucose are from the carbohydrate component of wheat but the high processing needed to produce glucose leads to the loss of all detectable gluten.[246] A 'low-gluten' claim can be made, which must contain no more than 200 ppm gluten.[245]

The fundamentals of identifying gluten-free foods requires knowledge of food labelling laws, food sources and food processing, and an interpretation of individual ingredients used to make whole foods, all of which vary from country to country.[246] Cross-contamination in food preparation or storage and when eating away from home where caterers (friends, family included) are not aware of all gluten sources can present hidden sources of gluten.[246] The complexity, cost and difficulty of maintaining adequate adherence to a GFD is reflected in the observations that more than half of coeliac disease patients fail to achieve disease remission.[241] Additionally, studies have found the number of coeliac patients maintaining a strict GFD to be as low as 50-70%.[247-250] In a Canadian survey, 44% of 2618 respondents indicated they found the GFD 'very' or 'moderately' difficult to follow.[251] Because gluten-containing foods are commonplace in our society, avoidance of gluten can have social effects (i.e., patients avoiding eating out or travelling).[251-253] Adherence to the diet has thought to also be impacted by the economic burden associated with the diet.[254] One US study found every gluten-free product cost almost three times as much as its wheat-based counterpart.[254] In the UK and other European countries, gluten-free foods are made available on prescription, which has been shown to aid compliance,[255] although this is not available elsewhere including

Australia. Other reported difficulties include accessibility (finding gluten-free food) and palatability (finding good quality gluten-free foods).[251]

Adherence to the GFD is known to benefit from initial education and explanation of dietary management, regular dietetic follow-up and active support group membership (e.g., The Coeliac Society of Australia, of which membership requires a medically certified letter stating GFD requirement).[131,246,252,256]

Markers to assess compliance with the GFD are usually based on a small bowel biopsy histology and serology, on the assumption that continuing villous atrophy and elevated coeliac-related antibodies reflect inadequate compliance. Some research groups have developed scales (derived from questionnaires) such as the 'Celiac Dietary Adherence Test' (CDAT).[257] These may be very useful for population studies of adherence to the diet, but their value in assessing the individual such as detecting minor dietary indiscretions is dubious. Detailed dietary history remains the most commonly used and possibly most accurate tool if expertly taken for assessing dietary compliance. There is no gold standard.

1.9.2 Nutritional implications of gluten avoidance

The growth in the demand for gluten-free products is not confined to coeliac patients and the emerging group of individuals who believe they have NCGS. It has been estimated that consumers of gluten-free products also include people with autism, ADHD or schizophrenia.[7] Other potential consumers of gluten-free products include health-conscious consumers who seek diversity in their diet, consumers interested in trying something different, or are fashion conscious (due to 'antiquity' reasons or following a dietary fad).[7]

Life-long dietary restrictions are inappropriate and unnecessary for people not medically requiring so.[258] Meeting nutritional goals has been found to be easier if following a well-planned GFD, taking into consideration any coexisting deficiencies, intolerances or other malabsorptive conditions.[246] Past research has raised concern of the GFD on lowering total carbohydrate intake

and grain food consumption.[259] Many studies have shown that substitution of staple foods with wheat-free alternatives not fortified or made from refined flour may yield inadequate intakes of energy and nutrient contents, especially B-group vitamins, iron, dietary fibre, folate, thiamin, riboflavin, niacin, calcium, zinc and complex carbohydrates.[246,259-262]

Nutrition education and several national dietary guidelines recommend to "eat plenty of cereal foods, preferably wholegrain and without added fat, salt or sugar".[263] Cereals, grains and their products provide around 30% of total energy intake in British adults, more than any other major food group.[263] Cereals and grains provide much more than carbohydrates, they are valuable sources of dietary fiber, B-vitamins, vitamin E, selenium, zinc, copper and magnesium.[264] Indeed, for Australians in the 1995 National Nutrition Survey, they were found to be the leading source of fibre, thiamin, magnesium and iron.[265] Epidemiology studies and scientific evidence have focused on the role of whole grains in preventing disease, particularly cardiovascular disease and cancer. In the late 1990s, five very large cohort studies[266-271] all showed consuming relatively large amounts of whole grain cereals were linked to lower rates of coronary heart disease (CHD).[263] The exact mechanisms linking grain cereals to disease prevention are not known, but most likely involve several protective factors including GI effects, antioxidant protection and intake of phytoestrogens.[264] Additionally, whole grain studies have reported beneficial changes in glucose/insulin responses, haemostatic factors and a cholesterol lowering effect.[264]

Cereals belong to the family of the *Gramineae,* also called *Poaceae* or grass family in which various subfamilies and tribes are distinguished (outlined in Figure 1.5). The most common and widely available grains (wheat, maize, rice) largely account for the general population's cereal-based nutritional intake. Other cereals include rye, oats and the ancient grains referred to as pseudocereals (defined as plants that produce fruits/seeds consumed as grains, though botanically are not true cereal grains; amaranth, quinoa and buckwheat). Table 1.7 describes these cereal grains in more detail and lists them as gluten- and non-gluten-containing grains. Comprehensive and complete FODMAP composition data of gluten-free and gluten-containing grains, especially processed cereal products is limited.

Another target of this project was to contribute to a greater understanding about the FODMAP content of gluten-free and gluten-containing grains by completing carbohydrate analysis of commonly consumed grains and cereal products. This information will contribute to a more complete understanding of gluten- and wheat-intolerances, and may also provide greater flexibility to the types of better-tolerated products that may be developed for wheat- and gluten-intolerant patients.

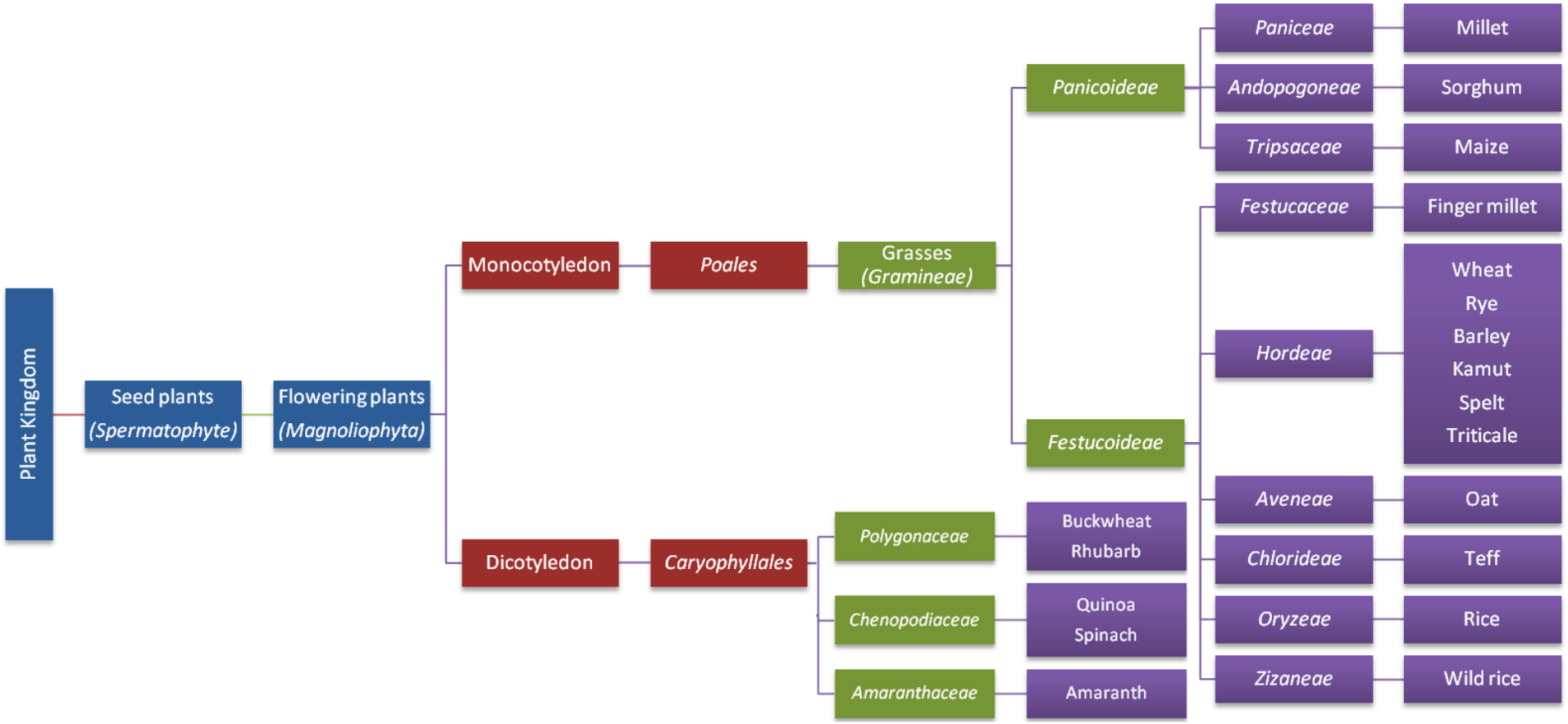

Figure 1.5 Cereal taxonomy[272]

Table 1.7 Description of common gluten and non-gluten containing grain cereals

GRAIN	CHARACTERISTICS	GLUTEN CONTENT
Major grains:		
Maize	Commonly known as corn, widely grown throughout the world for human consumption, animal feed and ethanol production	Gluten-free
Rice	Principle food for 50% of world's population; high in starch and low in protein[273]	Gluten-free
Wheat	One of the first grains to ever be cultivated by humans, which has evolved into the unhulled hexaploid used today, well known for its gluten content	Gluten-containing
Alternative ancient grains:		
Amaranth	This Ancient American crop is botanically not a true grain but has similar composition to the grain family, has 60 known species; higher in protein content than other grains, also high in dietary fibre, iron and calcium	Gluten-free
Barley	First domesticated in the Fertile Crescent (Israel, northern Syria, southern Turkey, eastern Iraq, and western Iran); currently a source of alcoholic beverages and fermented foods[274]	Gluten-containing
Buckwheat	Botanically not a true grain, which originates from East Asia; is high in calcium, iron, magnesium, phosphate potassium and zinc	Gluten-free
Kamut	An ancient relative of durum wheat with 20–40% more protein, higher in lipids, amino acids, vitamins and minerals than common wheat	Gluten-containing
Millet	Grass grown in the UK, USA and Australia mostly for animal feed; commonly consumed in Africa and Asia; high in antioxidant and B-vitamins	Gluten-free
Oats	Grass widely available for human and animal feed; high in β-glucans and soluble dietary fibre	Gluten-free
Quinoa	Originated from Andean region of South America and is high in protein	Gluten-free
Rye	Wholegrain best studied for its health benefits, containing high dietary fibre[275,276]	Gluten-containing
Sorghum	Grass grown in Australia mostly for animal feed; commonly consumed in Africa and Asia; high in antioxidants and insoluble fibre	Gluten-free
Spelt	Ancient hulled wheat, that may be high in vitamins and minerals[277,278]	Gluten-containing
Teff	Small grain mostly grown in South Africa, Ethopia and India; high dietary fibre	Gluten-free
Triticale	Hybrid of wheat and rye, grown mostly for animal feed	Gluten-containing

Chapter 2 – Aims and Hypotheses

Chapter 1 outlined the role of gluten in the pathophysiology of coeliac disease and the paucity of scientific evidence and understanding of gluten sensitivity in individuals without coeliac disease. The demand for gluten- and wheat-free foods and prescription of a GFD for GI and other symptoms continues to rise. However, it is not known whether it is the protein (gluten) or the carbohydrate (eg. fructan) component in wheat that is responsible for provoking GI symptoms. Fructans are one of the poorly absorbed, short-chain carbohydrates (termed Fermentable Oligo-, Di-, and Mono-saccharides And Polyols; FODMAPs) shown to induce GI symptoms in the majority of patients with IBS.

Chapters 4 – 8 describe a series of studies undertaken to explore and understand the components of wheat that may be responsible for 'non-coeliac gluten sensitivity' (NCGS).

Chapter 4: Quantification of fructans, galacto-oligosacharides and other short-chain carbohydrates in processed grains and cereals

Hypotheses

1. FODMAPs are naturally present in commonly consumed grains and cereal products.

2. Gluten-free products (normally based from corn or rice) have a lower overall FODMAP content than gluten-containing products (normally based on wheat, rye and barley).

Aims

1. To screen a variety of grains and processed cereal products for their FODMAP content.

2. To identify any patterns in the content of FODMAPs between foods with and without gluten.

Chapter 5: A survey: Self-diagnosis of non-coeliac gluten sensitivity by Australian adults

Hypotheses

1. In subjects who believe they have NCGS, these individuals have not had adequate investigations to exclude coeliac disease.

Aims

1. To characterise the sub-group of people on a gluten-free diet who believed they had NCGS.

Chapter 6: A double-blind randomised placebo-controlled trial (Study One): Non-coeliac gluten sensitivity may exist

Hypotheses

1. Gluten will induce symptoms and small intestinal injury in patients without coeliac disease in whom there has been a response of GI symptoms with the withdrawal of gluten from the diet.

Aims

1. To evaluate the effect of gluten on patients with GI symptoms who have symptomatically responded to a GFD in a randomised, placebo-controlled, double-blind trial on (i) GI symptoms, (ii) intestinal permeability, and (iii) other indices of small intestinal injury.

Chapter 7: A crossover double-blind randomised 3-arm trial (Study Two): Exploring the mechanisms and tolerance level in non-coeliac gluten sensitivity

Hypotheses

1. Non-coeliac gluten sensitivity is mediated by components in gluten

2. These components can (i) induce GI symptoms, (ii) effect cognitive function, (iii) induce systemic upset such as lethargy, without causing intestinal inflammation or immune effects

3. That gluten will increase levels of by-products of protein metabolism including faecal ammonia

4. These effects are dose-dependent.

Aims

1. In patients with non-coeliac IBS who are apparently gluten sensitive, we aim to assess the effect of wheat-gluten by comparing changes in:

(i) overall and individual GI symptoms (bloating, wind, abdominal pain and diarrhoea),

(ii) indices of mucosal inflammation and markers of immunological mechanisms,

(iii) measures of cognitive function and measures of fatigue and lethargy, and

(iv) by-products of protein metabolism including faecal ammonia.

2. Additionally, to establish the threshold of gluten tolerated by NCGS individuals.

Chapter 8: A rechallenge crossover double blind placebo-controlled trial (Study Three): Reproducing the effects of gluten and whey

Hypotheses

1. Both gluten and whey specifically trigger GI symptoms as well as stress and psychological responses in a proportion of (not necessarily the same) patients with NCGS.

Aims

1. To assess the effects of gluten and whey separately by comparing the changes (i) GI symptoms and fatigue, (ii) measures of psychological wellbeing, and (iii) cortisol secretion, in addition to (iv) reproducibility of effects.

Chapter 3 – General Methods

3.1 Ethics and facilities

Research described in this thesis was approved by the Eastern Health Research and Ethics Committee (Project no. E28/0607, E65/0910, E52/1011).

Subject recruitment was conducted in the Eastern Health Clinical School clinic rooms and Department of Gastroenterology clinic of Box Hill Hospital. Venesection was performed in the clinical rooms of the Eastern Health Clinical School, Box Hill Hospital, and laboratory work conducted in the Department of Gastroenterology, Monash University Research laboratory at Eastern Health Clinical School.

3.2 Carbohydrate analysis

Earlier studies have described and validated methodologies to quantify the major FODMAPs present in fruits and vegetables commonly consumed in Australia.[217,218] Total fructans were quantified using a commercially available kit that utilises an enzymic hydrolysis method.[218] The other major FODMAPs of interest, including fructose, lactose, sorbitol, mannitol, GOS- stachyose and raffinose and FOS- nystose and kestose, were measured using high-performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD).[217] There is a paucity of comprehensive food composition data that list the content of FODMAPs in processed grain and cereal products.

3.2.1 Food sample processing and extraction

Food sampling complied with Food Standards Australia New Zealand (FSANZ, Canberra, Australia). The food items chosen were a variety of breakfast cereals, grains, pasta, breads, commonly purchased biscuits and pulses. For commercial products, brands chosen included the most popular brand, a generic home-brand and one other. From 2007 to 2009, purchases were made in metropolitan Melbourne, Australia from a range of retail outlets that included supermarkets, markets and health stores. The description of food products and sampling details are given in Appendix 1.

Approximately 500 g ('as eaten' weight) of each food product was purchased. For grains, pasta and dried legumes, samples were prepared (e.g. soaking, boiling) as eaten and as directed on the packet label. These preparation methods are detailed in Appendix 1. Samples were then pooled (that is, 3 x 500 g = 3 kg) and thoroughly mixed. From this 3 kg pooled sample, 500 g was taken and blended in a food processor to a homogeneous consistency.

All low FODMAP meals and snacks used in the studies involving volunteers (Chapter 6 – 8) were prepared and a portion of each meal was blended in the food processor.

Approximately 100 g from the homogenized product/meal was taken for freeze-drying (Operon Freeze-drier, Thermoline Scientific). Extraction procedures were completed in triplicate as described in previous studies.[217] The dried sample was finely ground with a mortar and pestle. Approximately 1 g of sample was added to 100 ml of distilled water (80 °C) and stirred with heat (80 °C) for 15 min. The samples were filtered via Whatman filter paper (no 1). If the samples remained turbid they were then filtered through 0.22 µm sterile Millex GP syringe driven filter units (Millipore, Carrigtwohill, Co. Cork, Ireland) and through an OASIS HLB Cartridge (Waters, Milford, MA). New filter paper was used for samples that contained a high starch content. If immediate analysis was not possible, samples were stored frozen at -20 °C. If samples remained turbid, they were refiltered (after thawing if appropriate) before analysis.

3.2.2 Measurement of short-chain carbohydrates

The analytical technique was based on HPLC with an ELSD. The reagents and standards, HPLC apparatus (consisting of a ELSD Waters 2424, HPLC Pump Waters 515, Waters Autosampler 717 Plus, and a Waters Column Heater) and chromatographic procedure were kept consistent with previous studies.[217] Two separate columns were used for clear and accurate separation of the short-chain carbohydrates of interest. The first was the Waters Sugar Pak column with water as the mobile phase to separate glucose, galactose, fructose, mannitol and sorbitol. Free fructose (the fructose fraction present in excess of glucose) was calculated as it is well documented that fructose co-ingested with glucose enhances absorption.[279] The second column (Waters High-Performance Carbohydrate Column) with acetonitrile:water mix mobile phase, was used to separate lactose, longer chain FOS (nystose and kestose) and GOS (raffinose and stachyose). The elution profile of the standards used, relative standard deviations and also the detection and quantification limits have been detailed previously.[217]

Total fructan content was determined by the commercially available enzymatic kits (Megazyme Fructan HK Assay Kit; Megazyme International Ireland Ltd, Wicklow, Ireland; AOAC Method 999.03 and AACC Method 32.32) as per manufacturer's instructions. The measurement methods were consistent with those described previously.[217,218] The assay is based on the established enzymic hydrolysis method measuring total fructan.[280] This approach utilizes highly purified and specific enzymes to hydrolyze sucrose, starch, and fructans.

Average portion sizes were obtained from nutrition software program, FoodWorks Version 6 (Xyris Software Australia Pty Ltd) and used the household measures of 1 cup = 250 ml and 1 tablespoon = 15 ml.

3.3 Subject recruitment

Subjects who believe they have NCGS were recruited from Melbourne, Victoria and surrounding areas by flyers and advertisements placed in local clinic rooms (Breath Hydrogen Testing room and clinics around Eastern Health (Functional Gut Disorders Clinic at Box Hill Hospital), local newspapers, online newsletters (i.e., The Coeliac Society of Victoria, dietetic practices, gluten-free food stores, Monash University).

Inclusion and exclusion criteria are summarised in Table 3.1 The confirmation of not having coeliac disease was according to ESPGAN criteria (1990; see Table 3.2). Respondents meeting the eligibility criteria were supplied with a Patient Information and Consent Form. Initial biopsy and serology results, time and compliance on a GFD and current level of symptom control were recorded. Blood was collected for HLA-DQ genotyping and coeliac serology (Section 3.6.7). Subjects with inadequate symptom control or inadequate coeliac investigation were referred to their local doctor, Box Hill Hospital Coeliac or Functional Gut Clinic, or to a dietitian specialising in GI disorders, as appropriate, for further assessment.

Table 3.1 Inclusion and exclusion criteria for non-coeliac gluten sensitive (NCGS) participant recruitment

Inclusion criteria	• 16 years or older • Met Rome III criteria for IBS prior to implementation of GFD • Current gastrointestinal symptoms well-controlled on a GFD • Adherent to a GFD[#] for the previous 6 weeks • Coeliac disease excluded by either normal duodenal histology while consuming a gluten-containing diet *or* by having a genotype that is inconsistent with celiac disease (HLA-DQ2 and -DQ8 negative)
Exclusion criteria	• Other significant gastrointestinal disease (e.g., cirrhosis, inflammatory bowel disease) or other clinically significant co-morbidity • Intake of non-steroidal anti-inflammatory drugs • Use of systemic immunosuppressant medication (e.g., prednisolone, methotrexate, thiopurines, anti-tumour necrosis factor drugs) • Excessive alcohol intake • Psychiatric illness • Unable to give written/informed consent

[#] Gluten free diet (GFD) as based on a combination of (a) patients' subjective assessment of their gluten intake (see Appendix 2 for questionnaires used in Study One and Study Two) and (b) nutritionist's assessment of 7-day diet history (see Appendix 3 for food diary sheets). IBS, irritable bowel syndrome.

Table 3.2 Diagnosis of coeliac disease following ESPGAN criteria[138]

1. **Villous atrophy, crypt hyperplasia and raised intraepithelial lymphocytes consistent with coeliac disease whilst consuming gluten in the diet.**
2. **Positive coeliac serology (tTG-Ab currently most favoured) is supportive (but not essential)**
3. **Following a GFD, evidence of (i) recovery of intestinal histology, and/or (ii) clinical remission and/or (iii) normalization of coeliac serology**

3.4 Randomisation and blinding

Permuted randomisation blocks were randomly selected using a computer random sequence generated by http://www.randomization.com to develop the diet allocation randomisation codes used in Study One, Two and Three. In accordance with the CONSORT Statement[281] and guidelines of randomised controlled trials, all study personnel and subjects were blinded to the diet assignment for the duration of the study, including laboratory analyses and data entry. A colleague kept the study codes.

3.5 Design of diets

All meals and snacks provided were gluten-free and low FODMAP. Commercially available gluten-free bread and muffin mixes (Well and Good P/L, Classic Bread Mix and Muffin Mix, Noble Park, Victoria, Australia) went through carbohydrate analyses (previously discussed in Section 3.2) prior to being used for Study One. The FODMAP composition database was used to compile recipes for a gluten-free, low FODMAP 7-day diet plan for Study Two, described in Chapter 7 and a gluten-free, low FODMAP 3-day diet plan for Study Three, described in Chapter 8.

For Study Three, publicly available resources describing the content of natural food chemicals including salicylates, amines, and glutamate were used to ensure the recipes also met the description of being 'low to moderate' in food chemical content, in accordance with the moderate approach described by the Allergy Unit, Royal Prince Alfred Hospital (RPAH; Sydney, Australia) Elimination Diet.[225]

All meal plans and dietary records were analysed by using the nutrition analysis program, FoodWorks Professional 2009 (Xyris Software, QLD, Australia). Appendix 4 and 5 detail the meal plans for Study Two and Three, respectively. Appendix 6 and 7 provide the macro- and micro-nutrient breakdown of the diets compiled for Study Two and Three, respectively.

3.5.1 Protein challenge

Gluten was added to the study foods, which were prepared in gluten-free ovens and conditions. The quantity of 16 g per day was used, following the Australian Therapeutic Guidelines which describes an adequate gluten challenge protocol as the equivalent of four to six slices of bread (16-20 g gluten) per day for at least six weeks, prescribed for patients gluten-loading prior to being tested for coeliac disease.[201]

For Study One, the gluten used was commercially available, carbohydrate depleted wheat gluten (Gemtec 1160; Manildra Group, Auburn, Australia). For Study Two and Three, the gluten used was also commercially available, carbohydrate depleted wheat gluten, but from a different supplier (Vital Wheat Gluten; Penford Australia Ltd, Tamworth, Australia).

Protein characterisation studies were completed by Dr Ferenc Bekes (George Weston Foods, Enfield, Australia). Size exclusion-high performance liquid chromatography (SE-HPLC) was used to characterise the protein distribution and the lipid content determined using reverse-phase high-performance liquid chromatography (RP-HPLC). The results of these protein analyses for gluten are shown in Table 3.3.

Table 3.3 Percentage distribution of the gluten used shown on reversed-phase high-performance liquid chromatography (RP-HPLC) and percentage distribution of the protein content on the basis of size-exclusion high-performance liquid chromatography (SE-HPLC)

		Study One	Study Two & Three
Gluten distribution	Protein	91.7	0.6
	Crude fiber	1.1	6.9
	Lipid	1.9	75.0
	Starch	1.8	1.8
	Ash	3.5	15.6
Protein distribution	Gliadin	52.0	53.4
	Glutenin	45.7	40.0
	Non-gluten protein (albumin / globulin)	2.3	6.6

In Study Two and Three, a commercially available whey protein isolate was used (RESOURCE® Beneprotein Instant Protein Powder; Nestle Healthcare Nutrition, Inc., Minneapolis, USA). The pure whey protein was lactose-free and low-FODMAP, measured following analyses methodologies described in Section 3.2.

3.5.2 Composition of the low FODMAP diets

Fructose was either in a 1:1 ratio with glucose or present in less concentration than glucose for each meal and snack. Low lactose milk, yoghurt and hard cheese were used to minimize lactose intake. Gluten-free bread, breakfast cereal, crackers and pasta were included. All recipes excluded garlic and onion, and included the lowest FODMAP vegetables such as carrots and potato. Low FODMAP fruits allowed included kiwifruit and oranges.

3.5.3 Composition of the low chemical diet

All recipes excluded fruits and included the lowest chemical vegetables such as potato, celery and carrots. Only fresh beef, chicken without skin or stuffing, fresh white fish and fresh eggs were used. Artificial colours, preservatives, herbs and spices, fats and oils were screened and only used according to that described by the RPAH Elimination Diet.[225]

3.6 Endpoints and measurements

Study specific measurements are outlined in the respective chapters. Below are some used consistently throughout.

Compliance with the study treatment was assessed by an unused food count and a tick box for amount consumed. Compliance with the GFD was judged on food diary entries and on specific questioning at the time of review.

3.6.1 Dietary adherence

Various methods of assessing dietary adherence were used:

Seven-day food diary analysis: Patients were provided with a seven-day recording diary card and instructions for its completion. Recorded information was checked at review consultation and assessed for intake of gluten-containing foods (dietary compliance) by a nutritionist or dietitian. See Appendix 3 for sample diary card. This was completed in Study One, Two and Three.

Specific questioning: Patients were asked directly about any gluten consumed, either accidentally or intentionally, by completing a questionnaire (Appendix 2) containing specific questions about gluten consumption during the study period, including "Did you ever deliberately eat gluten", "On how many occasions did you deliberately eat gluten?", "What did you eat on these occasions?" and, "What was the reason for eating gluten?". This was completed in Study One and Two.

Validated compliance questionnaire: A verified flow chart established by Biagi and colleagues was used in Study Two, which gives a GFD compliance numerical score.[282] The questionnaire is based on four simple questions with a five-level score (0–IV), which from a clinical point of view can be grouped into three levels of classifications (see Table 3.4).

Table 3.4 Classifications for evaluation of gluten-free diet compliance[282]

Score	Classification
0 or I	Do not follow a strict GFD
II	Follow a GFD but with important errors that require correction
III or IV	Follow a strict GFD

3.6.2 Gastrointestinal symptoms

The 100 mm visual analogue scale (VAS) is frequently used to assess subjective phenomena such as symptoms[283] and was used for monitoring changes in GI symptoms during the clinical studies detailed in Chapters 6, 7 and 8.

The VAS can be adapted for any symptom, and involves the subject placing a mark at a point relevant to their degree of symptom severity, where zero indicates "no symptom at all", and one hundred indicates "severe or worst symptom". A ruler is then used to calculate the marked score. The questions asked using the VAS were overall GI symptoms, abdominal pain/discomfort, abdominal bloating/distenstion, passage of wind (i.e., flatulence), satisfaction with stool consistency, tiredness and lethargy, and nausea (see Appendix 8).

In Study One only, a global symptom question was used in addition to the VAS, "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.

3.6.3 Fatigue and other symptoms

Severity of fatigue was evaluated by the Daily-Fatigue Impact Scale (D-FIS),[284] a questionnaire containing eight items investigating fatigue impact on cognition, physical functioning and daily activities. Answers are given following a 5-point Likert-like scale, where zero equates to "no problem" and four to "extreme problem". A global score is derived from the sum of the ordinal scores obtained for each item. Scores of more than 10 are consistent with the reports of subjects within the first 6 days of the onset of an acute flu-like illness. Scores of more than 20 are associated with a high likelihood of time lost from work.

3.6.4 Physical activity and sleep

Validated accelerometry was used to objectively assess intensity and duration of physical activity and sleep patterns (ActiGraph GT3X accelerometer, LLC, Fort Walton Beach, Florida, USA).[285,286] The ActiGraph has shown high agreement with energy expenditure and measures of time spent in different intensities for adults[287,288] and when compared with polysomnographic measures of sleep in adults.[289,290]

Actigraphic raw data were translated into sleep measures using the ActiLife Data Analysis Software (Version 5), the parameters monitored are defined in Table 3.5. Information is reported in the form of step and activity counts for a specified time period (i.e., epoch). The activity count data is then converted into energy expenditure output, based on population-specific equations (i.e., Freedson equation),[291] and default cut-points to classify activity as sedentary, light, lifestyle, moderate, vigorous or very vigorous intensity (these categories are defined in Table 3.5). Bouts were defined as being moderate intensity or higher and for a minimum duration of 10 min.

Data recorded on the first and last days were discarded for each participant due to incompleteness on these days. Only participants with at least four complete days of accelerometer data (including one weekend day) were included in the analyses, consistent with recommendations that four days is the minimum acceptable amount to typify usual activity.[292] Days in which total accelerometer counts were less than 10,000 or exceeded 20,000,000 were also excluded from the analyses, as this indicated a possible malfunction of the accelerometer.[293]

Participants provided details on activity during non-wearing periods, as the monitor could not be worn during bathing and aquatic activities. Instructions were explained on how to wear the accelerometer (around the right hip using an elasticised belt) and also in the supplied "activity log" given to participants (see Appendix 9).

Table 3.5 Definition of categories used for accelerometer analysis

Characteristic	Description
Physical activity category:	*Cut point in counts per minute:*
Sedentary	0 – 100
Light intensity	101 – 760
Lifestyle	761 – 1951
Moderate intensity	1952 – 5724
Vigorous intensity	5725 and higher
Sleep parameter:	
Latency	Minutes taken to fall asleep (sleep onset latency)
Efficiency	Sleep efficiency score generated from analysis using the Sadeh algorithm[289,294]
Time in bed	Minutes spent in bed
Total sleep time	Sleep duration (minutes)

3.6.5 Cognitive function

Cognitive assessment used the Subtle Cognitive Impairment Test (SCIT; Version 1.0, April 12th, 2008), which measures the rate at which information is processed from iconic to short-term memory.[295] The SCIT is a brief (3-4 min) computerised test in which participants were asked to indicate which of two parallel vertical lines (joined together to form a U) in the target stimulus was shorter (left or right). The choice was indicated by pressing the corresponding button on a Cirque Easy Cat two-button touchpad. Presentations occurred in the following order: a focal point (a small cross); a short period of a blank screen; the target stimulus requiring a response; a pattern mask, presented to prevent any further visual processing of the target stimulus (see Figure 3.1).

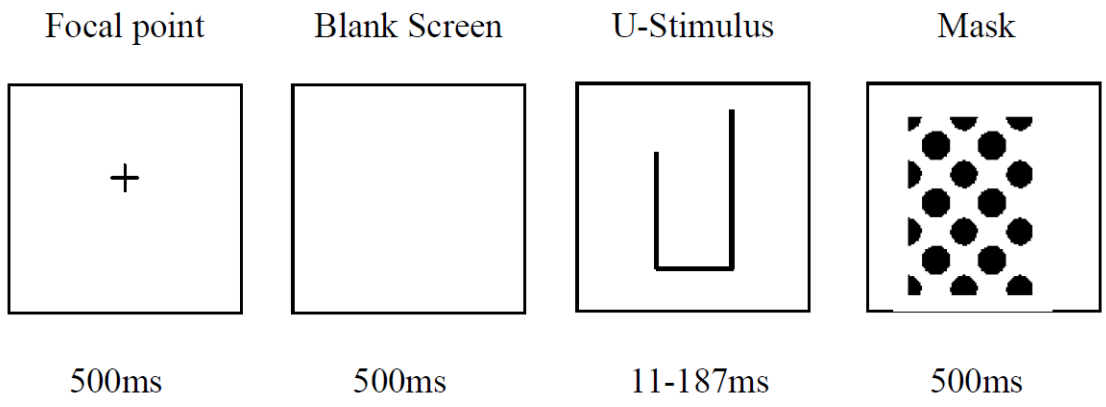

Figure 3.1 Examples of subtle cognitive impairment test stimuli

The stimulus exposure durations ranged from 11 to 187 ms, giving nine different exposure durations. Performance data on error-rate (errors in recognition of target stimulus; SCIT-E) and response time (time taken to respond to the target stimulus in ms; SCIT-RT) were recorded for each presentation. All stimuli were presented on a Phillips Brilliance 15A 15" Flat Screen monitor, using a Hewlett-Packard Mini P3 1.1 GHz PC with an ATI Radeon 6000 graphics card.

For data analyses, the SCIT data collected at the four exposure durations at the head of the data curve (11–77 ms) were combined (SCIT-E_H, SCIT-RT_H), and the remaining five exposure durations at the tail of the data curve (99–187 ms) were pooled (SCIT-E_T, SCIT-RT_T) to increase statistical power of comparisons.[296]

3.6.6 Gliadin-specific T-cell responses

Immunological readout (IFN-γ) for gliadin-specific T-cells via enzyme-linked immunospot (ELISpot) assays was performed following previous methodologies,[111,112] and using kits (Mabtech, Nacka Strand, Sweden) and 96-well plates (Multiscreen® Filter Plates; Millipore, Bedford, MA, USA) with isolated peripheral blood mononuclear cells (PBMC).

Antigens and materials - Synthetic gliadin peptides were kindly prepared and provided by Dr Jason Tye Din of Walter and Eliza Hall Institute of Medical Research (WEHI; Parkville, Victoria, Australia). Table 3.6 describes the key characteristics and preparation.

All peptides were 16mer in length, fractionated, treated and underwent quality control for purity by reverse phase-HPLC and identity by mass spectrometry (MS). Tetanus toxoid (tet tox) (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia) (10 light forming units/mL) and Phytohemagglutinin (PHA) (Commonwealth Serum Laboratories, Melbourne, Australia) (2.5 μg/mL) were used as positive control antigens. All antigens were stored at -80 ºC until use.

Table 3.6 Gliadin preparation

Peptide	Description	Company	Concentration in asssay
CT-Gliadin	Chymotrypsin (CT) digested ICN gliadin	Mimotopes (Clayton, Australia)	100 µg/mL
Deamidated CT-gliadin	tTG treated CT-digested ICN gliadin	Mimotopes	100 µg/mL
Gliadin peptide 1	High quality NPL01 in DMSO[#]	Pepscan (Lelystad, Netherlands)	25 µg/mL, 50 µg/mL
Gliadin peptide 2	High quality NPL02 in DMSO[#]	Pepscan	25 µg/mL, 50 µg/mL

[#] High quality indicates purity >70% where peptides were dissolved in sterile Dimethyl sulfoxide (DMSO) solvent (Sigma D5879, St Louis, USA) to 100 mg/mL

Custom templates were designed, where two wells were reserved for positive controls and one for a negative control consisting of medium alone. Antigens were prepared at five-times final assay concentration with PBS in 96-well round bottomed plates (Microtest™, Becton Dickinson, USA) stored frozen at -80 ºC and thawed immediately prior to use in the ELISpot assay. Remaining peptide was refrozen or later use, where each template contained enough antigen to test four separate ELISpot plates (4 × 25 µL) with 25 µL spare to allow for evaporative losses.

Tissue culture solutions and media were kindly prepared and provided by Dr Jason Tye Din (WEHI, Parkville, Australia).

PBMC preparation - Venesection was performed in the Eastern Health Clinical School using a 21 Guage Butterfly needle (BD Vacutainer® push button blood collection set, Franklin Lakes, USA) drawn into heparinised tubes (Vacuette® 4 mL Lithium Heparin tubes with Gel; Grenier Bio-One, Chonburi, Thailand) the morning prior to (Day 0) and 6 days after commencing each treatment arm. Whole blood was stored at room temperature (RT) and processed within 4 hours. PBMCs were isolated by Histopaque (Histopaque®-1077, Sigma-Aldrich Inc., St Louis, USA) density gradient centrifugation using Leucosep tubes (Greiner Bio-One GmbH, Frickenhausen, Germany). Leucosep tubes were pre-prepared by centrifuging 15 mL Histopaque at RT for 20 sec at 2,000 × *rpm* below the porous membrane and stored at RT wrapped in aluminium foil. Twenty mL of heparinised venous blood was poured over the Leucosep filter and centrifuged at 1,800 × *rpm* for

20 min at RT with brake off. The PBMC and plasma remaining above the filter were then decanted, resuspended in sterile PBS at RT and spun at 1,200 × *rpm* for 7 min. The cell pellet was resuspended in PBS and rewashed. The pellet was suspended in complete medium so that PBMCs were approximately 500×10^6 cells/well. Counting was performed by Box Hill Hospital Pathology using an Automated Hematology System (Sysmex XE-5000, Mundelein, USA).

The ELISpot assay - A 96-well Polyvinylidene fluoride (PVDF) membrane ELISpot plate (Multiscreen® Filter Plates; Millipore, Carrigtwohill, Ireland) was coated with anti-cytokine high affinity monoclonal antibody to IFN-γ at 1:100 concentration (50 µL/well) diluted in PBS and wrapped in foil overnight at 4 ºC. Immediately prior to use, each plate was washed three times with sterile PBS and non-specific binding blocked by addition of RPMI (Roswell Park Memorial Institute) medium (Sterile RPMI-1640; Sigma-Aldrich, St Louis, USA) with 10% inactivated foetal calf serum (FCS) for 2 h at 37 ºC. Peptide or antigen were then added to each well (25 µL), before adding isolated PBMCs (100 µL) and incubated overnight at 37 ºC in a 5% CO_2 incubator. The following day, cells were removed by washing once with cold distilled water then three times with PBS with 0.05% TWEEN-20 (TWEEN®-20 dry powder dissolved in deionised water; Sigma-Aldrich, St Louis, USA) and three times with PBS. Biotinylated anti-cytokine antibody diluted in PBS with 0.5% inactivated FCS was added (50 µL/well) and incubated for 2 h at RT. Following a further round of washing, Streptavidin conjugated with enzyme ALP was added (50 µL/well) and incubated for 1 h at RT. A precipitating developer substrate (BCIP-NBT) was first filtered through a 0.22 µm filter (Millipore Millex-GP syringe driven filter unit, Carrigtwohill, Ireland) and diluted 1:1 with distilled water before being added (50 µL/well) until the development of spots at the site of responding cells. Developing was terminated by washing under cold tap water when spots were fist visible or at 6 min. After removing the plastic backing the 96-well plate was allowed to dry overnight. Spot forming units (SFU) in individual wells were counted using an automated ELISPOT reader (AID ELISPOT Reader System, AID Autoimmun Diagnostika GmbH; Strassberg, Germany).

For data analyses, ELISpot results were reported as raw SFU unless otherwise stated. Mean SFU was calculated from duplicate wells. A positive or significant response was more than 10 SFU/well and also more than three-fold change from Day 0, after correction for blanks (medium alone), as based on previous studies,[111,112] For ELISpot analyses, results were reported as raw SFU unless otherwise stated. Mean SFU was calculated from duplicate wells.

3.6.7 Coeliac disease measurements

3.6.7.1 HLA typing

HLA-DQB1 and *HLA-DQA1* alleles were determined using the polymerase chain reaction-sequence-specific oligonucleotide hybridisation method by the Red Cross Immunogenetics Service, Melbourne Pathology (or affiliated Sonic Pathology branches).

3.6.7.2 Coeliac serological markers

Serum was analysed for antibodies to whole gliadin (IgA and IgG) and deamidated gliadin (IgA and IgG) by ELISA (enzyme-linked immunosorbent assay) using commercially available assays (INOVA Diagnostics, San Diego, USA). All tests were performed in conjunction with a total-IgA level to exclude any subjects with selective IgA deficiency.

3.6.8 Other biomarkers

3.6.8.1 Human ß-defensin-2

Stool samples were analysed for human ß-defensin-2 (HßD-2), an anti-microbial peptide affecting the barrier function of epithelial cells, by ELISA using commercially available assays (Immundiagnostik AG, Bensheim, Germany). Preliminary studies revealed that this was the most sensitive kit. Extracts were prepared by manually weighing stool samples within 80-120 mg and adding a specified volume of the supplied washing buffer, maintaining a constant dilution factor of 1 : 50. Samples were vortexed well and 1 mL then centrifuged at 13,000 × *rpm* for 5 min at RT. Fifty µL of the supernatant was combined with 50 µL buffer and used in the assay, performed according to the kit's procedure.

3.6.8.2 Calprotectin

Stool samples were analysed for human calprotectin, a calcium-binding protein indicative of neutrophils cytosol and therefore bowel inflammation, by ELISA using commercially available assays (BÜHLMANN Laboratories AG, Schönenbuch, Switzerland). Extracts were prepared using the faecal sample preparation kit (BÜHLMANN Laboratories AG, Schönenbuch, Switzerland), where by approximately 80 mg was pressed into the hollow cavity in the base cap and vortexed well with 4 mL of the supplied extraction buffer (a spiral coil within the tube assists homogenisation). Any samples hard in consistency or inconsistent in result were repeated with a new sample, which was allowed to stand with the buffer for at least 15 min before homogenisation. Two mL of this mixed solution was centrifuged at 3,000 × *g* for 5 min at RT, of which 1 mL of the supernatant was used in the assay, performed according to the kit's procedure.

3.6.8.3 Eosinophil Cationic Protein

Serum was analysed for human Eosinophil Cationic Protein (ECP), a basic protein located within the eosinophil primary matrix that has degranulation activity and is associated with inflammation, by ELISA using commercially available assays (Cuasbio Biotech Co., Ltd, Newark, USA). Samples were prepared with a biotin-conjugated antibody preparation specific for ECP and an enzyme-conjugated Avidin, which were then added to microtiter plate pre-coated with an antibody specific to ECP, in accordance with the manufacturer's instructions

3.6.9 Faecal examination

3.6.9.1 Faecal collection and analysis

To assess the colonic fermentation of protein (such as undigested gluten) in the colon, three-day total faecal output from day 5-7 was collected during each diet treatment week. Volunteers were provided with labelled containers and a -20 °C portable freezer (WAECO Model CD F35 Pack Cool Freeze Fridge, Varsity Lakes, Australia). Volunteers were asked to collect all output during the three-day period and note the date and time of collection on each container and place them immediately into the freezer. Subjects recorded the details of any samples missed whilst away from home (e.g., at workplace).

Samples were weighed and described according to the validated Kings Stool Chart (King's College London), which characterises samples based on stool consistency, weight and frequency.[297] Scores are weighted such that an increase in faecal frequency alone results in a higher score than a change in faecal consistency alone, which in turn results in a higher score than an increase in faecal weight alone.[298] Diarrhoea is classified by a daily faecal score of 15 or more. Samples were then pooled on ice, and aliquots frozen at -80 °C until time of analysis. Before any measurement, samples were thawed at 4 °C.

3.6.9.2 Faecal pH

The pH of faecal samples (approximately 20 g faeces) was measured using a pH electrode probe (Mettler Toledo InLab® pH Combination Electrode, Schwerzenbach, Switzerland) and portable electrode meter (Mettler Toledo AG FiveGo™ Duo reader, Schwerzenbach, Switzerland) at RT. Sterilisation was maintained between samples.

3.6.9.3 Faecal ammonia

Ammonia concentration in faeces, a by-product of protein metabolism, was measured using commercially available assays (Megazyme Australia Pty Ltd, North Rocks, Australia) for total ammonia (Ammonia *Rapid* Kit). Samples were prepared by adding approximately 0.5 g faeces to centrifuge tubes containing 4 mL of 3% trichloroacetic acid, to minimise ammonia losses. The contents were incubated for 5 min at room temperature, followed by centrifugation at 2,000 × *g* for 10 min at RT. Two mL of the supernatant was removed and added to 60 µL of 10 M potassium hydroxide to neutralise the pH, and filtered through a 0.22 µm filter (Millipore Millex-GP syringe driven filter unit, Carrigtwohill, Ireland). This sample was then used in the assay, performed according to the kit's procedure.

3.6.9.4 Faecal gliadin-specific peptides

To assess for indigested gliadin-derived peptides, faecal samples (collection described in Section 3.6.8) underwent analysis of proteins using the established tool of mass spectrometric peptide mapping,[299] which involves detection and quantification of gliadin-derived peptides via multiple reaction monitoring (MRM) conducted on a triple quadruple mass spectrometer.

Pepsin and/or trypsin digested peptides were separated using reverse-phase high-performance liquid chromatography (RP-HPLC; Agilent 1200 Infinity Series LC, Agilent Technologies Inc., Santa Clara, California, USA) with a gradient from 0-60% acetonitrile. The peptides released were directly analysed by being coupled to a triple quadruple mass spectrometer (Agilent 6430 Series Triple Quadrupole LC/MS Systems, Agilent Technologies Inc., Santa Clara, California, USA)

through a ChipCube interface (Agilent 1260 Infinity HPLC-Chip Cube Interface G4240A, Agilent Technologies Inc., Santa Clara, California, USA). Transitions were divided across three separate methods to enable sufficient duty cycle in the mass spectrometer and used theoretically calculated collision energies. Transitions from the four major proteins in the gluten mixture were targeted. As this was an exploratory measure, analysis was completed in only 5 samples from the participants who had the highest symptom response to the high-gluten treatment week (from Chapter 8 only).

3.6.10 Mental health

Emotional states of anxiety and depression subscales were assessed by the State-Trait Personality Inventory (STPI). The STPI is a 80-item self-report inventory which consists of eight ten-item subscales measuring anxiety, anger, and curiosity rated as a trait (felt generally) and as a state (how participants feel at that moment).[300] The depression subscales were evaluated in separate factor analyses, termed depression-present (dysthymia) and depression-absent (euthymia) items. Table 3.7 describes the STPI state and trait depression and curiosity scales and subscales in more detail.[301]

Table 3.7 Descriptions and guidelines for interpreting the STPI Depression and Curiosity Scales[301]

STPI scale	Interpretation
State-Depression	Measures the intensity of depressive feelings and cognitions at a particular time. Individuals with high S-Dep scores experienced relatively intense feelings of sadness and gloom at the time the test was administered.
S-Dysthymia	Measures the intensity of depressive feelings and cognitions at a particular time.
S-Euthymia	Measures the strength of positive feelings and cognitions experienced at a particular time that indicate the absence of depression.
Trait-Depression	Measures how often cognitions and feelings relating to depression are experienced over time. Persons with high T-Dep scores experience more frequent and intense cognitions and feelings of depression, which may be reflected in deep despair and hopelessness.
T-Dysthymia	Measures how frequently cognitions and feelings indicating the presence of depression are experienced over time.
T-Euthymia	Measures how often positive cognitions and feelings indicating the absence of depression are experienced over time.
State-Curiosity	Measures the intensity of feelings and cognitions relating to curiosity and interest in exploratory behaviour at a particular time. Individuals with high S-Cur scores experienced increased inquisitiveness and desire to engage in exploratory behaviours at the time the test was administered.
Trait-Curiosity	Measures how often feelings of curiosity, inquisitiveness, and interest in exploring the environment are experienced over time. Persons high in T-Cur experience more frequent interest and desire to engage in exploratory behaviour in a wide variety of situations.

3.6.11 Salivary cortisol

Salivary cortisol is frequently used as a biomarker of psychological stress,[302] as it is an accurate and practical alternative to blood determinations.[303] Because several factors may influence concentrations (i.e., contaminating substances in saliva, diurnal rhythm of cortisol, sample storage), participants were provided with clear instructions (see Appendix 10) on collection, including sample collection taken at standardised times (in the evening at 2030 h). The Salimetrics Oral Swab (SOS; Salimetrics™, State College, USA) was used to collect saliva samples and stored inside a Swab Storage Tube (clear sterile plastic tube; Salimetrics™, State College, USA). All saliva samples were transported on ice and frozen at -20 °C until being assayed externally (Stratech Scientific APAC Pty Ltd, Sydney, Australia) by competitive immunoassay using commercially available kits (Salimetrics™, State College, USA).

3.7 Statistical analysis

Study specific techniques are outlined in the respective chapters. Statistical programs used included; GraphPad Prism® (Version 5.02 for Windows, GraphPad Software, San Diego California USA), the R Statistical Software Package (R Development Core Team, R: A Language and Environment for Statistical Computing, Vienna, Austria) and SPSS® (SPSS for Windows, Rel. 16.0.1 2007, Chicago: SPSS Inc.). Two-tailed P values at or below 0.05 were considered statistically significant.

Chapter 4 – Quantification of fructans, galacto-oligosacharides and other short-chain carbohydrates in grains and cereals

4.1 Background and aims

Grains and cereals are major sources of carbohydrate in the human diet.[38] In addition to the provision of energy, grain and cereal derived carbohydrates also have wide ranging effects on physiological processes important for maintaining health and disease prevention.[38] Dietary carbohydrates can be broadly divided into two major categories, the short-chain carbohydrates (SCC) including sugars, polyols and oligosaccharides, and the long-chain carbohydrates including starch, resistant starch (RS) and non-starch polysaccharides (NSP).[38]

The important roles grain and cereal-derived long-chain carbohydrates, NSP and RS, have on bowel health are well established.[38] Many of the beneficial effects relate to the process of colonic fermentation.[304] During fermentation the colonic microflora ferment the undigested carbohydrate to produce by-products including gases (hydrogen, methane and carbon dioxide) and short-chain fatty acids (SCFA; acetate, propionate and butyrate).[304] Major physiological benefits of long-chain carbohydrates relate to effects on faecal bulking, faster colonic transit time and slight acidification of the luminal milieu.[206]

There is now evidence for an important role for SCC in the health of the GI tract. Some SCC selectively stimulate the growth and activity of beneficial colonic bacteria, in particular *bifidobacteria* and *lactobacillus*.[31] These dietary SCC are called 'prebiotics' and include fructans (fructo-oligosaccharides (FOS) and inulin) and galacto-oligosaccharides (GOS).[31] Fructans (FOS and inulin) and GOS satisfy the strict criteria for being prebiotics, being a non-digestible food

ingredient that selectively stimulates the growth and or activity of desirable bacteria in the gut.[31] A wide range of benefits have now been attributed to these carbohydrate prebiotics[24,31,305-307] ranging from acting as adhesion particles for pathogenic bacteria and thus, reducing the risk of gastrointestinal infection,[305] improving laxation,[307] increasing calcium absorption,[43] maintaining a functional gut mucosal barrier,[308] and stimulating the gastrointestinal-immune system.[31] There is also some evidence in animal models that the immune-enhancing effects of prebiotics may reduce the risk of colon cancer.[306] Other benefits include lowering blood glucose levels[42] and decreasing levels of serum cholesterol, triacylglycerols and phospholipids.[309,310]

Nevertheless, despite the clear evidence of health benefits for some SCC there is a proportion of the general population who are 'intolerant' to the malabsorption of SCC in the small intestine. IBS is known to affect 5-12% of the general population[311,312] and is characterised by functional gut symptoms including abdominal pain, bloating, flatus and altered bowel habits.[313] We have grouped these potentially problematic SCC together and named them FODMAPs (Fermentable Oligosaccharides, Disaccharides, Monosaccharides, And Polyols).[314] FODMAPs are found in a wide variety of foods and include lactose (in milk), fructose in excess of glucose (in pears, apples and honey), fructans and FOS (in artichoke, garlic, onions, rye, wheat), GOS (stachyose and raffinose in pulses), and sugar polyols (mannitol and sorbitol in stone fruits and artificial sweeteners).[217,218,314]

Malabsorption of FODMAPs can occur for a number of reasons, including the absence of the luminal enzymes capable of hydrolyzing the glycosidic bonds contained in the carbohydrates (e.g. fructans, FOS and GOS), the absence or low activity of brush border enzymes, (e.g. lactase) or presence of low-capacity epithelial transporters (fructose, GLUT2, GLUT5).[213] The prevalence of fructose malabsorption can be as high as 34-61% of the population whether healthy or with gastrointestinal disorders,[214] but will depend on the dose of fructose given.[211,315]

Fructose is a major FODMAP present in the Western diet and is absorbed across the villous epithelium via low capacity and carrier-mediated facilitated diffusion involving GLUT-5 transporters.[213] The absorption of free fructose is markedly enhanced in the presence of glucose via GLUT-2.[213] Consequently the balance of fructose to glucose in a food has the potential for influencing malabsorption. If fructose is present in excess of glucose then the risk of fructose malabsorption is greater.[213]

FODMAPs are very rapidly fermented by intestinal bacteria producing carbon dioxide, hydrogen and/or methane gas.[200] In addition, we have recent evidence that malabsorbed SCC are osmotically active and increase the volume of fluid entering the bowel.[195] In healthy people, this may provide a natural laxative effect. In IBS sufferers, this action may contribute to diarrhoea, additionally the fermentation of these carbohydrates and associated gas production can lead to luminal distension and symptoms of abdominal pain, bloating and flatulence.[196,200,316]

There is now high quality evidence that restriction of dietary FODMAPs leads to symptomatic improvement of overall gut symptoms, gut pain, bloating and wind in the majority of patients with IBS.[196,200,314,316] This dietary approach, however, requires knowledge of comprehensive FODMAP food composition data. Our earlier studies have described methodologies to quantify the major FODMAPs present in fruits and vegetables commonly consumed in Australia.[217,218] Total fructans were quantified using a commercially available kit that utilises an enzymic hydrolysis method.[218] The other major FODMAPs of interest including fructose, lactose, sorbitol, mannitol, GOS- stachyose and raffinose and FOS- nystose and kestose were measured using high performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD).[217]

There is a paucity of comprehensive food composition data that list the content of FODMAPs and naturally found prebiotics in processed grain and cereal products.

It was hypothesised that:

(i) FODMAPs are naturally present in commonly consumed grains and cereal products.

(ii) Gluten-free products (normally based from corn or rice) have a lower overall FODMAP content than gluten-containing products (normally based on wheat, rye and barley).

The overall aim of this study was to quantify the major FODMAPs including the prebiotics (fructans and GOS) present in a wide range of processed grain-, cereal- and pulse- products that are commonly consumed. An additional aim was, to identify any patterns in the content of FODMAPs between foods with and without gluten.

4.2 Methods

4.2.1 Food sample processing and extraction

The methods were consistent with those described previously[217,218] and is further detailed in Chapter 3, Section 3.2. Food sampling complied with Food Standards Australia New Zealand (FSANZ, Canberra, Australia). The food items chosen were a variety of breakfast cereals, grains, pasta, breads, commonly purchased biscuits and pulses. Brands chosen included the most popular brand, a generic home-brand and one other. From 2007 to 2009, purchases were made in metropolitan Melbourne, Australia from a range of retail outlets that included supermarkets, markets and health stores. The description of food products and sampling details are given in Appendix 1.

Approximately 500 g ('as eaten' weight) of each food product was purchased. For grains, pasta and dried legumes, samples were prepared (e.g. soaking, boiling) as eaten and as directed on the packet label. These preparation methods are detailed in Appendix 1. Samples were then pooled (that is, 3 x 500 g = 3 kg) and thoroughly mixed. From this 3 kg pooled sample, 500 g was taken and blended in a food processor to a homogeneous consistency and approximately 100 g was taken for freeze-drying (Operon Freeze-drier, Thermoline Scientific). Extraction procedures were completed in triplicate as described in previous studies.[217] The dried sample was finely ground with a mortar and

pestle. Approximately 1 g of sample was added to 100 ml of distilled water (80 °C) and stirred with heat (80 °C) for 15 min. The samples were filtered via Whatman filter paper (no 1). If the samples remained turbid they were then filtered through 0.22 µm sterile Millex GP syringe driven filter units (Millipore, Carrigtwohill, Co. Cork, Ireland) and through an OASIS HLB Cartridge (Waters, Milford, MA). New filter paper was used for samples that contained a high starch content. If immediate analysis was not possible, samples were stored frozen at -20 °C. If samples remained turbid, they were refiltered (after thawing if appropriate) before analysis.

4.2.2 Measurement of short-chain carbohydrates

The analytical technique was based on HPLC with an ELSD. The reagents and standards, HPLC apparatus (consisting of a ELSD Waters 2424, HPLC Pump Waters 515, Waters Autosampler 717 Plus, and a Waters Column Heater) and chromatographic procedure were kept consistent with previous studies.[217] Two separate columns were used for clear and accurate separation of the SCC of interest. The first was the Waters Sugar Pak column with water as the mobile phase to separate glucose, galactose, fructose, mannitol and sorbitol. Free fructose (the fructose fraction present in excess of glucose) was calculated as it is well documented that fructose co-ingested with glucose enhances absorption.[75,279] The second column (Waters High-Performance Carbohydrate Column) with acetonitrile:water mix mobile phase, was used to separate lactose, longer chain FOS (nystose and kestose) and GOS (raffinose and stachyose). The elution profile of the standards used, relative standard deviations and also the detection and quantification limits have been detailed previously.[217]

Total fructan content was determined by the commercially available enzymatic kits (Megazyme Fructan HK Assay Kit; Megazyme International Ireland Ltd, Wicklow, Ireland; AOAC Method 999.03 and AACC Method 32.32) as per manufacturer's instructions. The measurement methods were consistent with those described previously.[217,218] Briefly, the assay is based on the established enzymic hydrolysis method measuring total fructan.[280] This approach utilizes highly purified and specific enzymes to hydrolyse sucrose, starch, and fructans.

Average portion sizes were obtained from nutrition software program, FoodWorks Version 6 (Xyris Software Australia Pty Ltd) and used the household measures of 1 cup = 250 ml and 1 tablespoon = 15 ml.

4.3 Results

4.3.1 Elution profile of standards, wholegrain bread and muesli

Examples of chromatogram profiles for sugar standards are shown for both columns in Figure 4.1. Examples of chromatogram profiles via both columns are shown for wholegrain bread (Figure 4.2) and muesli (Figure 4.3).

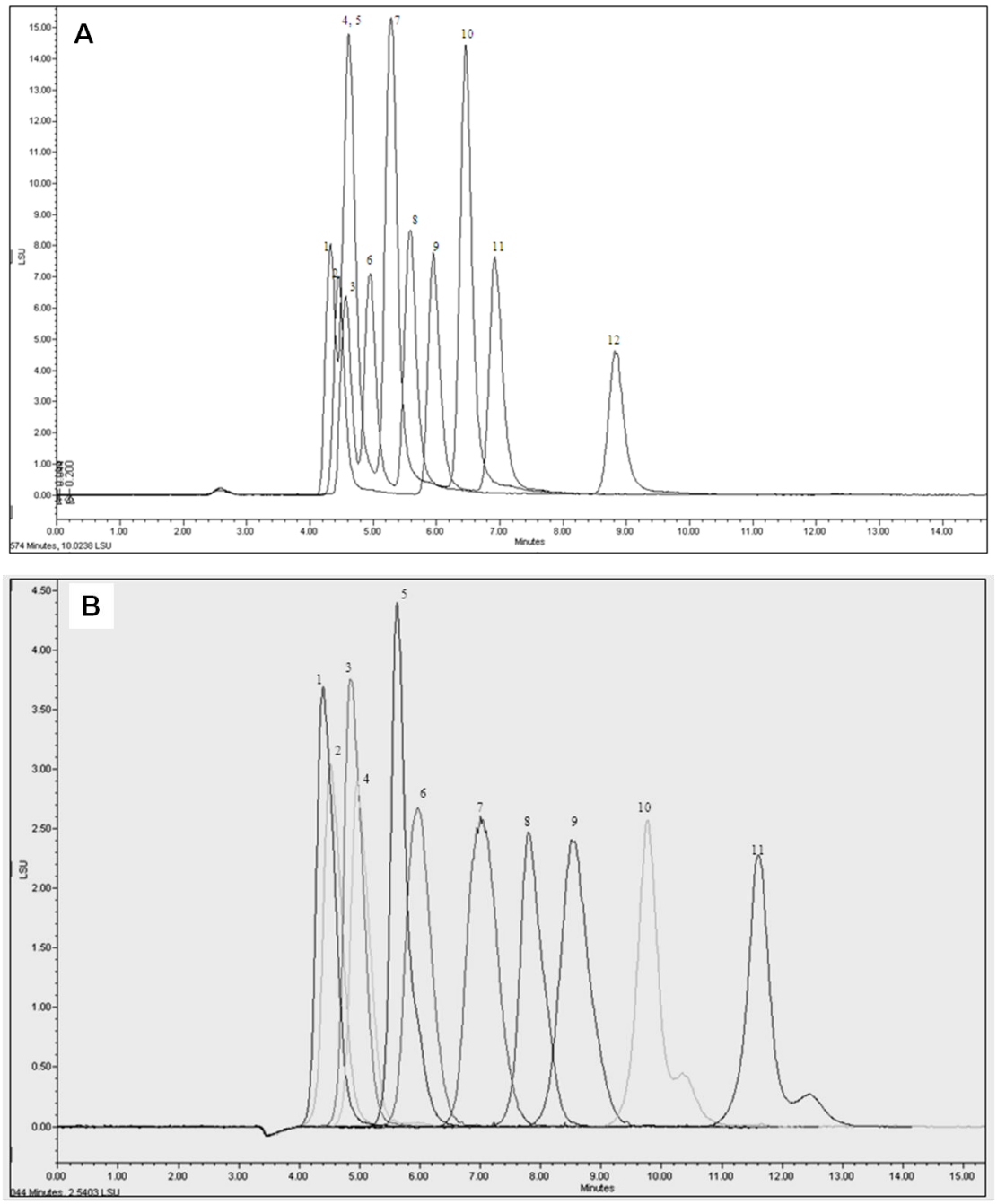

Figure 4.1 HPLC with ELSD chromatogram profile illustrating the location of standard sugars using (A) the High-Performance Carbohydrate column with acetonitrile:water (75:25, v/v) as the mobile phase (peaks: 1, fructose; 2, glucose; 3, sorbitol; 4, mannitol; 5, galactose; 6, sucrose; 7, maltose; 8, lactose; 9, nystose; 10, raffinose; 11, kestose; 12, stachyose) and (B) the Sugar-Pak column with water as the mobile phase (peaks: 1, nystose; 2, stachyose; 3, kestose; 4, raffinose; 5, sucrose; 6, lactose; 7, glucose; 8, galactose; 9, fructose; 10, mannitol; 11, sorbitol).

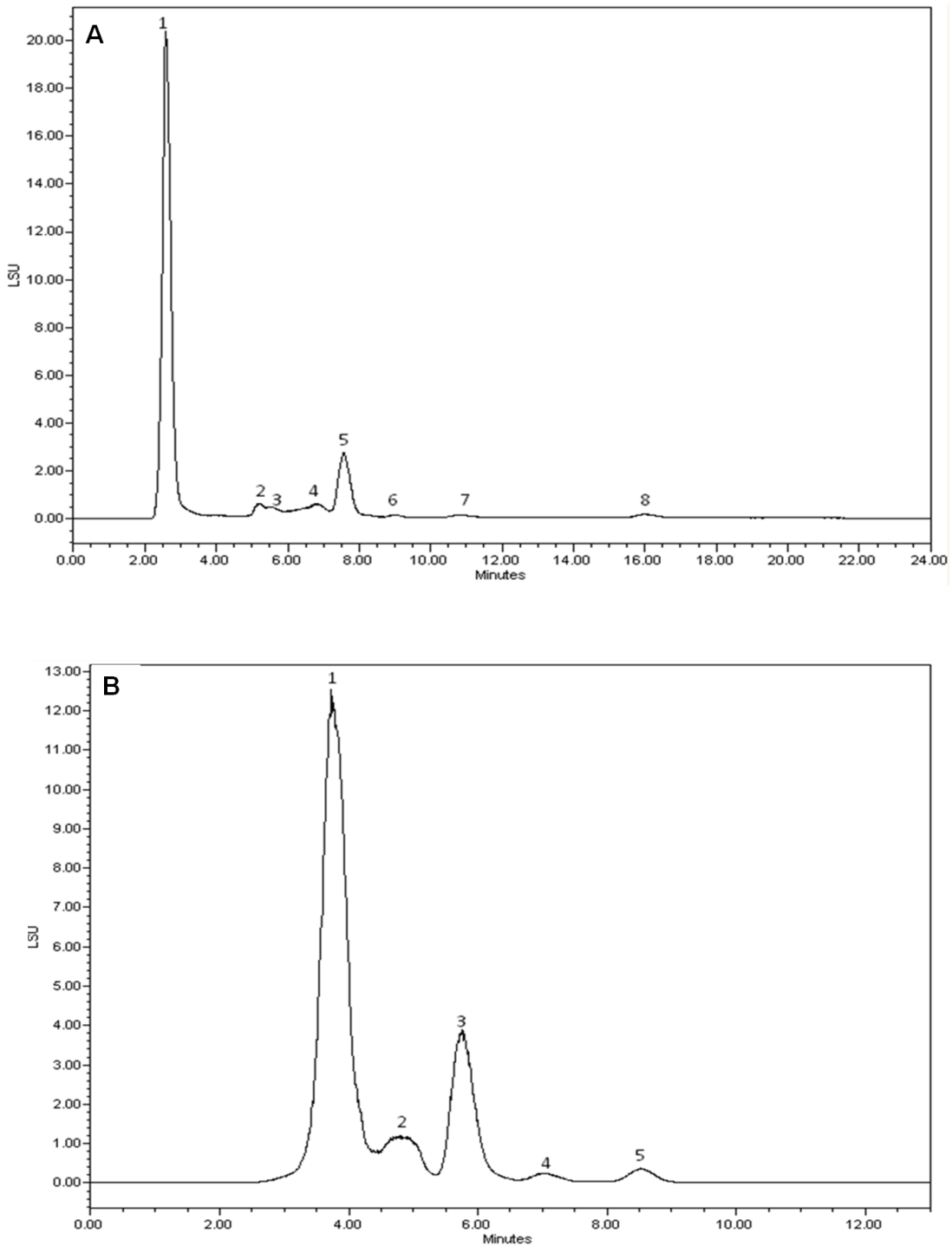

Figure 4.2 HPLC with ELSD chromatogram profile of wholegrain bread using (A) High-Performance Carbohydrate column (peaks: 1, unretained compound; 2, fructose; 3, glucose; 4, unknown sugar; 5, sucrose; 6, raffinose; 7, unknown sugar; 8, stachyose) and (B) Sugar-Pak column (peaks: 1, raffinose and stachyose; 2, unknown sugar; 3, sucrose; 4, glucose; 5, fructose).

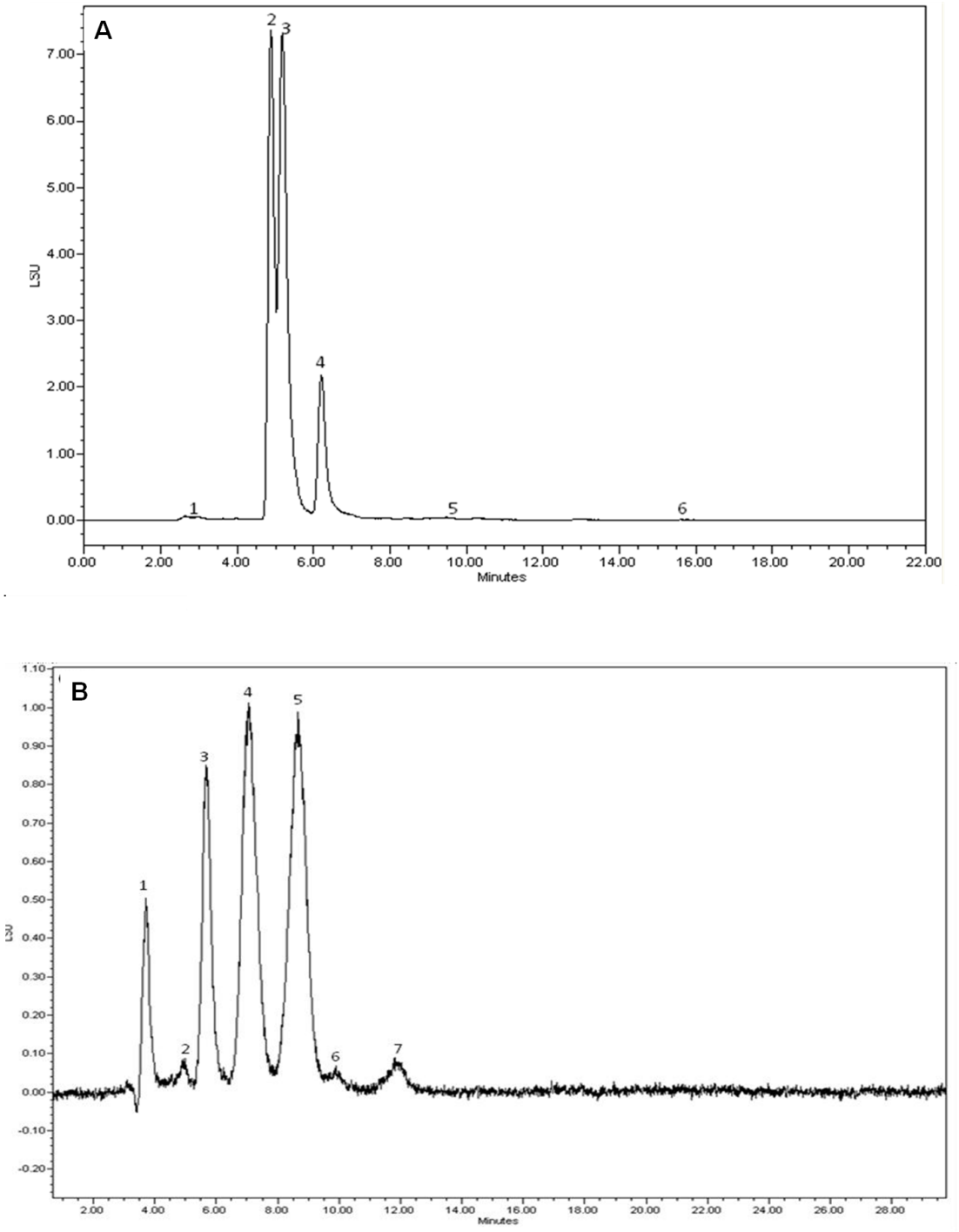

Figure 4.3 HPLC with ELSD chromatogram profile of muesli using (A) High-Performance Carbohydrate column (peaks: 1, unretained compound; 2, fructose; 3, glucose/mannitol/sorbitol; 4, sucrose; 5, raffinose; 6, stachyose) and (B) Sugar-Pak column (peaks: 1, unretained compound; 2, unknown sugar; 3, sucrose; 4, glucose; 5, fructose; 6, unknown sugar; 7, sorbitol).

4.3.2 Content of short-chain carbohydrates in cereal grain products

Quantities of SCC measured via HPLC with ELSD, as well as total fructans (measured enzymatically) in common grain and cereal products are given in Tables 4.1 – 4.5. Fructans were the major SCC present in eight common Australian grains and pastas (Table 4.1). The grains and pastas with the highest amount of fructans (g/portion as eaten) included gnocchi 1.19 g > couscous 1.12 g > wheat pasta 0.50 g > gluten-free pasta 0.24 g > quinoa pasta 0.22 g. Traces of fructose were detected in all samples except for gluten-free pasta, but fructose was found in quantifiable amounts of (g/portion as eaten) only in gnocchi (0.28 g), quinoa pasta (0.05 g) and couscous (0.02 g). No sample contained fructose in excess of glucose. Rice (brown rice, white rice, rice noodles) did not contain any quantifiable levels of SCC.

4.3.3 Content of short-chain carbohydrates in bread products

Quantities of SCC contained in ten common Australian breads are presented in Table 4.2. All breads contained fructose and glucose. The most common SCC present was fructans but other FODMAPs included excess fructose, sorbitol, mannitol and raffinose. The highest amount of total fructans (g/portion as eaten) were in dark rye 0.60 g > rye sourdough bread 0.54 g > rye 0.44 g > wheat multigrain 0.38 g > wheat wholegrain 0.36 g > white wheat bread 0.33 g > wheat wholemeal 0.23 g. Fructose occurred in excess of glucose (g/100 g as eaten) in all breads except multigrain and sourdough, the highest levels (g/portion as eaten) were in gluten-free (0.12 g) and rye breads (0.11 g). Mannitol was detected in six varieties but was quantified only in sourdough bread. Wheat, rye, spelt-based breads all contained raffinose. The highest quantifiable amounts of raffinose were found in multigrain (0.26 g) and light rye sourdough (0.17 g). Stachyose was only found in wholegrain (0.19 g) and wholemeal bread (0.15 g). The results for separate brands of spelt breads are also presented in Table 4.2. One bread containing 25 % spelt flour contained raffinose (0.14 g/portion) and the lowest total fructan content (0.07 g/portion) of any of the breads tested.

4.3.4 Content of short-chain carbohydrates in breakfast cereal products

The content of nine common Australian breakfast cereals are shown in (Table 4.3). The most common SCC measured was total fructans. The cereals with the highest amount of total fructan (g/portion as eaten) included gluten-free muesli 0.96 g > All-Bran 0.76 g > muesli 0.69 g > wheat-free muesli 0.59 g > wholegrain wheat biscuit 0.62 g > ready-to-eat mixed grain flakes with fruit and nuts 0.61 g > corn flakes 0.32 g > Rice Bubbles 0.31 g > oats 0.11 g. Oats were the only cereal not to contain fructose or glucose. Fructose occurred in excess of glucose (g/portion as eaten) in All-Bran (0.18 g), wheat-free muesli (0.18 g) and Rice Bubbles (0.04 g). No cereal contained lactose and the only sugar polyol found was sorbitol in two of the three muesli varieties analysed (gluten-free 0.49 g and plain 0.07 g). The cereals with the highest amount of raffinose on a per portion basis were All-Bran (0.43 g) and wheat-free muesli (0.29 g).

4.3.5 Content of short-chain carbohydrates in biscuit and snack products

Fifteen common Australian biscuits and snacks were analysed and results are shown in Table 4.4. The principal SCC content was fructans and these included rye crispbread 0.92 g > muesli bar with fruit 0.81 g > fruit-filled biscuits 0.63 g > pretzels 0.29 g > chocolate chip 0.21 g > and to plain rice cakes 0.09 g > plain potato chips 0.05 g. All biscuits/snacks contained fructose and glucose, where the highest amounts of excess fructose (g/portion as eaten) included plain corn thins (0.03 g) and plain rice cakes (0.03 g). Lactose was detected in five samples, flavoured corn thins had the highest (0.06 g). GOS (raffinose or stachyose) was not found in quantifiable levels. Rye crispbread was the only biscuit to contain both of the FOS (0.22 g nystose and 0.17 g kestose). Kestose was also found in pretzels (0.10 g).

4.3.6 Content of short-chain carbohydrates in pulse and LSA products

Thirteen common Australian pulses and LSA are presented in Table 4.5. The most common SCC measured in pulses was the GOS- raffinose and stachyose, and total fructans. All pulses contained GOS (raffinose and stachyose) and generally contained more stachyose than raffinose. The pulses with the highest total fructan content (g/portion as eaten) included split peas 0.66 g > red kidney beans 0.51 g > lima beans 0.27 g > soya beans 0.26 g. Although fructose and glucose were detected in all pulses, excess fructose (g/portion as eaten) was only found in four bean mix (0.49 g), chickpeas (0.03 g), canned lentils (0.01 g) and split peas (0.01 g). Both sugar polyols (g/portion as eaten) measured were present together in boiled lentils only, with green lentils slightly higher (0.04 g sorbitol and 0.27 g mannitol) than red lentils (0.02 g sorbitol and 0.10 g mannitol). Mannitol was also detected at low levels in lima beans and canned lentils.

4.3.7 Comparison of current data with other published results

The carbohydrate composition for twelve common foods obtained during the present study was compared with results published by other investigators where samples were collected and tested in Europe and North America[33,317-321] are shown in Table 4.6. The foods chosen were wheat pasta, white rice, white bread, rye bread, All-Bran, corn flakes, quick oats, Rice Bubbles, chocolate chip biscuit, rye crispbread, chickpeas, and split peas. Although a variety of methods were used to measure sugars, there was generally good agreement between the databases.

Table 4.1 Short-chain carbohydrates separated via HPLC with ELSD and total fructans as measured enzymatically in common Australian grains and pasta (g/100g fresh weight sample and g/average portion[d])

	Short-chain carbohydrates via HPLC with ELSD										Total fructans (via enzymic assay)
Food	Mono[b]- and di[c]- saccharides				Sugar Polyols[b]		GOS[c]		FOS[c]		Total fructan
	fructose	glucose	excess fructose[a]	lactose	sorbitol	mannitol	raffinose	stachyose	nystose	kestose	
Couscous, cooked											
- g/100g as eaten wt	0.01	0.03	0	nd	nd	tr	nd	nd	nd	nd	0.73
- g/154g (1 cup)	0.02	0.05	0	nd	nd	tr	nd	nd	nd	nd	1.12
Noodles, rice stick											
- g/100g as eaten wt	tr	tr	0	nd	nd	nd	nd	nd	nd	tr	nd
- g/220g (1 cup)	tr	tr	0	nd	nd	nd	nd	nd	nd	tr	nd
Pasta, gluten free											
- g/100g as eaten wt	nd	0.22	0	nd	nd	nd	nd	nd	nd	nd	0.19
- g/127g (1 cup)	nd	0.28	0	nd	nd	nd	nd	nd	nd	nd	0.24
Pasta, gnocchi											
- g/100g as eaten wt	0.14	0.21	0	nd	nd	nd	nd	nd	nd	nd	0.60
- g/199g (1 cup)	0.28	0.42	0	nd	nd	nd	nd	nd	nd	nd	1.19
Pasta, wheat											
- g/100g as eaten wt	tr	tr	0	nd	nd	nd	nd	nd	nd	nd	0.34
- g/148g (1 cup)	tr	tr	0	nd	nd	nd	nd	nd	nd	nd	0.50
Pasta, quinoa											
- g/100g as eaten wt	0.03	0.54	0	nd	nd	nd	nd	nd	nd	nd	0.14
- g/154g (1 cup)	0.05	0.83	0	nd	nd	nd	nd	nd	nd	nd	0.22
Rice, brown											
- g/100g as eaten wt	tr	tr	0	nd	nd	nd	nd	nd	nd	nd	tr
- g/180g (1 cup)	tr	tr	0	nd	nd	nd	nd	nd	nd	nd	tr
Rice, white											
- g/100g as eaten wt	tr	tr	0	nd	nd	nd	nd	nd	nd	nd	nd
- g/190g (1 cup)	tr	tr	0	nd	nd	nd	nd	nd	nd	nd	nd

[a] Excess fructose = fructose – glucose; [b] fructose, glucose, sorbitol and mannitol data were obtained from the Sugar Pak Column (column 1); [c] data for lactose, GOS (raffinose and stachyose) and FOS (nystose and kestose) were obtained using the High Performance column (column 2); [d] average portion sizes were obtained from FoodWorks Version 6; nd, not detected; tr, trace amounts detected only.

Table 4.2 Short-chain carbohydrates separated via HPLC with ELSD and total fructans as measured enzymatically in common Australian breads (g/100g fresh weight sample and g/average portion[d])

	Short-chain carbohydrates via HPLC with ELSD										Total fructans (via enzymic assay)
Food	Mono[b]- and di[c]- saccharides				Sugar Polyols[b]		GOS[c]		FOS[c]		Total fructan
	fructose	glucose	excess fructose[a]	lactose	sorbitol	mannitol	raffinose	stachyose	nystose	kestose	
Gluten free											
-g/100g as eaten wt	0.46	0.22	0.24	nd	tr	tr	0.14	nd	0.09	0.07	0.19
-g/52g (2 slices)	0.24	0.11	0.12	nd	tr	tr	0.07	nd	0.05	0.04	0.10
Rye											
- g/100g as eaten wt	0.38	0.11	0.27	tr	nd	nd	0.24	nd	nd	nd	1.05
- g/42g (1 slice)	0.16	0.05	0.11	tr	nd	nd	0.10	nd	nd	nd	0.44
Rye, dark											
- g/100g as eaten wt	0.38	0.11	0.27	nd	tr	tr	0.27	nd	nd	nd	1.42
- g/42g (1 slice)	0.16	0.05	0.11	nd	tr	tr	0.11	nd	nd	nd	0.60
Rye, Sourdough, light											
- g/100g as eaten wt	0.11	0.39	0	nd	nd	0.16	0.33	nd	nd	nd	1.07
- g/50g (2 slices)	0.06	0.20	0	nd	nd	0.08	0.17	nd	nd	nd	0.54
Spelt, 100% spelt flour											
- g/100g as eaten wt	0.16	0.12	0.04	nd	nd	nd	tr	nd	nd	nd	0.20
- g/52g (2 slices)	0.08	0.06	0.02	nd	nd	nd	tr	nd	nd	nd	0.10
Spelt, 25% spelt flour											
- g/100g as eaten wt	0.13	0.10	0.03	nd	nd	nd	0.26	nd	nd	nd	0.14
- g/52g (2 slices)	0.07	0.05	0.02	nd	nd	nd	0.14	nd	nd	nd	0.07
Wheat, Multigrain											
- g/100g as eaten wt	0.19	0.22	0	nd	nd	nd	0.38	nd	nd	nd	0.56
- g/68g (2 slices)	0.13	0.15	0	nd	nd	nd	0.26	nd	nd	nd	0.38
Wheat, White											
- g/100g as eaten wt	0.26	0.10	0.16	tr	tr	tr	0.20	nd	0.11	nd	0.68
- g/49g (2 slices)	0.13	0.05	0.08	tr	tr	tr	0.10	nd	0.05	nd	0.33
Wheat, Wholegrain											
- g/100g as eaten wt	0.27	0.19	0.08	nd	nd	tr	0.23	0.36	nd	nd	0.69

- g/52g (2 slices)	0.14	0.10	0.04	nd	nd	tr	0.10	0.19	nd	nd	0.36
Wheat, Wholemeal											
- g/100g as eaten wt	0.26	0.12	0.14	tr	tr	tr	0.19	0.31	0.15	nd	0.48
- g/48g (2 slices)	0.12	0.06	0.07	tr	tr	tr	0.09	0.15	0.07	nd	0.23

[a] Excess fructose = fructose – glucose; [b] fructose, glucose, sorbitol and mannitol data were obtained from the Sugar Pak Column (column 1); [c] data for lactose, GOS (raffinose and stachyose) and FOS (nystose and kestose) were obtained using the High Performance column (column 2); [d] average portion sizes were obtained from FoodWorks Version 6; nd, not detected; tr, trace amounts detected only.

Table 4.3 Short-chain carbohydrates separated via HPLC with ELSD and total fructans as measured enzymatically in common Australian breakfast cereals (g/100g as eaten sample and g/average portion[d])

	Short-chain carbohydrates via HPLC with ELSD										Total fructans (via enzymic assay)
Food	Mono[b]- and di[c]- saccharides				Sugar Polyols[b]		GOS[c]		FOS[c]		Total fructan
	fructose	glucose	excess fructose[a]	lactose	sorbitol	mannitol	raffinose	stachyose	nystose	kestose	
All-Bran®											
- g/100g as eaten wt	1.07	0.57	0.56	nd	nd	nd	1.32	nd	0.66	tr	2.35
- g/32.5g (0.5 cup)	0.35	0.19	0.18	nd	nd	nd	0.43	nd	0.21	tr	0.76
Corn flakes											
- g/100g as eaten wt	1.13	1.33	0	nd	nd	nd	tr	nd	nd	nd	1.07
- g/30g (1 cup)	0.34	0.40	0	nd	nd	nd	tr	nd	nd	nd	0.32
Muesli											
- g/100g as eaten wt	12.57	16.13	0	nd	0.12	nd	0.34	tr	nd	nd	1.26
- g/55g (0.5 cup)	6.91	8.87	0	nd	0.07	nd	0.19	tr	nd	nd	0.69
Muesli, gluten free											
- g/100g as eaten wt	16.81	18.98	0	nd	0.89	nd	0.33	tr	0.38	nd	1.74
- g/55g (0.5 cup)	9.25	10.44	0	nd	0.49	nd	0.18	tr	0.21	nd	0.96
Muesli, wheat free											
- g/100g as eaten wt	0.32	nd	0.32	nd	nd	nd	0.52	tr	nd	0.13	1.08
- g/55g (0.5 cup)	0.18	nd	0.18	nd	nd	nd	0.29	tr	nd	0.07	0.59
Oats, dry											
- g/100g as eaten wt	nd	nd	0	nd	nd	nd	0.34	tr	nd	nd	0.32
- g/34g (0.5 cup)	nd	nd	0	nd	nd	nd	0.12	tr	nd	nd	0.11
Ready-to-eat mixed grain flakes with fruit and nuts (Sustain™)											
- g/100g as eaten wt	5.27	5.67	0	nd	nd	nd	0.28	tr	nd	nd	2.04
- g/30g (1 cup)	1.58	1.70	0	nd	nd	nd	0.08	tr	nd	nd	0.61
Rice Bubbles®											
- g/100g as eaten wt	0.29	0.17	0.12	nd	nd	nd	nd	nd	nd	nd	1.04
- g/30g (1 cup)	0.09	0.05	0.04	nd	nd	nd	nd	nd	nd	nd	0.31
Wholegrain wheat biscuit (Weetbix®)											
- g/100g as eaten wt	0.56	0.56	0	nd	nd	nd	0.31	tr	nd	nd	2.05

- g/30g (2 biscuits)	0.17	0.17	0	nd	nd	nd	0.09	tr	nd	nd	0.62

[a] Excess fructose = fructose – glucose; [b] fructose, glucose, sorbitol and mannitol data were obtained from the Sugar Pak Column (column 1); [c] data for lactose, GOS (raffinose and stachyose) and FOS (nystose and kestose) were obtained using the High Performance column (column 2); [d] average portion sizes were obtained from FoodWorks Version 6; * not freeze-dried; nd or 0, not detected; tr, trace amounts detected only.

Table 4.4 Short-chain carbohydrates separated via HPLC with ELSD and total fructans as measured enzymatically in common Australian biscuits and snacks (g/100g fresh weight sample and g/average portion[d])

	Short-chain carbohydrates via HPLC with ELSD										Total fructans (via enzymic assay)
Food	**Mono[b]- and di[c]- saccharides**				**Sugar Polyols[b]**		**GOS[c]**		**FOS[c]**		**Total fructan**
	fructose	**glucose**	**excess fructose[a]**	**lactose**	**sorbitol**	**mannitol**	**raffinose**	**stachyose**	**nystose**	**kestose**	
Biscuit, chocolate chip											
- g/100g as eaten wt	0.17	0.12	0.05	0.28	0.08	nd	nd	nd	nd	nd	1.82
- g/11.6g (1 biscuit)	0.02	0.01	0.01	0.03	0.01	nd	nd	nd	nd	nd	0.21
Biscuit, cream filled, chocolate coating											
- g/100g as eaten wt	0.21	0.23	0	0.34	nd	nd	nd	nd	nd	nd	0.96
- g/19.4g (1 biscuit)	0.04	0.04	0	0.07	nd	nd	nd	nd	nd	nd	0.19
Biscuit, fruit filled											
- g/100g as eaten wt	21.07	22.22	0	nd	nd	nd	nd	nd	nd	nd	4.61
- g/13.6g (1 biscuit)	2.87	3.02	0	nd	nd	nd	nd	nd	nd	nd	0.63
Biscuit, savoury, plain											
- g/100g as eaten wt	0.29	0.19	0.10	nd	tr	tr	nd	nd	nd	nd	0.77
- g/17.8g (2 biscuits)	0.05	0.03	0.02	nd	tr	tr	nd	nd	nd	nd	0.14
Biscuit, savoury, wholemeal											
- g/100g as eaten wt	0.22	0.14	0.08	nd	nd	nd	nd	nd	nd	nd	0.55
- g/17.8g (2 biscuits)	0.04	0.02	0.01	nd	nd	nd	nd	nd	nd	nd	0.10
Biscuit, savoury, rye crispbread											
- g/100g as eaten wt	0.19	0.18	0.01	nd	nd	nd	nd	nd	1.11	0.84	4.60
- g/20g (2 biscuits)	0.04	0.04	0.00	nd	nd	nd	nd	nd	0.22	0.17	0.92
Biscuit, shortbread											
- g/100g as eaten wt	0.19	0.13	0.06	0.20	nd	nd	nd	nd	nd	nd	1.25
- g/12.9g (1 biscuit)	0.02	0.02	0.01	0.03	nd	nd	nd	nd	nd	nd	0.16
Biscuit, sweet, plain											
- g/100g as eaten wt	0.46	0.47	0	nd	0.12	nd	nd	nd	nd	nd	1.00
- g/14g (1 biscuit)	0.06	0.07	0	nd	0.02	nd	nd	nd	nd	nd	0.14

Chips, potato, plain											
- g/100g as eaten wt	0.64	0.40	0	nd	tr	nd	nd	nd	nd	nd	0.22
- g/22g (1 cup)	0.14	0.09	0	nd	tr	nd	nd	nd	nd	nd	0.05
Corn thins, plain											
- g/100g as eaten wt	0.25	tr	0.25	nd	tr	nd	tr	nd	nd	nd	1.35
- g/11.6g (2 biscuits)	0.03	tr	0.03	nd	tr	nd	tr	nd	nd	nd	0.16
Corn thins, flavoured (sour cream & chives)											
- g/100g as eaten wt	0.18	0.21	0	0.53	tr	nd	tr	nd	nd	nd	1.58
- g/11.6g (2 biscuits)	0.02	0.02	0	0.06	tr	nd	tr	nd	nd	nd	0.18
Muesli bar, plain with dried fruit											
- g/100g as eaten wt	4.82	7.46	0	nd	nd	nd	nd	nd	nd	nd	2.53
- g/32g (1 bar)	1.54	2.39	0	nd	nd	nd	nd	nd	nd	nd	0.81
Pretzels											
- g/100g as eaten wt	0.24	0.2	0.04	nd	0.13	nd	nd	nd	nd	0.48	1.40
- g/21g (0.5 cup)	0.05	0.04	0.01	nd	0.03	nd	nd	nd	nd	0.10	0.29
Rice cakes, plain											
- g/100g as eaten wt	0.23	tr	0.23	nd	nd	nd	nd	nd	nd	nd	0.78
- g/11.6g (2 thins)	0.03	tr	0.03	nd	nd	nd	nd	nd	nd	nd	0.09
Rice cakes, flavoured (sour cream & chives)											
- g/100g as eaten wt	tr	tr	0	0.37	nd	nd	nd	nd	nd	nd	1.60
- g/11.6g (2 thins)	tr	tr	0	0.04	nd	nd	nd	nd	nd	nd	0.19

[a] Excess fructose = fructose – glucose; [b]fructose, glucose, sorbitol and mannitol data were obtained from the Sugar Pak Column (column 1); [c] data for lactose, GOS (raffinose and stachyose) and FOS (nystose and kestose) were obtained using the High Performance column (column 2); [d] average portion sizes were obtained from FoodWorks Version 6; * not freeze-dried; nd or 0, not detected; tr, trace amounts detected only.

Table 4.5 Short-chain carbohydrates separated via HPLC with ELSD and total fructans as measured enzymatically in common Australian pulses[d] and LSA (g/100g fresh weight sample and g/average portion[e])

	Short-chain carbohydrates via HPLC with ELSD										Total fructans (via enzymic assay)
Food	Mono[b]- and di[c]- saccharides				Sugar Polyols[b]		GOS[c]		FOS[c]		Total fructan
	fructose	glucose	excess fructose[a]	lactose	sorbitol	mannitol	raffinose	stachyose	nystose	kestose	
Beans, mixed, canned											
- g/100g as eaten wt	0.71	0.22	0.49	nd	nd	nd	0.10	0.51	nd	nd	0.11
- g/100g (0.5 cup)	0.71	0.22	0.49	nd	nd	nd	0.10	0.51	nd	nd	0.11
Borlotti beans, canned											
- g/100g as eaten wt	tr	tr	0	nd	nd	nd	0.48	0.52	nd	0.10	0.13
- g/91g (0.5 cup)	tr	tr	0	nd	nd	nd	0.44	0.47	nd	0.09	0.12
Butter beans, canned											
- g/100g as eaten wt	0.14	0.15	0	nd	nd	nd	0.05	0.37	0.14	0.08	0.14
- g/70g (0.5 cup)	0.10	0.11	0	nd	nd	nd	0.04	0.26	0.10	0.06	0.10
Chickpeas, canned											
- g/100g as eaten wt	0.14	0.1	0.04	nd	nd	nd	0.11	0.08	0.07	nd	0.16
- g/86.5g (0.5 cup)	0.12	0.09	0.03	nd	nd	nd	0.10	0.07	0.06	nd	0.14
Haricot beans, boiled											
- g/100g as eaten wt	0.05	0.05	0	nd	nd	nd	0.25	0.84	nd	nd	0.26
- g/100g (0.5 cup)	0.05	0.05	0	nd	nd	nd	0.25	0.84	nd	nd	0.26
Red kidney beans, boiled											
- g/100g as eaten wt	0.03	0.04	0	nd	nd	nd	0.28	1.16	nd	0.51	0.54
- g/95g (0.5 cup)	0.03	0.04	0	nd	nd	nd	0.23	1.10	nd	0.48	0.51
Lentils, green, boiled											
- g/100g as eaten wt	nd	nd	0	nd	nd	nd	0.05	0.41	nd	nd	0.22
- g/92.5g (0.5 cup)	nd	nd	0	nd	nd	nd	0.05	0.38	nd	nd	0.20
Lentils, red, boiled											
- g/100g as eaten wt	nd	nd	0	nd	nd	nd	0.06	0.40	0.17	nd	0.14
- g/92.5g (0.5 cup)	nd	nd	0	nd	nd	nd	0.06	0.37	0.16	nd	0.13
Lentils, canned											

- g/100g as eaten wt	0.05	0.04	0.01	nd	nd	tr	0.03	0.19	nd	nd	0.15
- g/92.5g (0.5 cup)	0.05	0.04	0.01	nd	nd	tr	0.03	0.18	nd	nd	0.14
Lima beans, boiled											
- g/100g as eaten wt	0.05	0.14	0	nd	nd	0.06	0.18	1.16	nd	0.05	0.29
- g/91.5g (0.5 cup)	0.05	0.13	0	nd	nd	0.05	0.16	1.06	nd	0.05	0.27
LSA (Linseed Sunflower Almond mix)											
- g/100g as eaten wt	tr	tr	0	nd	nd	nd	0.58	0	nd	nd	0.85
- g/12.2g (1 tbs)	tr	tr	0	nd	nd	nd	0.07	0	nd	nd	0.10
Soya beans, boiled											
- g/100g as eaten wt	0.07	0.10	0	nd	nd	nd	0.15	0.64	tr	nd	0.30
- g/85g (0.5 cup)	0.06	0.09	0	nd	nd	nd	0.13	0.54	tr	nd	0.26
Split peas, boiled											
- g/100g as eaten wt	0.04	0.03	0.01	nd	nd	nd	0.33	1.55	nd	nd	0.73
- g/90g (0.5 cup)	0.04	0.03	0.01	nd	nd	nd	0.30	1.40	nd	nd	0.66

[a] Excess fructose = fructose – glucose; tr, trace amounts detected only; [b] fructose, glucose, sorbitol and mannitol data were obtained from the Sugar Pak Column (column 1); [c] data for lactose, GOS (raffinose and stachyose) and FOS (nystose and kestose) were obtained using the High Performance column (column 2); [d] pulses were soaked and boiled, or canned, drained as indicated; [e] average portion sizes were obtained from FoodWorks Version 6; * not freeze-dried; tbs, tablespoon; nd or 0, not detected.

Table 4.6 Comparison of current data with other published results (g/100g fresh weight sample)

	Short-chain carbohydrates via HPLC with ELSD										Total fructans (via enzymic assay)
Food	**Mono- and di- saccharides**				**Sugar Polyols**		**GOS**		**FOS**		**Total fructan**
	fructose	**glucose**	**excess fructose**	**lactose**	**sorbitol**	**mannitol**	**raffinose**	**stachyose**	**nystose**	**kestose**	
Grains & pasta											
Pasta, wheat											
current[a]	tr	tr	0	nd	nd	nd	nd	nd	nd	nd	0.34
others[b]	0	0	0	0	-	-	-	-	-	-	-
others[c]	0	0	0	-	-	-	-	-	-	-	-
Rice, white											
current[a]	tr	tr	0	nd	nd	nd	nd	nd	nd	nd	0
others[b]	0	0	0	0	-	-	-	-	-	-	-
others[c]	0.10	0.06	0	-	-	-	-	-	-	-	-
others[d]	nd	0	0	0	-	-	nd	nd	-	-	-
others[f]	-	-	-	-	-	-	-	-	-	-	0
Breads											
White											
current[a]	0.26	0.10	0.16	tr	tr	tr	0.23	nd	0.11	nd	0.68
others[b]	0.20	0.20	0	0	-	-	-	-	-	-	-
others[c]	0.73	0.37	0.36	-	-	-	-	-	-	-	-
others[d]	1.50	1.80	0	nd	-	-	nd	nd	-	-	-
Rye											
current[a]	0.38	0.11	0.27	tr	nd	nd	0.24	nd	nd	nd	1.05
others[b]	0.40	0.40	0	0	-	-	-	-	-	-	-
others[c]	0	0	0	-	-	-	-	-	-	-	-
others[g]	-	-	-	-	-	-	-	-	-	-	0.96
Breakfast cereals											
All-Bran®											
current[a]	1.07	0.57	0.56	nd	nd	nd	1.22	nd	0.66	tr	2.35
others[b]	1.70	1.20	0.50	0	-	-	-	-	-	-	-
others[e]	0.70	0.90	0	0	-	-	-	-	-	-	-
Corn flakes											

current[a]	1.13	1.33	0	nd	nd	nd	tr	nd	nd	nd	1.07
others[b]	2.40	2.40	0	0	-	-	-	-	-	-	-
others[d]	2.40	1.40	1.00	0	-	-	nd	nd	-	-	-
others[e]	2.90	1.60	1.30	0	-	-	-	-	-	-	-
Oats, dry											
current[a]	nd	nd	nd	nd	nd	nd	0.34	tr	nd	nd	0.32
others[b]	0	0	0	0	-	-	-	-	-	-	-
others[d]	nd	0	0	0	-	-	nd	nd	-	-	-
Rice Bubbles®											
current[a]	0.29	0.17	0.12	nd	nd	nd	nd	nd	nd	nd	1.04
others[b]	1.30	1.00	0.30	0	-	-	-	-	-	-	-
others[d]	0	0	0	0	-	-	nd	nd	-	-	-
others[e]	0.40	0.90	0	0	-	-	-	-	-	-	-
Biscuits & snacks											
Biscuit, chocolate chip											
current[a]	0.17	0.12	0.05	0.28	0.08	nd	nd	nd	nd	nd	1.82
others[b]	0.20	0.30	0	0.60	-	-	-	-	-	-	-
others[d]	0.30	0.70	0	1.80	-	-	nd	nd	-	-	-
Biscuit, savoury, rye crispbread											
current[a]	0.19	0.18	0.10	nd	nd	nd	nd	nd	1.11	0.84	4.60
others[b]	0.10	0.20	0	0.10	-	-	-	-	-	-	-
others[e]	0.90	0.50	0.40	0	-	-	-	-	-	-	-
others[g]	-	-	-	-	-	-	-	-	-	-	4.20
Pulses											
Chickpeas											
current[a], canned	0.14	0.1	0.04	nd	nd	nd	0.11	0.08	0.07	nd	0.52
others[b], canned	0	0	0	0	-	-	-	-	-	-	-
others[c], canned	0	0	0	-	-	-	-	-	-	-	-
others[d], boiled	0.10	0.10	0	0	-	-	0.40	0.50	-	-	-
Split peas											
current[a], boiled	0.04	0.03	0.01	nd	nd	nd	0.33	1.55	nd	nd	0.73
others[b], boiled	0	0	0	0	-	-	-	-	-	-	-
others[c], boiled	0	0	0	-	-	-	-	-	-	-	-
others[d], boiled	nd	nd	0	0	-	-	nd	nd	-	-	-

[a] Current study using HPLC with ELSD and Fructan Enzymic Assay; [b] Data obtained from NUTTAB database[321] using gas chromatography (GC) and HPLC; [c] Results[318] obtained using HPLC according to AOAC Method 982.14; [d] Data[319] based on analysis via HPLC or GC; [e] Data[320] obtained using enzymic, GC, HPLC; [f] Fructans DP2, DP3

and DP4 measured only;[317] [g] Total fructan content measured using AOAC Method 999.03;[33] excess fructose = fructose – glucose; * not freeze-dried; tr, trace amounts detected only; nd, analysed but not detected; – , not analysed.

4.4 Discussion

This study provides comprehensive information about the content of the major FODMAPs including the prebiotics (fructans and GOS) and other SCC in common processed grains, cereals and pulses. The results show clearly that fructans are the major SCC present in wheat-based grains, pasta, breads and breakfast cereals, while GOS and total fructan predominate in pulses. To our knowledge, there are no other composition tables that record quantitative information regarding mono- and di-saccharides, sugar polyols, GOS, FOS and total fructan in the same food samples.

Quantifying this number of SCC in foods requires a number of analytical approaches. We have used HPLC with ELSD to quantify most SCC as described in detail previously (Chapter 3, Section 2.2). Due to the problem of 'co-elution' of some carbohydrates using this approach, two separate columns with two different mobile phases were used to ensure that sugars of interest were well separated. Use of HPLC methodology alone, however, does not provide accurate quantitative information about the large fructan family where another approach involving enzymic hydrolysis is required.

Foods contain a complex mixture of fructans of different chain lengths including short-chains of DP (i.e., degree of polymerization) 2-9 units or FOS (including nystose GF_3 and kestose GF_2) as well as longer chain DP $\geq$ 10 or 'inulins'.[33] The HPLC method described here is only suitable for quantification of FOS- nystose and kestose and does not provide information about other longer-chain fructans DP $\geq$ 4. For this reason, the total fructans were measured using highly purified and specific enzymes to hydrolyse and remove sucrose, starch, free fructose and glucose followed by fructanase to hydrolyse all fructans present in the sample.[218] This study showed clearly that total fructans in cereal grains products were highest in couscous (1.12 g/portion), muesli (0.96 g/portion) and dark rye bread (0.6 g/portion) and lowest in rice and rice products (0-trace levels/portion).

The results from this study show clearly that cereal grain products can contain a number of different types of short chain carbohydrates. The quantity and type of SCC present will in turn depend on the nature of the grain-ingredient used in the manufacture of these products. For example, products made from rice tend to be very low in FODMAP or prebiotic carbohydrates, while products made using durum wheat (e.g. couscous, wheat pasta) and rye (sourdough bread, savoury rye crispbread) tended to be high. This clearly demonstrates that levels can be manipulated in cereal grain products through choice of grain ingredient.

Indeed the differences between the breads analysed in this study and other studies[322] may be the consequence of the different types of flours used in the baking; for example the addition of soy flour to a bread ingredient will boost the GOS content. It is well known that soy products including soy flour will be high in GOS.[323] In addition, the use of varying sourdough cultures in bread making may alter the total FODMAP content. During the sourdough bread making process, the sourdough culture fermentation can produce mannitol as a by-product,[324] but also significantly degrade the rye fructan.[276]

Other types of food processing may also affect quantities of SCC measured in food. There were differences between boiled and canned lentils in the levels of raffinose and fructan, possibly due to effects of cooking. Cooking can reduce or increase GOS contents depending on the extent of ‘leaching’ of the water-soluble GOS into the surrounding cooking solution or possibly release of bound GOS within the food matrix.[325]

The high FODMAP products had a tendency to be gluten-containing and products with a low FODMAP content were mostly gluten-free (highlighted in Figure 4.4A). Recent analyses of unprocessed grains and flours has since been undertaken (Figure 4.4B).[326] These findings confirmed fructans and GOS being the FODMAP most present in raw grains. It also confirmed that common gluten-free grains (e.g., maize, rice, buckwheat, quinoa) are lower in FODMAP content, especially when compared to gluten-containing grains (e.g., wheat, rye).

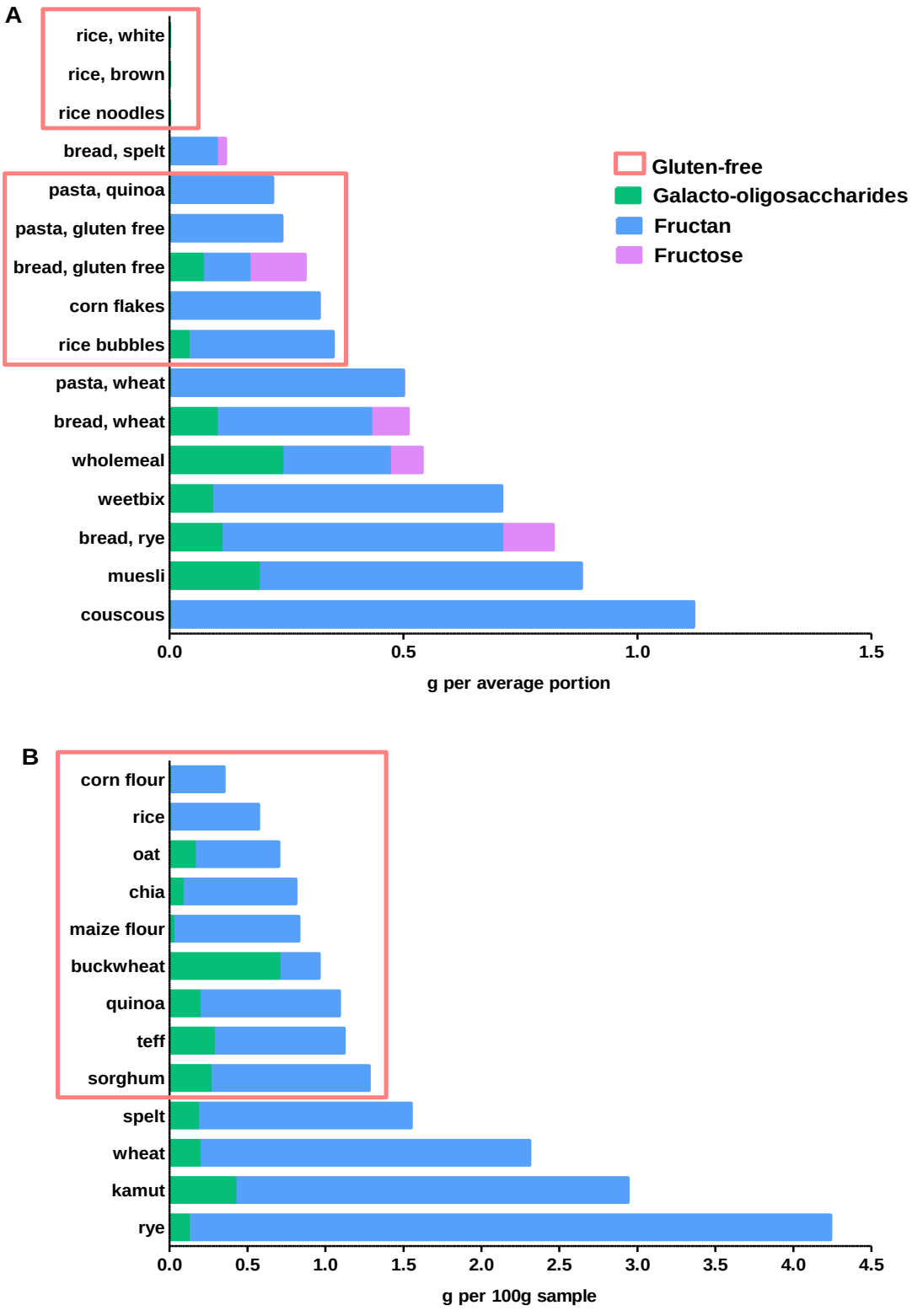

Figure 4.4 FODMAP analysis of (A) cereal grain products (g/average portion), and (B) raw grains and flours (g per 100g sample).[326]

With the increasing evidence supporting the benefits of dietary SCC, the information produced here may be used as a guide for people wanting to increase their natural dietary sources of these prebiotic-carbohydrates.[24,305,327] The majority of inulin and FOS research focuses on the plant species suitable for industrial functional food and non-food application. This current data highlights that fructans (FOS and inulin) are also naturally present in significant amounts in other dietary sources. The benefits derived from these carbohydrates rely upon the rapid fermentation and the subsequent expansion of bacterial populations, especially of *bifidobacteria* and *lactobacilli* in the gut.[31] A dose range of 3.5-7 g per day of these fructans as pure powders has been shown to produce these types of physiological benefits.[25] This current study clearly shows the foods that will be naturally high in prebiotic fructans and GOS include dark rye, couscous and pulses. Although no studies exist to support the bifidogenic effects of unfortified foods, a dose of 3.5 g per day could be reached easily by one average portion of couscous (1.12 g), borlotti beans (1.03 g), pasta (0.50 g) and two slices of dark rye bread (1.2 g).

Conversely, the data derived from this present study may also be used in conjunction with our earlier work describing the FODMAP content of fruits and vegetables[217,218] to provide a more complete dietary strategy to reduce functional gut symptoms in patients with IBS.[75] IBS is a common disorder in our community and affects one in seven adults.[313] Short-chain carbohydrates provide unabsorbed substrates to be fermented by colonic microflora producing gases (H_2, CO_2, CH_4) and greatly contributing to gastrointestinal symptoms such as wind, bloating and pain in IBS.[64,196,213,314] This study followed past observations[217] that some foods contain a number of FODMAPs, the load of which may be particularly problematic for IBS patients. For example, the muesli samples contained excess fructose, sorbitol, GOS as well as total fructans. Other grains and cereal products that contained more than one type of FODMAP included breads, all breakfast cereals, all biscuits and snacks and all pulses. High FODMAP-containing foods that should be avoided for individuals with IBS include couscous, wheat pasta, rye-products, All-Bran and pulses.

Interestingly, of the breads tested in this study, spelt bread had the lowest levels of fructans. Spelt bread made from 100% Australian spelt flour (with no addition of soy flour) had the lowest levels

of total FODMAPs and only trace amounts of GOS- raffinose. This observation may help to explain, in part at least, why spelt bread is often reported to be well tolerated by people with functional gut disorders.[328] The low level of fructans and total FODMAP contained in spelt bread compared with other breads reduce unabsorbed carbohydrates to be fermented by the colonic microflora producing less gastrointestinal symptoms.

While it should be noted that all products used in this study were sampled in Australia, and that food products from other countries may yield different results, nevertheless, there was good agreement between the results obtained in this present study with data collected and tested in Europe and North America (see Table 4.7).

4.5 Conclusions

In conclusion, this study provides comprehensive information about the major poorly absorbed SCC and total fructan content of common processed grains, cereals and pulses. This data expands our knowledge about SCC food composition, detailing the natural food sources of prebiotics (FOS, GOS). There is a high frequency of co-existence of gluten and high-FODMAP contents in cereal and grain products, and gluten-free products tend to be low in FODMAP content. This observation may help explain, in part at least, the symptom improvement that individuals with IBS experience while following a ‘gluten-free’ diet. The role of gluten, however, needs to be investigated. This information will assist in defining the role of FODMAPs in health as well as refining the dietary approach for the management of individuals with GI symptoms associated with IBS.

Chapter 5 – Self-diagnosis of non-coeliac gluten sensitivity

5.1 Background and aims

The most well understood gluten intolerance is coeliac disease, an immune-mediated GI disease estimated to affect 1% or more of Western populations.[329] It occurs when genetically susceptible patients are exposed to dietary gluten, the major protein in wheat, rye, barley and related grains, activating a specific immune response. Poorly digested gluten peptides are deamidated by intestinal tissue transglutaminase,[330] eliciting a T-cell response. These events lead to small intestinal villous atrophy, intraepithelial lymphocytosis, and the development of GI symptoms.[21]

More than 99.6% of individuals with coeliac disease possess the human leukocyte antigen (HLA)-DQ2 and/or HLA-DQ8.[128] The serological tests, deamidated gliadin, endomysial and tissue transglutaminase antibodies, and, more recently, HLA status are important screening tools for coeliac disease and are pivotal in determining who should undergo endoscopy and biopsy.[331] The only known treatment is a lifelong, strict GFD.[246] Since the GFD usually normalises serological markers and leads to healing of the small intestine in more than 50% of patients, disease investigation prior to removal of gluten from the diet is essential. The risks and complications if left untreated include higher mortality, increased risk of malignancy, growth impairment in children, infertility, osteoporosis and autoimmune disease, as recently reviewed.[92]

Many of the GI symptoms seen in coeliac disease (such as diarrhoea, bloating, gut pain) can mimic IBS, a disorder characterised by a lack of biomarkers and based on symptom diagnostic criteria.[70] There is an emerging belief that gluten intolerance might mediate IBS symptoms.[332] The existence of non-coeliac gluten sensitivity (NCGS), defined as those without coeliac disease but whose GI symptoms improve on a GFD, has been hypothesised and will be studied in detail in the following chapters (Chapters 6, 7 and 8). There are no other controlled clinical studies, and related scientific evidence assessing the effects of gluten outside of coeliac disease have focussed on animal models or cancer cell lines.[181,184,185] Regardless, the GFD is prescribed by alternative health practitioners and recommended on internet sites. This prescription of the GFD for gut and other symptoms may lead to missing the diagnosis of true coeliac disease, putting some patients at risk of adequate management and screening of associated complications (i.e., reduced bone health)[333] if left untreated. Although, only few studies suggest that undiagnosed coeliac disease is associated with long-term mortality,[134,334] other studies show no risk increase.[333]

The GFD is not simple and is generally inappropriate for patients to be on life-long, because it is markedly restrictive and presents challenges when eating at places other than home. It can be two to three times more expensive than that of a standard diet[254] and may also be nutritionally inadequate, especially fiber and B-vitamins.[259,335] It has been estimated in Australia, that for every person who has diagnosed coeliac disease, there are twenty others eating gluten-free food.[7] Such trends are occurring worldwide and are not restricted to adults, for example approximately 5% of New Zealand children avoid gluten, prevalence five times higher than that of actual doctor-diagnosed coeliac disease.[227] However, there is a paucity of information investigating in this non-coeliac population as to why individuals choose to follow the GFD and whether they have had coeliac disease formerly excluded. It is unknown how well subjects who believe they have NCGS understand the GFD and their adherence to the diet.

It was hypothesised that, in subjects who believe they have NCGS, that although these individuals believe they do not have coeliac disease, they have not had adequate investigations to confirm this is so. The aims of the present study were to characterise the sub-group of people on GFD who believed they had NCGS.

5.2 Methods

5.2.1 Subjects

From January 2010 to February 2011 in metropolitan Melbourne, Australia, flyers distributed through websites, local clinic rooms and a local newspaper advertised for adults who believed they had NCGS. The flyer was advertised in the context of a clinical trial for volunteers wanting to participate in a research study (Study Two discussed in Chapter 7) and clearly stated the inclusion criteria (for example, living in Melbourne; had coeliac disease ruled out; have currently well controlled symptoms; follow a GFD; aged 16 years or older) and that participation would involve consuming gluten, taking blood samples and collecting faecal samples.

5.2.2 Protocol

Respondents were asked to fill out a questionnaire (not-validated), consisting of 23 items, divided into three dimensions of symptoms, diet and coeliac disease investigation (see Appendix 11). The advertising, questionnaire and protocol were approved by the Eastern Health Research and Ethics Committee.

5.2.3 Statistical analyses

Data were analysed descriptively. Proportions were compared using Chi-square analysis or Fisher's exact test. Time on a GFD was compared using Mann-Whitney test. P-value of 0.05 or less was considered statistically significant.

5.3 Results

5.3.1 Survey population

Of 233 respondents to advertising, 132 completed and returned the survey. The mean age of respondents was 40 (range 16 – 84) years and 117 were female.

5.3.2 Details of symptoms

The range and frequency of symptoms described by respondents experienced after consuming gluten is shown in Figure 5.1. 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). Participants were then asked whether they currently felt in control of their symptoms, of which 24% answered “no”, 3% answered “sometimes”, 17% answered “mostly” and 56% answered “yes”. Under a third (29%; n = 38) of participants reported having had a hydrogen breath test, 23 (61%) of whom reported positive results for fructose and nine (24%) for lactose.

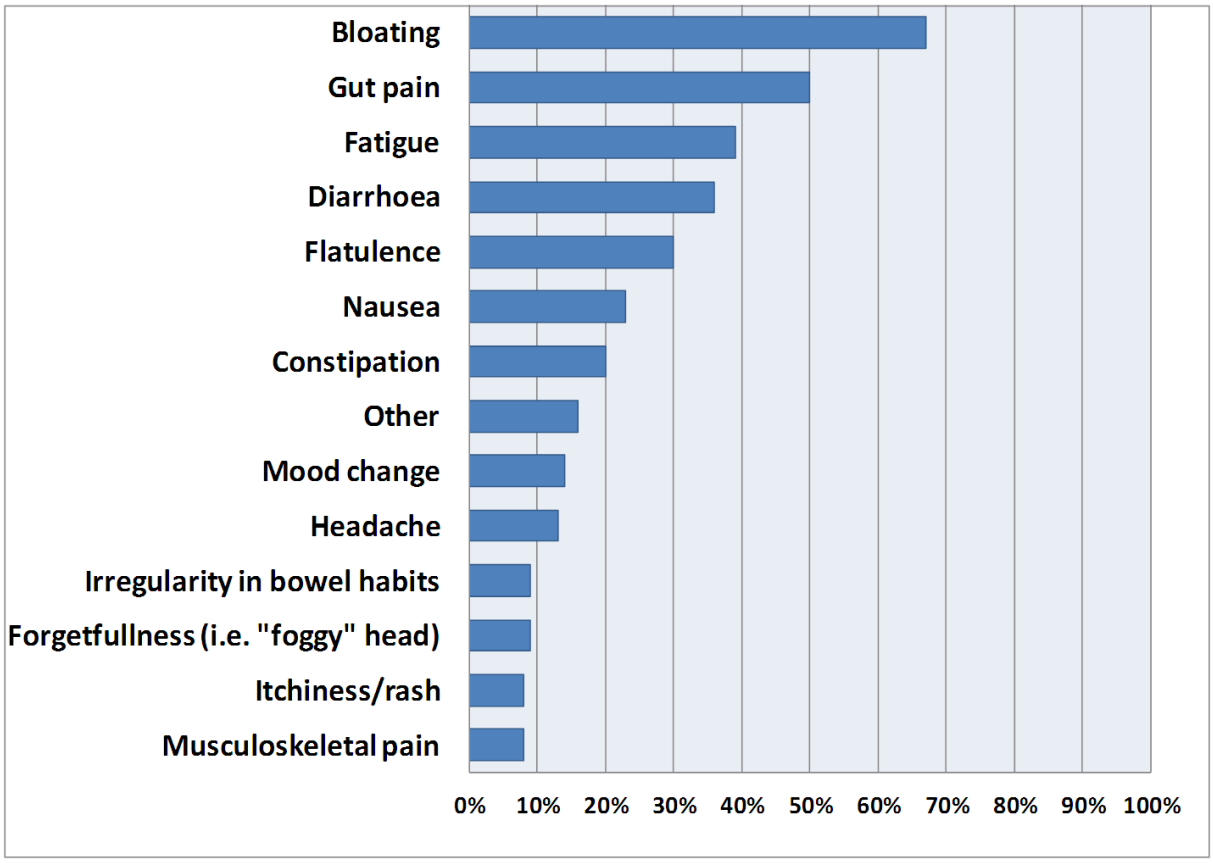

Figure 5.1 Most common symptoms related to gluten intake reported by participants (n=132). "Other" refers to descriptions of flu-like symptoms, dry retching, congestion, reflux, mouth symptoms, belching, shivers/shudders, sore throat, dizziness, poor balance, dry eyes, locomotion, blood nose, hot flushes, hiccups and sweats.

5.3.3 Adherence of the gluten-free diet

Participants were asked whether they believed they follow a strict GFD, answering "no" (21%), "sometimes" (8%), "mostly" (17%) or "yes" (54%). The median time for all respondents to be following a GFD was 24 (range 0.25 – 360) months. The reasons why participants initiated the GFD varied. It was self-initiated in 45%, and prescribed by alternative health professionals in 22%, dietitians in 19% or general practitioners in 14%.

5.3.4 Diagnostic investigations of coeliac disease

Investigations performed for the diagnosis of coeliac disease are shown in Figure 5.2. No investigation whatsoever (HLA status, antibody testing or biopsy) had been performed in 14% of the respondents. Of the 63 participants who had duodenal biopsies, 35% (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 of at least four weeks of 16 g gluten per day (according to Australian guidelines).[201] Only 40% (n = 25) 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 six participants having more than one biopsy.

In total, only 23% met the description of being NCGS. The remaining had inadequate exclusion of coeliac disease (73%), and/or uncontrolled symptoms despite gluten restriction (27%), and/or was not following a GFD (29%).

Coeliac disease was inadequately excluded in 44 of the 60 (73%) participants who self-initiated the GFD, compared with 21 of the 28 (75%) initiated by alternative health practitioners, 12 of the 26 (46%) initiated by dietitians and 11 of the 18 (61%) initiated by general practitioners (p=0.064; Chi-square). The only statistical significant difference in inter-group comparisons was for dietitians versus self-initiated (p=0.016; Fisher's exact).

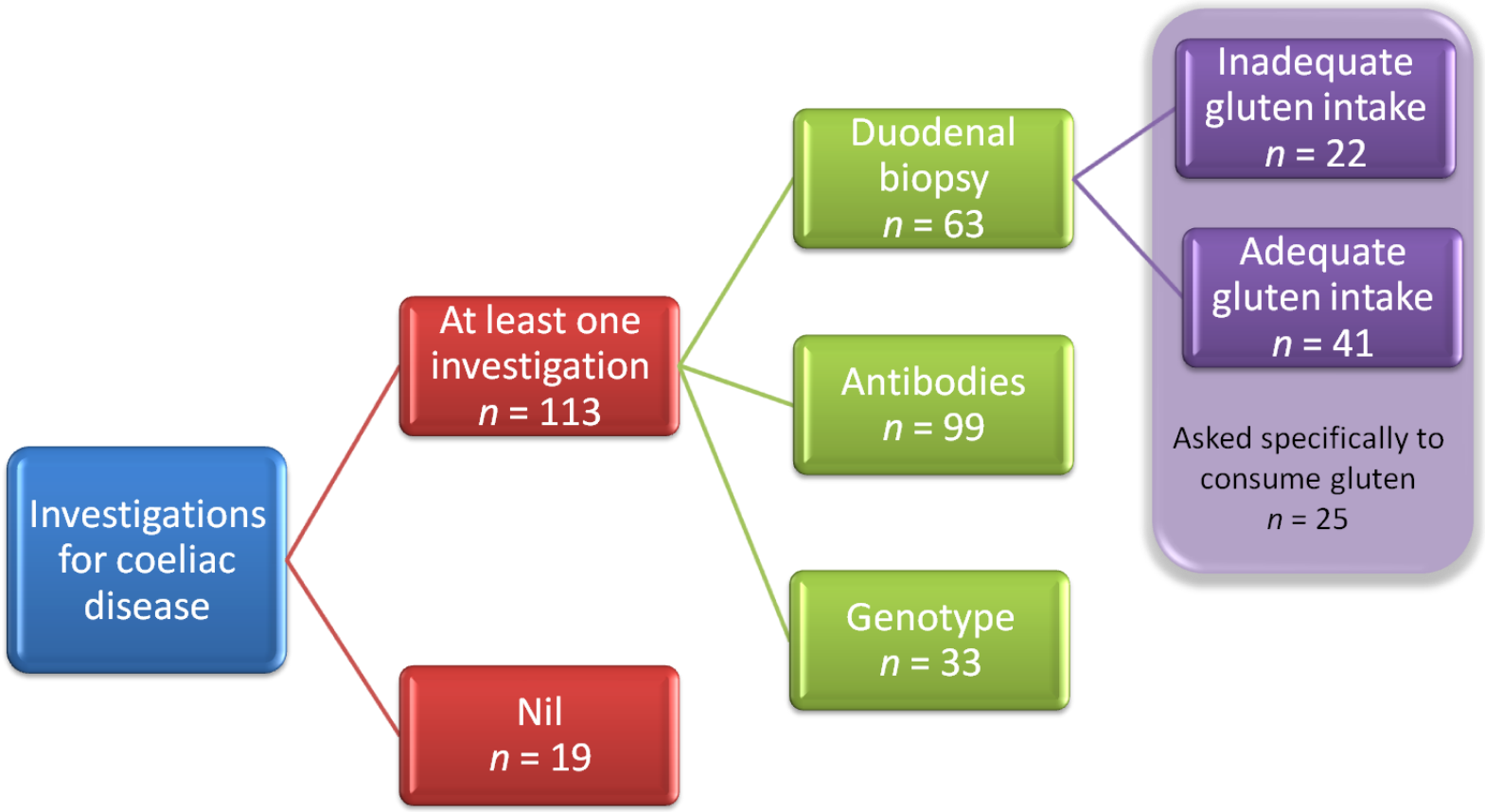

Figure 5.2 Investigations for coeliac disease in participants following a gluten-free diet (n = 132)

5.5 Discussion

Although our understanding of coeliac disease has considerably improved both clinically and pathologically during recent decades, the evidence behind NCGS remains scarce and incomplete. This survey has provided the first data to characterise and contribute to our understanding of self-diagnosis of NCGS by Australian adults.

It is estimated that around one million Australians are now eating gluten-free foods, presumably for perceived or potential health benefits.[7] This survey targeted a sub-group who use a GFD for relief of their GI symptoms. While it might have been anticipated that there would have been a greater number of respondents, the survey did form part of recruitment for volunteers for a more intense research study, which may have been a deterrent. Although participants were almost exclusively women with a mean age of 40 y, until more comprehensive data on the underlying background rate of NCGS exists, comparisons cannot be made to show whether these respondents are representative of the overall population who consider themselves to have NCGS.

These results were based on participant interpretation, and are limited by not having access to medical or dietary histories. More detailed assessments including biopsy reports were only followed up for those meeting all inclusion criteria and expressing interest to participate in the research study.

Although 54% believed they were following a strict GFD, the accuracy of this and whether they have a detailed understanding of what entails being gluten-free (including basic knowledge of gluten containing cereals, label reading, hidden sources and cross-contamination issues) would only be confirmed with dietetic assessment. A somewhat surprising finding was that one in four participants judged him/herself to be gluten-sensitive and, despite gluten avoidance, remained markedly symptomatic. Although some patients with true coeliac disease may have unresolved symptoms even when gluten-free, there are other dietary components such as fermentable

carbohydrates that have been shown to contribute to the generation of symptoms in patients with IBS,[51,196] which warrant investigation.

Coeliac disease is under-diagnosed as up to 90% of the coeliac population in Western countries remains unrecognised.[136,336,337] A recent meta-analysis suggested that patients with IBS are four times as likely to suffer from coeliac disease than the general population.[338] In addition coeliac patients may present with a wide range of symptoms and clinical manifestations, varying in severity.[137] Therefore, there is no justification for the 67% of survey participants to have inadequate exclusion of coeliac disease. We acknowledge without access to the patients' records, we cannot be certain that more investigations were actually undertaken. As many of the participants were recommended a GFD, it is hoped that coeliac disease was indeed in the mind of the health care personnel caring for them.

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 and were significantly less likely to teach the GFD without first excluding coeliac disease than those who self-initiated. The importance of excluding coeliac disease cannot be underplayed. Being definitively diagnosed with coeliac disease ensures ongoing monitoring for associated conditions, memberships to support groups, health fund assistance and in many European countries, a subsidy for the GFD.

The gold standard in the diagnosis of coeliac disease and best clinical practice remains duodenal biopsy, although new European Society for Paediatric Gastroenterology, Hepatology and Nutrition recommendations suggest this may not always be the case.[139] 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. For this reason, patients should not commence a GFD and, ideally, should be gluten-loaded prior to being tested for coeliac disease. However, the dose of gluten needed and the length of time of such loading has not been well studied. The Australian Therapeutic Guidelines recommend an adequate

challenge protocol to be the equivalent of four to six slices of bread (16-20 g gluten) per day for at least six weeks.[201] More than one in three patients in the present study had inadequate gluten intake (of at least four weeks of 16 g gluten per day) at the time of endoscopy, but 40% of those patients who underwent endoscopy 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 that is 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.

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. It must be a key strategy to get information and education about this aspect to patients, dietitians, general practitioners and gastroenterologists.

5.6 Conclusions

In conclusion, the practice of initiation of a GFD without adequate exclusion of coeliac disease is common. The belief by one in four individuals that they are gluten-sensitive despite uncontrolled symptoms seems illogical, but most patients appear to be well versed in the GFD. This is a new research area requiring evidence to confirm the existence of NCGS, and to establish a biomarker and clinical tools to definitively characterise and fully understand NCGS.

Chapter 6 – Study One: Non-coeliac gluten sensitivity may exist

6.1 Background and aims

In clinical practice, some patients have symptoms of IBS that respond well to a GFD but they have no markers of coeliac disease. The published scientific literature is largely devoid of the so-called "non-coeliac gluten sensitivity" (NCGS) or "wheat intolerance", yet they are widely believed to be very common.[181,182,339] In the evaluation of exclusion diets, wheat has been found to be one of the most common factors inducing GI symptoms,[171] but it is not known whether gluten is the responsible agent, since wheat, the major cereal removed from the GFD, contains other components that include other proteins, lipids and carbohydrates. Of particular importance are fructans which are poorly absorbed carbohydrates (one of the fermentable, poorly absorbed, short chain carbohydrates termed FODMAPs – Fermentable Oligo- Di- and Mono-saccharides And Polyols)[9] and can induce symptoms themselves.[51,196]

The role of gluten in coeliac disease is clear. The toxic peptide sequences have been defined [87,340], 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).[126] This process initiates a cascade of events resulting in mucosal inflammation, small intestinal villous atrophy,[88] increased intestinal permeability,[89] malabsorption of macro and micronutrients,[91] 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.[182-185,187,244,339] Whether

gluten itself can contribute to GI symptoms and/or induce injury to the proximal small intestine in non-coeliac patients has never been directly assessed.

It was hypothesised that:

(i) Gluten can cause GI symptoms in patients without coeliac disease; and

(ii) Gluten does so by causing intestinal injury and/or inflammation in such subjects.

The aims of Study One were to examine these hypotheses and to preliminary screen for potential mechanisms. 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 GFD.

6.2 Methods

6.2.1 Subjects

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. Thirty-nine subjects meeting the inclusion/exclusion criteria (see Chapter 3, Section 3.3) gave written, informed consent. Briefly, the inclusion criteria were age greater than 16 years, symptoms of IBS fulfilling Rome III criteria that have improved on a GFD, and adherence to the diet for at least six weeks immediately prior to screening. Adherence was assessed by patients completing a GFD compliance and knowledge questionnaire at baseline (see Appendix 2). 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 GI 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.

6.2.2 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 GFD 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 Section 6.2.4) every day for six weeks (shown in Figure 6.1).

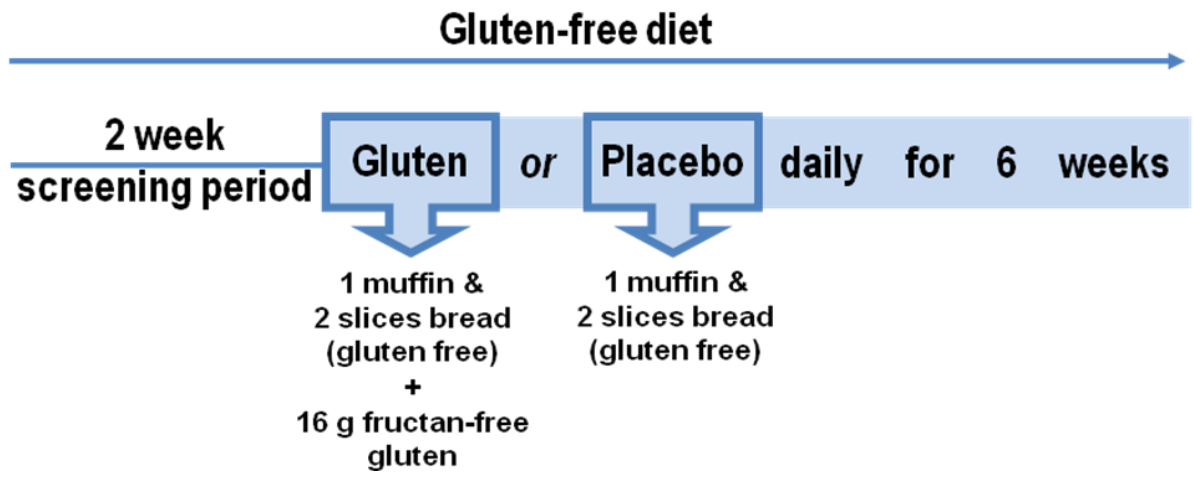

Figure 6.1 Study One protocol outline

Both the patient and the investigators evaluating the patient were blinded to the study treatment. Food diaries (Appendix 3) 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.

6.2.3 End-points

The primary end-point 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. The secondary end-points were the change in overall and individual GI symptoms as assessed by the VAS (see Chapter 3, Section 6.2), and changes in biomarkers (see below).

6.2.4 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 (Chapter 3, Section 5.1). To assess FODMAP content, the gluten was analysed as previously described (Chapter 3, Section 2.2) 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 (shown in Figure 6.2). Compliance with the study treatment was assessed by an unused food count at weeks three and six. Compliance with the GFD was judged on food diary entries and on specific questioning at review.

Figure 6.2 Study One study foods

6.2.5 Measurements

6.2.5.1 Gastrointestinal symptoms

Gastrointestinal symptoms were assessed by participants completing weekly diary cards, which contained the question for the primary end-point detailed previously (see above Section 6.2.3) and used a 100 mm VAS, with zero representing no symptoms, to score the presence and severity of overall abdominal symptoms, abdominal pain, bloating, wind, tiredness and nausea (see Appendix 8). At the end of each study week and for three weeks after completion of the study intervention, symptoms were evaluated.

6.2.5.2 Potential biomarkers

At weeks zero and six, serum, urine and stool samples were collected for analysis. 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.[187] 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.[217] 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.

6.2.6 Statistical analyses

Power calculations were based upon a placebo effect using a similar end-point and re-challenge methodology of about 20%[187] 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 previous Chapter 5, Section 2.1).

To determine the relationship between tolerable symptoms ("yes" / "no") over the six weeks, a generalized estimating equation,[84] was utilised (primary end-point). 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.

6.3 Results

6.3.1 Study population

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 6.3. Following randomisation, five patients had to be withdrawn. Thus, 34 patients completed the study as per protocol; 19 received gluten and 15 received placebo.

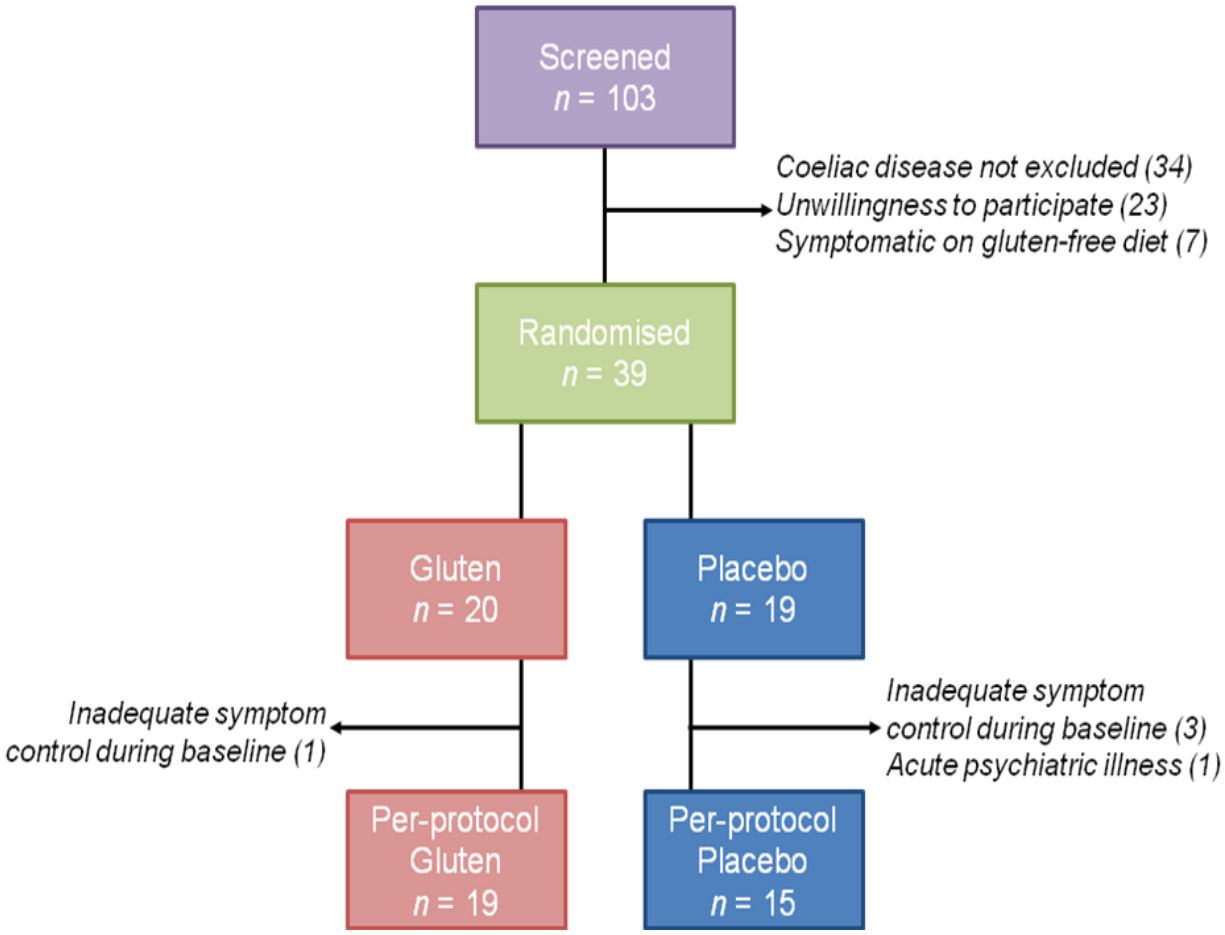

Figure 6.3 Recruitment pathway and reasons for screen failure and withdrawals.

The details of those patients are shown in Table 6.1. 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.

6.3.2 Dietary compliance

All patients adhered to the GFD 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).

Table 6.1 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)	40 (29–55) years	49 (33–51) years
Men	16%	7%
Median BMI (range)	23 (18–41) kg/m^2	22 (18–33) kg/m^2
Predominant bowel habit:		
Constipation	16%	20%
Diarrhoea	58%	33%
Alternating	26%	47%
HLA type:		
DQ2 or DQ8 positive	53%	60%
Elevated serum coeliac antibodies (percentage of patients (mean (SEM) units/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

6.3.3 Effect on gastrointestinal symptoms

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 end-point 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 6.4, 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. The differences were compared at week one by an independent samples t-test, in which overall symptoms (p=0.047), abdominal pain (p=0.016), bloating (p=0.031), satisfaction with stool consistency (p=0.024) and tiredness (p=0.001) were statistically significant but wind (p=0.053) and nausea (p=0.120) were not. The differences were also compared over the entire study period using a linear mixed effects model, in which abdominal pain (p=0.016), satisfaction with stool consistency (p=0.032), and tiredness (p=0.001) were statistically significant but overall symptoms (p=0.147), wind (p=0.083) and nausea (p=0.690) were not.

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 6.4). 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. 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).

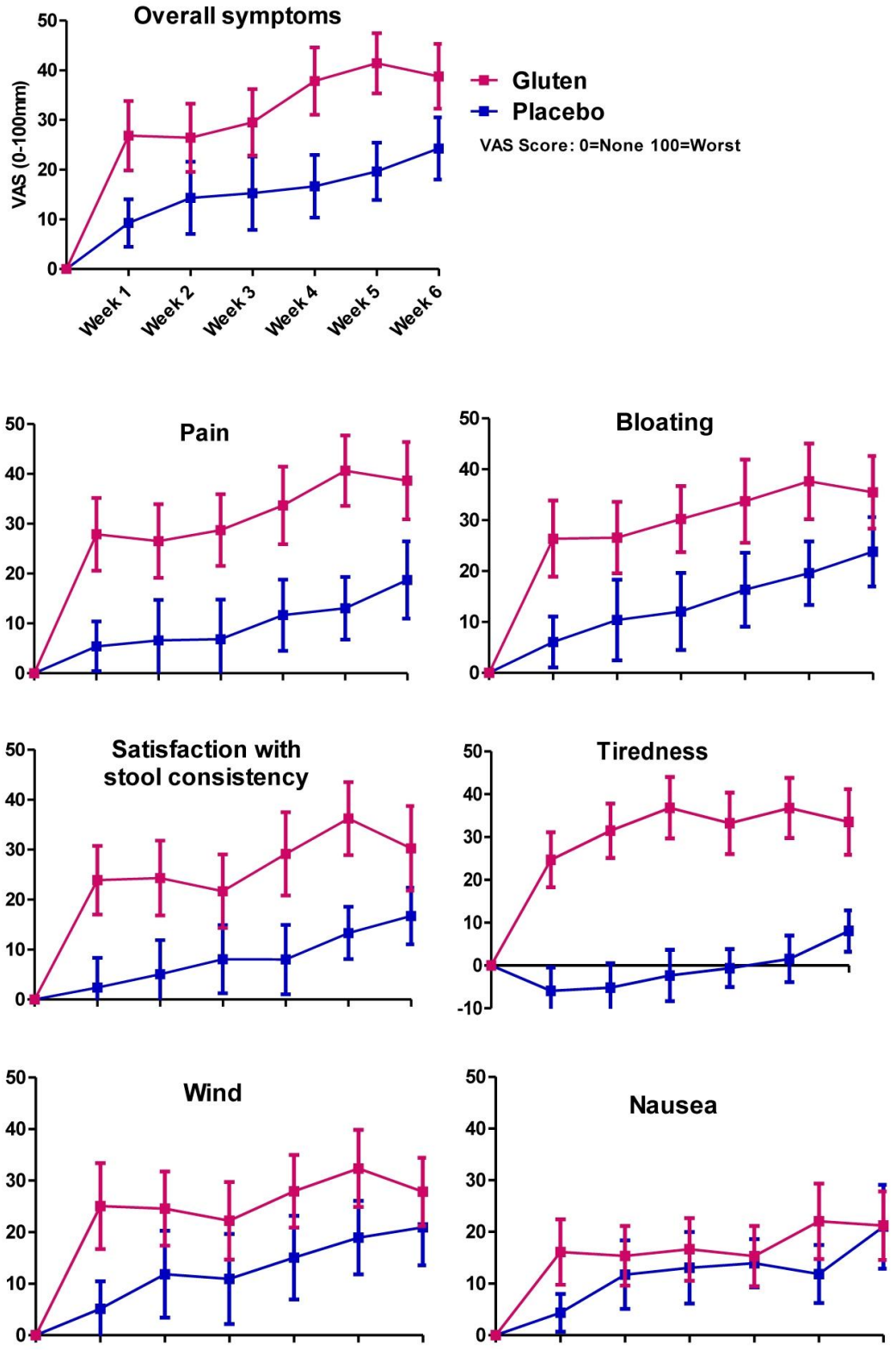

Figure 6.4. Change in symptom severity from baseline in the gluten and placebo-treated groups over the 6-weeks of the study. Data shown represent the mean change for the subjects remaining on study therapy at each time point.

6.3.4 Effect on biomarkers

As shown in Table 6.2, 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.

No differences in the response of biomarkers to gluten exposure were noted according to HLA-D status.

Table 6.2 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 $p \geq 0.1$).

Biomarker	Gluten (n=19)			Placebo (n=15)		
	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)*

6.4 Discussion

Gluten sensitivity in people without coeliac disease is a controversial issue and has been described as the "no man's land of gluten sensitivity".[179] 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[341] 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 GFD 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[341] 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[342] 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,[343,344] 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.[345] 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)[9] 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.[9] Alternatively, gluten may mediate cholinergic activation as has been shown in murine models of gluten sensitivity.[346] 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 .

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,[184] induce apoptosis,[186,347] and increase oxidative stress[187] in Caco-2 (human colon adenocarcinoma) monolayers, a surrogate model for the human gut epithelium. In addition, gliadin may inhibit RNA and DNA synthesis.[244] 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.[196] 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 NCGS 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 GFD, 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.

6.5 Conclusions

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 GFD 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 NCGS is, how it can be reliably identified and what its underlying mechanisms are, warrant further evaluation.

Chapter 7 – Study Two: Understanding non-coeliac gluten sensitivity

7.1 Background and Aims

Study One produced, for the first time, strong evidence that NCGS may exist. The results highlighted several key questions and the need to identify clinical biomarker(s) that would enable NCGS to be more fully characterised and understood.

(i) Are the findings reproducible?

The primary objective of Study One (a randomised controlled trial of a single dose of gluten without a controlled background in parallel groups) was to examine the hypothesis that gluten is capable of inducing gut symptoms in people without coeliac disease, thereby supporting (but not proving) the existence of NCGS. These initial findings must be reproduced to move towards proving the concept.

(ii) What are the mechanisms of action?

Study One was not designed to look for exhaustive mechanistic associations, but represented a preliminary screen for gross markers of intestinal damage. Due to the exploratory nature of Study One, there was limited sensitivity of the chosen laboratory markers. Associations with inflammation, specific immunological activation or changes in barrier function were assessed (albeit crudely) with the induction of symptoms. These results did not differentiate between the gluten and placebo groups, giving prominence to measuring more rigorous, sensitive and invasive tests in subsequent larger studies.

Study One showed for study participants randomised to gluten, that clear, consistent and statistically significant differences in GI symptoms appeared to have a rapid onset of action (within 1 week). A striking difference in fatigue levels was also shown, highlighting that potential mechanisms of a systemic process and functional gut symptoms also require attention. This may include stimulation of the enteric nervous system and an increased sensitivity of visceral nociception.[346] To understand this involvement of extra-intestinal manifestation and a potential systemic inflammatory basis, more detailed measurements of cognitive function, fatigue and lethargy are required.

Gluten is one of the more poorly digestible proteins, where the gliadin component contains proteolytic-resistant proline-and glutamine-rich peptides that persist to elicit the immune response in coeliac disease. The fate of gluten peptides in the small intestine of NCGS individuals is not known. Such peptides might also potentially be available to intestinal bacteria in the biofilm of the distal small intestine or within the colon and may trigger symptoms through downstream effects of bacterial metabolism.

(iii) What is the quantity of gluten responsible?

The only available treatment for coeliac disease is life-long strict avoidance of gluten-containing foods (including wheat, rye and barley). While the average daily gluten intake in a Western diet is 10-20 g/d,[28] just 50 mg is the minimum quantity of gluten needed to induce damage to the lining of the small intestine in people with coeliac disease.[238] There seems to be a threshold, rather than dose-dependent effect. It is possible that, in those with NCGS, there is clearer dose-dependence implying that they can tolerate low doses of gluten (e.g., 2 g/d) and do not require the strict GFD required by coeliac patients.

(iv) Is it really gluten – what about another dietary trigger of gastrointestinal symptoms?

Wheat and other grains contain significant quantities of poorly absorbed short-chain carbohydrates (FODMAPs) that are capable of inducing gut symptoms themselves.[196] FODMAPs are found in a wide variety of foods, discussed in Chapter Four, including lactose (in milk), excess fructose (in pears, apples), fructans and FOS (in artichoke, garlic, onions, wheat and rye), GOS (in legumes, some breads), and sugar polyols (in stone fruits, sourdough bread and artificial sweeteners). FODMAPs are osmotically active and rapidly fermented by intestinal bacteria, which may trigger GI symptoms in some individuals such as those with IBS.[205,206] Although the study foods (gluten-free bread and muffins) provided to subjects in Study One were devoid of FODMAPs, the participant's individual background dietary FODMAP intake, prior to and during the study, were not analysed in detail or controlled. Since it is possible that FODMAP intake may have changed or have been different between the gluten and placebo-treated groups, this area requires attention.

It was hypothesised that, in subjects who have NCGS:

- components in gluten induce GI symptoms, affect cognitive function, and induce systemic disturbances such as lethargy, without causing intestinal inflammation or immune effects;
- gluten increases levels of by-products of protein metabolism including faecal ammonia; and
- the effects of gluten are dose-dependent.

The overall aim of Study Two was to examine these hypotheses by undertaking a controlled feeding trial (randomised, crossover, double-blind design) of three diets differing in gluten content – placebo, high-gluten (16 g/d) and low-gluten (2 g/d) – for seven days each in patients with IBS in whom GFD had led to an improvement of symptoms and coeliac disease has been excluded. The specific aims were to assess the effect of wheat-gluten by comparing changes in overall and individual GI symptoms (bloating, wind, abdominal pain and diarrhoea), indices of mucosal inflammation and markers of immunological mechanisms**,** measures of cognitive function and measures of fatigue and lethargy, and faecal by-products of protein metabolism.

7.2 Methods

7.2.1 Subjects

Patients were recruited between January 2010 and January 2011 via advertisements in e-newsletters and community newspapers in metropolitan Melbourne, by referrals from private dietetic practice or gastroenterology clinics, and by inviting past volunteers to return. Any person who expressed interest was asked to complete a recruitment survey (see Appendix 11), assessing key criteria including symptoms, diet and coeliac disease investigations. This survey is discussed in detail in Chapter 5 (page 91). Only patients who reported good symptom control were deemed eligible. Forty subjects meeting the inclusion/exclusion criteria (see Chapter 3, Section 3.3) gave written, informed consent. Prior to commencing the study, subjects were asked to keep a 7-day food and symptom diary and complete a food frequency questionnaire. This was to allow their usual gluten and FODMAP intake, and symptom level to be assessed. Only patients following a GFD commenced participation.

7.2.2 Study protocol

A randomised, placebo-controlled, double-blinded crossover study design of 9 weeks' duration (including a one-week baseline period where the subjects recorded their usual diet and symptoms, followed by a two-week run-in period at the start) was conducted (see Figure 7.1). All participants, regardless of previous knowledge, underwent consultation at the completion of their one-week baseline, and prior to beginning the run-in period, for education on the low FODMAP diet. Patients were randomised according to a computer-generated order, held by an independent observer. Patients received one of three diet treatments (high-gluten, low-gluten or placebo) for one week, followed by a washout period of at least 2-weeks (or until symptoms induced during the previous dietary challenge resolved), before crossing over to the next diet. Both the patient and the investigators evaluating the patient were blinded to the study treatment. Participants were asked to follow a diet that was low in FODMAPs and gluten-free throughout the study.

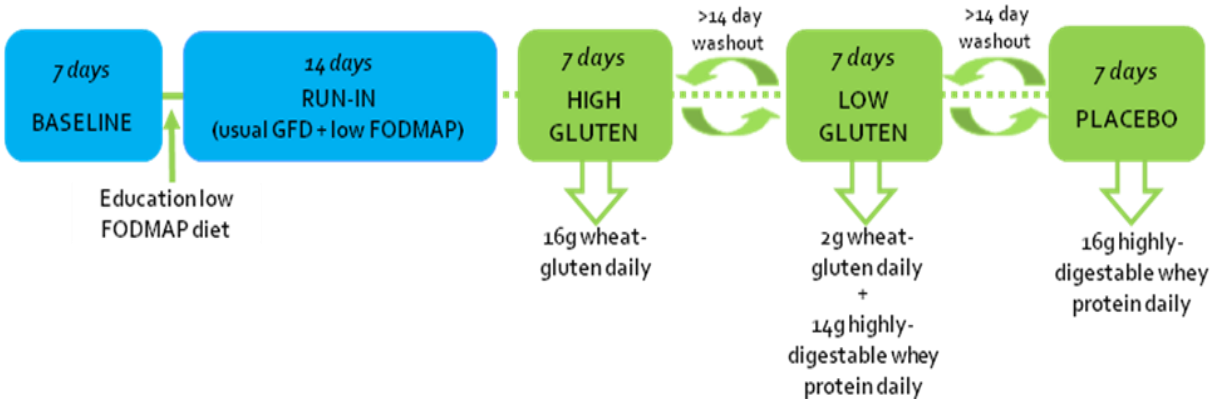

Figure 7.1 Study Two protocol outline

Patients unable to continue the study due to intolerable symptoms were permitted to cease the study food of that particular treatment arm but continue to collect data as per day six (symptom assessment, physical activity and cognitive studies, blood and stool samples collected) and collect symptom and food diaries when not on the study diet. Patients then went onto resume any remaining treatment arms following the allocated washout period. The protocol was approved by Eastern Health Research and Ethics Committee and also registered with Australia and New Zealand Clinical Trials Register (ANZCTR): ACTRN12610000524099.

7.2.3 End-points

The primary end-point was the comparison of the change from run-in in mean of the overall symptom score measured on the VAS after one week of study diet.

The secondary end-points were:

- The proportion of participants demonstrating an increase in the overall symptom score measured on the visual analogue scale of at least 20 mm after one week of study diet.
- The proportion of participants demonstrating an increase in individual symptom scores measured on the visual analogue scale of at least 20 mm after one week of study diet.
- The change in symptom scores (individual and overall) at one week compared with run-in symptom levels.
- Changes during dietary treatment in biomarkers and by-products of protein metabolism.
- Magnitude of gluten-specific T-cell responses following gluten challenge.
- Change and comparison over the treatment arms in scores on D-FIS, cognitive function tests and activity levels.

7.2.4 Study food preparation

The background diet was gluten-free and low in FODMAPs using current knowledge of FODMAP food composition (see Chapter 3, Section 3.5 for details on diet design). Total protein levels were balanced with a highly digestible, lactose-free whey protein isolate (see Section 3.5.1 for details on whey and gluten composition). During the three treatment periods, the background diet had the following incorporated:

- High-gluten (16 g whole wheat gluten per day); or
- Low-gluten (2 g whole wheat gluten per day and 14 g per day whey protein isolate); or
- Placebo (16 g per day whey protein isolate)

All main meals were supplied to the subjects. Fresh fruit, salad and vegetables were supplied by the participants themselves, following suggestions provided. A sample meal plan is given in

Appendix 4. The meal plan was adequate in macronutrients, micronutrients and provided 8 MJ energy daily (Appendix 6 for the average daily nutritional breakdown). Volunteers with smaller energy requirements were advised to consume fewer snacks and given smaller portions of the meals, but the same proportion of gluten was added. Volunteers with larger energy requirements were provided with additional low FODMAP, gluten-free meals and snacks. Lactose-free milk and yoghurts were used throughout.

Meals were similar between 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. This ensured that volunteers were blinded to the test diets. All food was prepared by the investigator and Research Chef, assisted by two hospitality students, in the kitchens of Monash University and of the Research Chef. Meals were packed individually in food-grade foil containers or bags sealed with a cryovac Orved VM-12 vacuum sealer (Orved®, Musile di Piave, Italy) to extend shelf life and quality of the food. All meals were kept frozen and reheating instructions were provided to the volunteers. All food was provided free of charge. 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 week.

7.2.5 Measurements

Medical history and examination, and HLA genotyping (if not already done) were completed at baseline. During each of the treatment periods, several indices were measured requiring faecal and blood collection. In addition, questionnaires assessing GI symptoms and fatigue (D-FIS), and the Subtle Cognitive Impairment Test (SCIT) were completed, and an accelerometer was worn for 7 days (see below). Table 7.1 shows a timeline for when data or samples were collected.

Table 7.1 Overview of assessments made, during each diet treatment period (shaded boxes indicate when assessment was performed)

DAY	*0*	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Diet treatment		■	■	■	■	■	■	■
Symptom (GI, D-FIS) questionnaires*	■	■	■	■	■	■	■	■
Accelerometer*		■	■	■	■	■	■	■
Faecal collection						■	■	■
Blood sample*	■						■	
SCIT*							■	

* Assessment also made during baseline period. GI, gastrointestinal; D-FIS, Daily-Fatigue Impact Scale; SCIT, Subtle Cognitive Impairment Test.

7.2.5.1 Dietary adherence

A verified flow chart established by Biagi and colleagues was used, which gives a numerical score to adherence to the GFD.[282] Such adherence was cross-checked with dietary assessment of participants' baseline 7-day food diary.

7.2.5.2 Gastrointestinal symptoms

Gastrointestinal symptoms were assessed by the participant completing daily diary cards, which used a 100 mm VAS (see Chapter 3, Section 6.1 and Appendix 8) to score the presence and severity of overall abdominal symptoms, abdominal pain, bloating, wind, satisfaction with stool consistency, tiredness and nausea. Gastrointestinal symptom cards were completed throughout the study (i.e., baseline, run-in, three diet treatments weeks, washout weeks). Clinical significant change of symptoms was arbitrarily defined as a change of at least 20 mm.

7.2.5.3 Fatigue and other symptoms

Severity of fatigue was evaluated by the Daily-Fatigue Impact Scale (D-FIS),[284] a questionnaire containing eight items investigating fatigue impact on cognition, physical functioning and daily activities. Scores were measured as described in Chapter 3, Section 6.2. Fatigue symptom cards were completed throughout the study (i.e., baseline, run-in, three diet treatments weeks, washout weeks).

7.2.5.4 Physical activity and sleep

Accelerometry was used to objectively assess physical activity and sleep patterns (ActiGraph GT3X accelerometer, LLC, Fort Walton Beach, Florida, USA),[285,286] described in Chapter 3, Section 6.3. The participants were asked to wear the accelerometer for seven consecutive days, at all times during the baseline week and during each treatment arm.

7.2.5.5 Cognitive function

The SCIT was used to measure cognitive function. This test is described in Chapter 3, Section 6.4. Participants received training and practice on the SCIT prior to commencement of the experiment. Testing was completed once during baseline and then once during each diet treatment week (at day 6). Testing times were all before midday to minimise circadian effects.

7.2.5.6 Gliadin-specific T-cell responses

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.[111,112] Commercially-available kits were used (Mabtech, Nacka Strand, Sweden) in which isolated peripheral blood mononuclear cells (PBMC) were studied in 96-well plates (Multiscreen® Filter Plates; Millipore, Bedford, MA, USA) with. The methods are described in detail in Chapter 3, Section 6.5. Blood was taken from patients on day 0 and day 6 of each treatment week.

7.2.5.7 Other biomarkers

Inflammatory and immune markers were evaluated as described in detail in Chapter 3, Section 6.7. All assays were performed in duplicate; if there were readings the assay was repeated in triplicate. The manufacturer's reference ranges were used to determine the classification of the result, unless otherwise stated. The biomarkers examined were as follows:

- Coeliac serological markers - Serum was analysed for antibodies to whole gliadin (IgA and IgG) and deamidated gliadin (IgA and IgG) by enzyme-linked immunosorbent assay[244] using commercially available assays (INOVA Diagnostics, San Diego, USA). All tests were performed in conjunction with a total-IgA level to identify subjects with selective IgA deficiency. Serology was performed using blood taken from patients during Baseline and on day 6 of each treatment week.

- Human ß-defensin-2 - Stool samples collected by patients during day 5-7 of each treatment week were analysed for human ß-defensin-2 (HßD-2), an anti-microbial peptide affecting the

barrier function of epithelial cells. This was performed using a commercially-available ELISA (Immundiagnostik AG, Bensheim, Germany) where extracts were prepared using the supplied buffer and the assay completed as per manufacturer's instructions. Results were expressed as ng/mL.

- Calprotectin - Stool samples collected by patients during day 5-7 of each treatment week were analysed for human calprotectin, a calcium-binding protein indicative of neutrophils cytosol and therefore bowel inflammation. This was performed by ELISA using commercially available assays (BÜHLMANN Laboratories AG, Schönenbuch, Switzerland). Extracts were prepared using the faecal sample preparation kit (BÜHLMANN Laboratories AG, Schönenbuch, Switzerland). Results were expressed as μg/g.

- Eosinophil Cationic Protein - Serum from peripheral blood taken from patients on day 6 of each treatment week was analysed for human Eosinophil Cationic Protein (ECP), a basic protein located within the eosinophil primary matrix that has degranulation activity and is associated with inflammation. This was assessed by ELISA using commercially available assays (Cuasbio Biotech Co., Ltd, Newark, USA) in accordance with the manufacturer's instructions. Briefly, samples were prepared with a biotin-conjugated antibody preparation specific for ECP and the assay carried out in accordance with the manufacturer's instructions. Results were expressed as ng/mL.

7.2.5.8 Faecal examination

To assess the colonic fermentation of protein (such as undigested gluten) in the colon, three-day total faecal output from day 5-7 was collected during each diet treatment week. Volunteers were provided with labelled containers and a -20 °C portable freezer (WAECO Model CD F35 Pack Cool Freeze Fridge, Varsity Lakes, Australia). Volunteers were asked to collect all output during the three-day period, avoiding urine contamination. The date and time of collection was noted on each container, which was then placed immediately into the portable freezer. Subjects recorded the

details of any samples missed whilst away from home (i.e., at workplace). The samples were assessed as follows:

- Samples were weighed and described according to the validated Kings Stool Chart (King's College London. Diarrhoea was classified by a daily faecal score of 15 or more.

- The pH of faecal samples (approximately 20 g faeces) was measured using a pH electrode probe (Mettler Toledo InLab® pH Combination Electrode, Schwerzenbach, Switzerland) and portable electrode meter (Mettler Toledo AG FiveGo™ Duo reader, Schwerzenbach, Switzerland). The samples were warmed to RT and the pH electrode calibrated at this temperature before taking measurements.

- The faecal concentration of ammonia, a by-product of protein metabolism, was measured using commercially available assays (Megazyme Ammonia *Rapid* Kit; Megazyme International Ireland Ltd, Wicklow, Ireland) for total ammonia.

- To assess if undigested fragments of gliadin-derived peptides reached the faeces, faecal samples underwent analysis using the established tool of mass spectrometric peptide mapping.[299] This was performed by pepsin and/or trypsin digested peptides being separated using reverse-phase high-performance liquid chromatography (RP-HPLC; Agilent 1200 Infinity Series LC, Agilent Technologies Inc., Santa Clara, California, USA) with a gradient from 0-60% acetonitrile. The peptides released were directly analysed by being coupled to a triple quadruple mass spectrometer (Agilent 6430 Series Triple Quadrupole LC/MS Systems, Agilent Technologies Inc., Santa Clara, California, USA) through a ChipCube interface (Agilent 1260 Infinity HPLC-Chip Cube Interface G4240A, Agilent Technologies Inc., Santa Clara, California, USA).

7.2.6 Statistical analyses

Per-protocol analyses were performed. Comparisons of symptom severity scores and measured indices 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 between baseline and run-in periods and Wilcoxon signed rank test to compare the non-parametric data. Spearman's correlations were used for associations between symptom severity and biomarkers. Two-tailed P-values at or below 0.05 were considered statistically significant.

7.3 Results

7.3.1 Study population

Just over half of respondents to the advertisements completed their recruitment surveys to assist in screening. The results of the survey are discussed in detail in Chapter Eight. Of those 149 subjects, 40 met the inclusion criteria and were enrolled. Nine patients returned from Study One. Subject flow is shown in Figure 7.2. Following randomisation, three patients had to be withdrawn due to poor symptom control. Thus, 37 patients completed the study as per protocol. The details of those patients recruited are shown in Table 7.2.

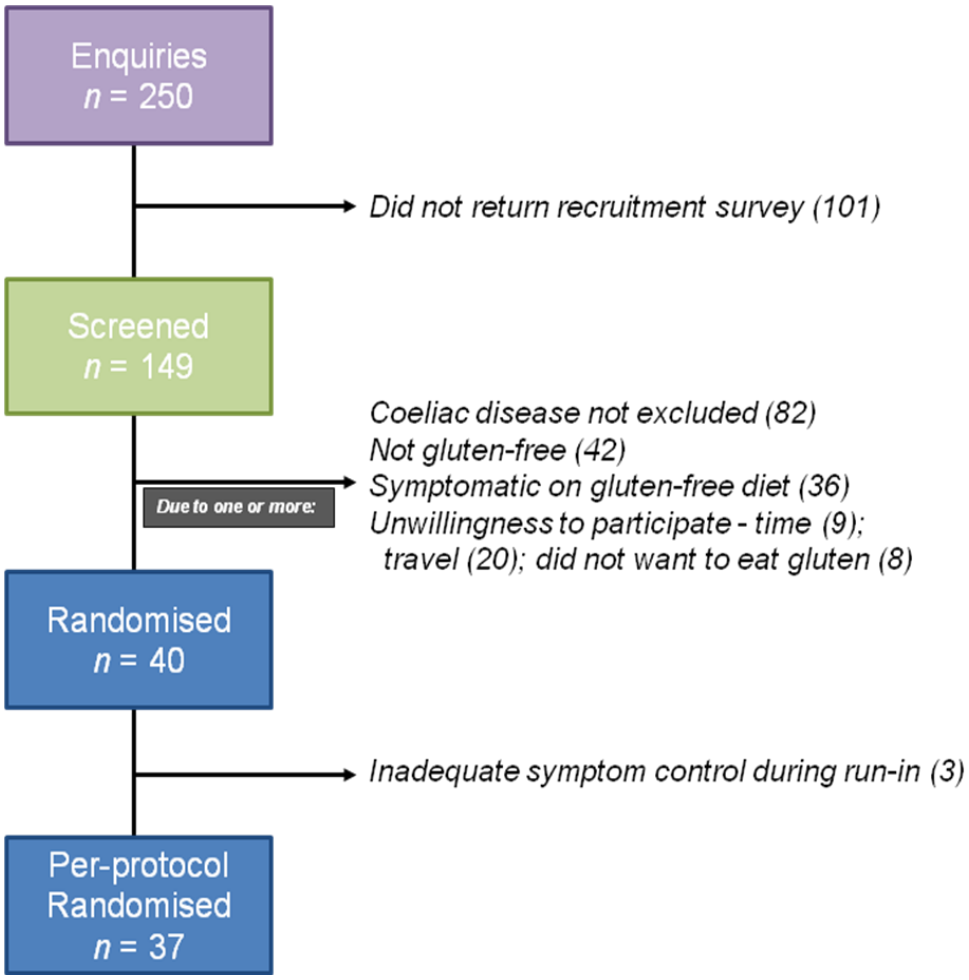

Figure 7.2 Recruitment pathway and reasons for screen failure

Table 7.2 Study subject characteristics

Characteristics	
Number of patients	37
Gender	6 male
Median age (range)	45 (24 – 61) years
Median BMI (range)	23 (17 – 38.6) kg/m^2
Current smokers	2
Moderate/heavy alcoholic drinkers	0
Education level	
Completed secondary school	8%
Post-school qualification	14%
University degree	78%
Predominant bowel habit	
Diarrhoea	43%
Constipation	35%
Mixed/Alternating	22%
HLA type	
DQ2 or DQ8 positive	57%
Elevated serum coeliac antibodies (mean ± SEM units/ml)	
Whole-gliadin [348]	37% (27 ± 3)
Whole gliadin (IgG)	5% (8 ± 1)
Deamidated gliadin [348]	13% (17 ± 1)
Deamidated gliadin (IgG)	5% (9 ± 2)

BMI, body mass index; HLA, human leukocyte antigen; Moderate/heavy drinker defined as five or more drinks per occasion.[349]

All participants were found to be adherent with the GFD for a median of 48 months prior to participation (see Table 7.3). During the baseline interview, 65% of participants described some other form of dietary intolerance, allergy or problem food (detailed in Table 7.3). 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, zinc, St John's Wort and horsetail.

Table 7.3 Dietary adherence to the gluten-free diet (GFD) and other food intolerances described by participants

Baseline dietary information		
Median 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	FODMAPs or FODMAP-containing food	43%
	Dairy (including lactose, casein, whey)	17%
	Food chemicals (e.g., amines, sulphites, benzoates)	8%
	Tomatoes	5%
	Other[a]	22%

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

7.3.2 Dietary adherence

All patients adhered to the GFD during the study. Each volunteer undertook all three diet treatment arms. Alcohol intake did not differ during the treatment periods (see Table 7.4). 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 patient 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 dietary consumption of each diet is detailed in Table 7.4. Five participants continued to consume their usual milk products (lactose containing) as they had previous negative lactose breath tests. Although all participants were educated on the low FODMAP diet, 22 participants reported having some knowledge of the diet prior to recruitment. There was a significant decrease in dietary fibre and FODMAP intake during the run-in, and also an average decrease in energy content from 7.9 MJ per day during baseline to 7.3 MJ per day during the run-in.

Table 7.4 Actual daily dietary intake during each phase of the study. 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 in the Chapter 3, Section 3.2. 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.

Dietary component	Baseline	Run-in	*P*-value	High-gluten	Low-gluten	Placebo	*P*-value
Energy, MJ	7.9 ± 0.3	7.3 ± 0.3	0.003	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 fibre, g	23 ± 2.3	19 ± 1.9	<0.0001	26 ± 0.6	26 ± 0.6	26 ± 0.6	NS
Carbohydrates	210 ± 8.7	183 ± 9.2	0.001	215 ± 5.3	221 ± 5.5	220 ± 5.3	NS
Monosaccharides							
Glucose, g	18 ± 1.6	15 ± 1.1	0.017	21 ± 1.0	23 ± 1.5	21 ± 1.2	NS
Fructose, g	15 ± 1.5	9.5 ± 0.7	0.001	12 ± 0.7	13 ± 0.9	12 ± 0.7	NS
Disaccharides							
Sucrose, g	28 ± 3.3	21 ± 2.7	0.001	24 ± 1.2	26 ± 1.6	25 ± 1.4	NS
Lactose, g	14 ± 2.0	9.8 ± 1.1	0.030	2.4 ± 0.7	3.3 ± 1.1	3.5 ± 1.2	NS
Sugar polyols							
Sorbitol, g	1.1 ± 0.2	0.4 ± 0.07	<0.0001	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.02	NS
Mannitol, g	0.4 ± 0.05	0.2 ± 0.03	0.011	0.4 ± 0.02	0.4 ± 0.02	0.4 ± 0.02	NS
Oligosaccharides							
Fructans, g	1.5 ± 0.1	1.2 ± 0.1	0.011	0.9 ± 0.03	0.9 ± 0.03	0.8 ± 0.03	NS
GOS, g	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	0.003	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

* NS, not significant

7.3.3 Effect on gastrointestinal symptoms

Compared with average of symptoms in the baseline period, average of symptoms from the second week of the low FODMAP run-in period were mostly significantly improved. As shown in Figure 7.3, this included overall symptoms, abdominal pain, bloating, satisfaction with stool consistency, wind and tiredness (all $p<0.0001$; Wilcoxon signed rank test; see Figure 7.3), 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 20 mm during the low FODMAP run-in period, from their baseline (Figure 7.4).

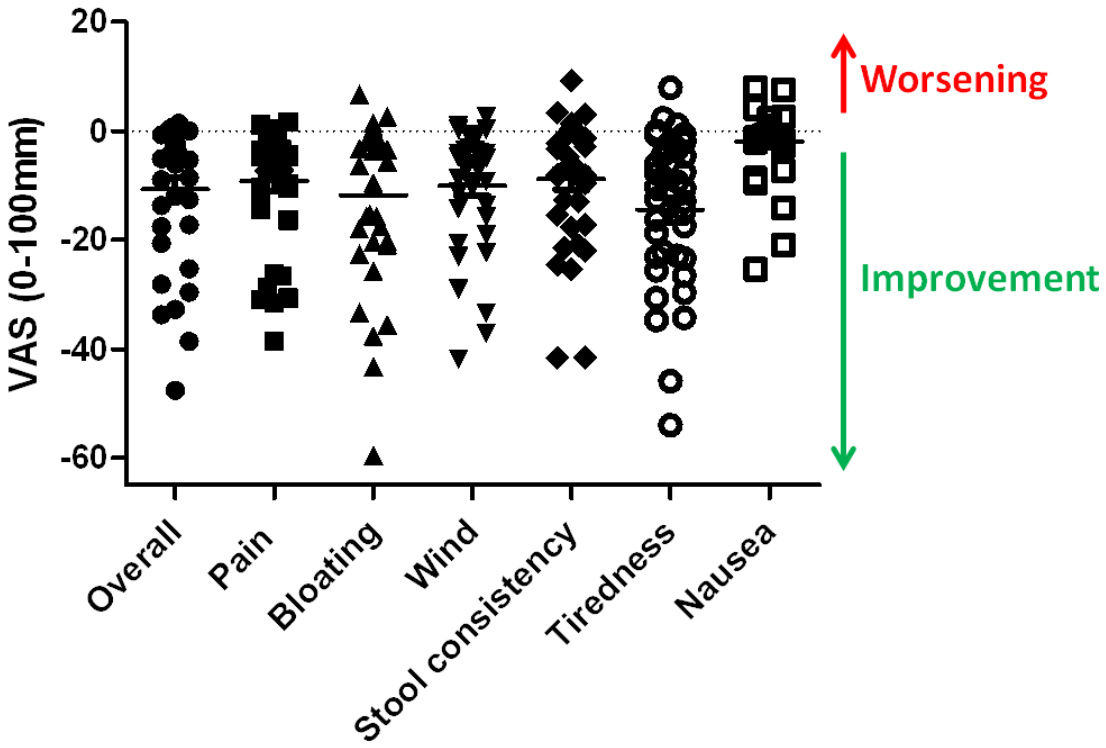

Figure 7.3 Change in mean symptom severity score from baseline (participant's usual gluten-free diet) to the run-in period, where low FODMAP diet was commenced. Data shown represent the mean. VAS, visual analogue scale.

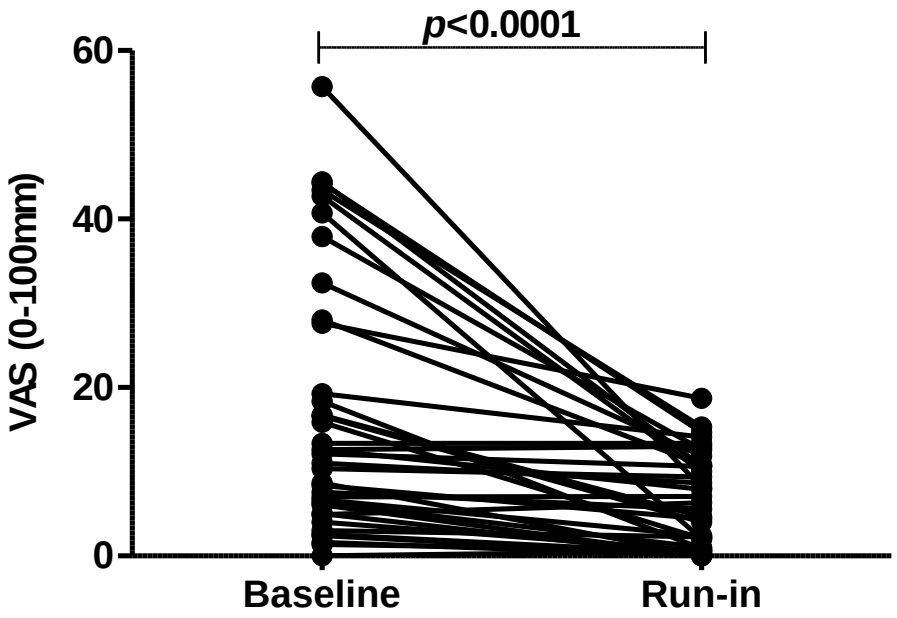

Figure 7.4 Individual responses in mean overall symptom severity score during the run-in period, where low FODMAP diet was commenced, compared with those in the baseline period, where participant's usual gluten-free diet was consumed. Scores were significantly greater during the baseline period ($p<0.0001$, Wilcoxon signed rank test). VAS, visual analogue scale.

Symptoms during the dietary interventional periods were expressed as change from the average scores during the last week of run-in. Overall symptoms and pain significantly worsened during each dietary treatment period, irrespective of the diet, as detailed in Table 7.5. Bloating and tiredness significantly worsened during low-gluten and placebo treatment arms only. As shown in Figure 7.5, there were differences in the symptoms induced across the three diets for overall, bloating, satisfaction with stool consistency and wind, but not for pain, tiredness and nausea.

Table 7.5 Symptom score changes (mean ± SEM, Wilcoxon signed rank test) from run-in period during each dietary treatment period.

		High-gluten		Low-gluten		Placebo	
	Run-in	**Day 7 change**	***P*-value**	**Day 7 change**	***P*-value**	**Day 7 change**	***P*-value**
Overall symptoms	6.4 ± 0.9	14.9 ± 3.7	0.027	18.7 ± 3.0	0.001	20.8 ± 3.68	0.001
Pain	5.6 ± 0.8	13.2 ± 3.5	0.041	16.4 ± 3.2	0.002	18.3 ± 3.69	0.004
Bloating	7.2 ± 1.9	13.6 ± 3.0	NS	15.2 ± 3.2	0.017	17.6 ± 4.14	0.005
Satisfaction with stool consistency	8.6 ± 1.5	9.4 ± 4.0	NS	15.6 ± 3.5	NS	14.2 ± 3.65	NS
Wind	6.7 ± 1.1	9.3 ± 2.3	NS	13.0 ± 3.2	NS	13.9 ± 3.33	NS
Tiredness	8.0 ± 1.3	16.3 ± 4.6	NS	16.3 ± 3.2	0.014	16.2 ± 3.73	0.049
Nausea	2.8 ± 0.7	3.9 ± 2.0	NS	4.5 ± 1.8	NS	6.5 ± 2.05	NS

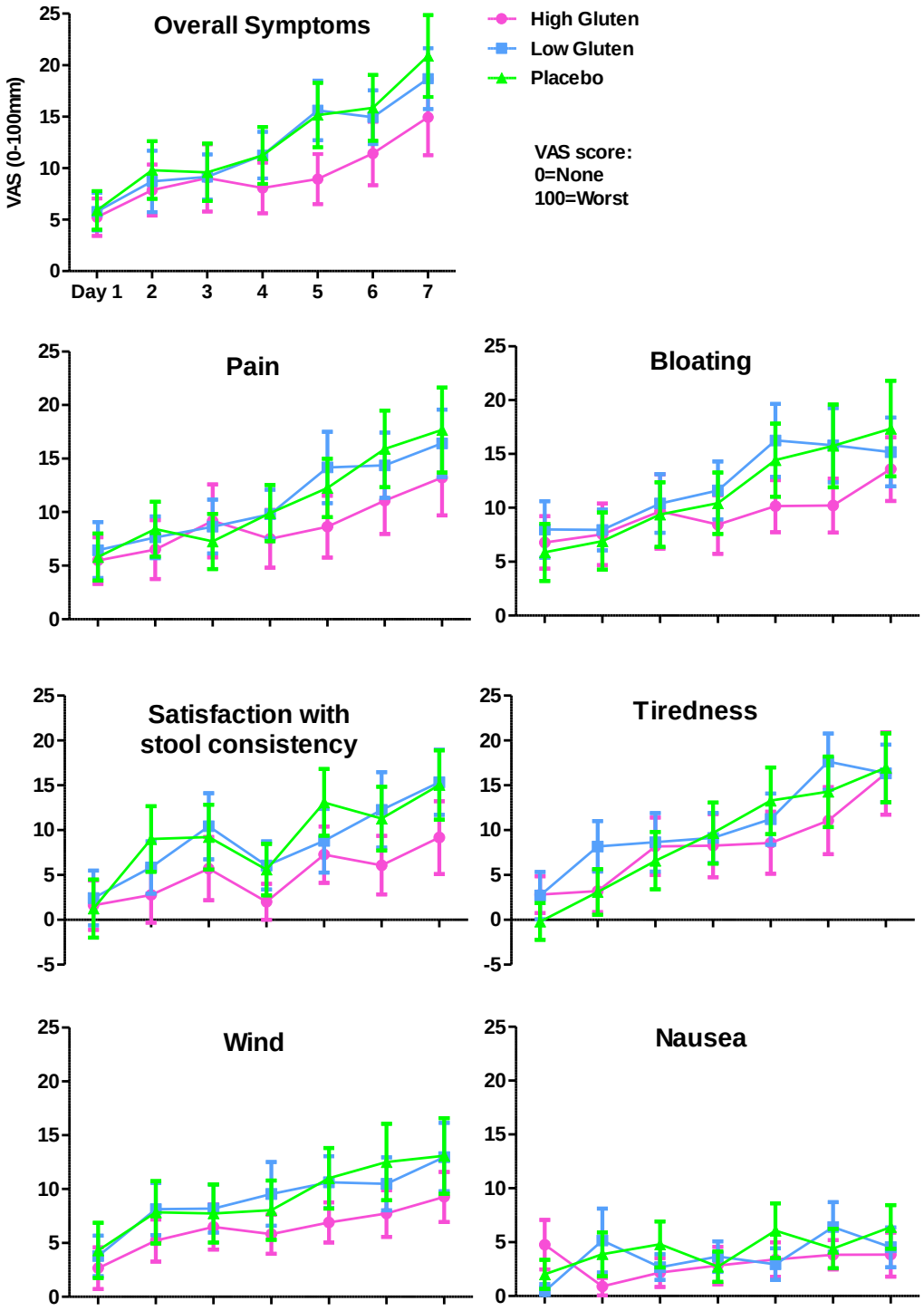

Figure 7.5 Change in symptom severity from run-in for each dietary treatment over 7-day study period. Data shown represent mean ± SEM. The differences across the treatment arms were compared by Friedman test, in which overall symptoms (p=0.001), bloating (p=0.016), satisfaction with stool consistency (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.

Possible explanations for the lack of gluten-specific induction of symptoms included that only some of the subjects actually had NCGS and that some had specific reactions to whey protein. In order to address these issues, those with a positive symptomatic response to gluten and whey were identified. Their results are shown in Figure 7.6.

- *Gluten-specificity:* Only six participants (16% of total cohort) had an average increase on the VAS for overall abdominal symptoms of more than 20 mm on the high-gluten arm compared those during the run-in period. Only one of these also had a positive response to the low-gluten arm. Three also had a positive response to the no-gluten arm, which contained whey 16 g/d. One responded in all three arms (Figure 7.6A). Thus, gluten-specificity of symptomatic responses was observed in only three subjects (8% of the total cohort).

- *Whey-specificity:* Eleven participants (30%) had a positive response in overall symptom severity in the no-gluten, whey (16 g/d) arm, eight of whom also reacted to the low-gluten, whey (14 g/d). Only one of these eight responded to the high-gluten arm (Figure 7.6B). Thus, seven subjects (19% of the total cohort) had whey-specific symptomatic responses

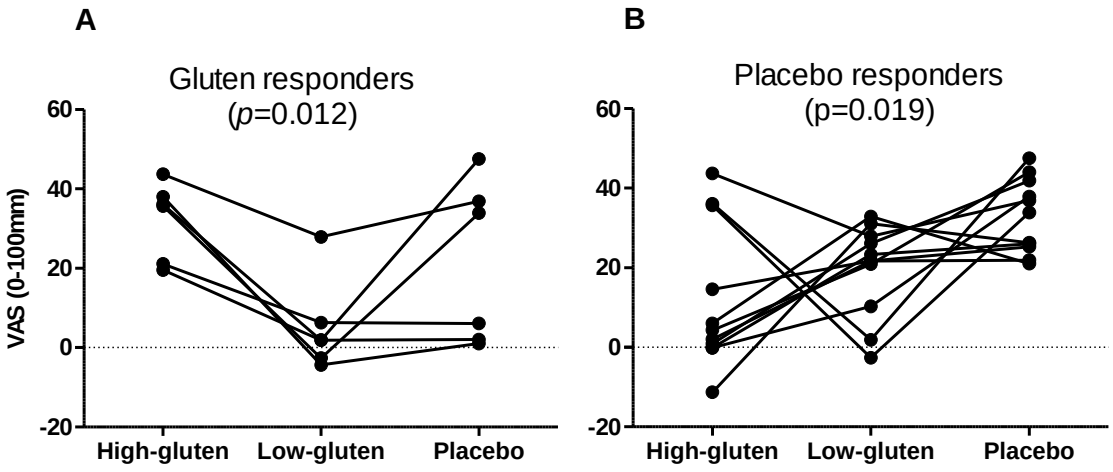

Figure 7.6 Average change in overall symptom severity for high-gluten, low-gluten and placebo treatment arms. Participants were defined as being a responder if they had an average increase of at least 20 mm for high-gluten (A; n = 6) or placebo (B; n = 11) arms from run-in. The differences were compared by Friedman test. VAS, visual analogue scale.

Several patient-related factors were examined in terms of their association with symptomatic responses to the diets. 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).

The influence of the order of the dietary intervention was also examined. As shown in Figure 7.7, the first intervention significantly induced greater symptomatic changes than the second or third challenges, regardless of what it contained (i.e., high-gluten, low-gluten or placebo).

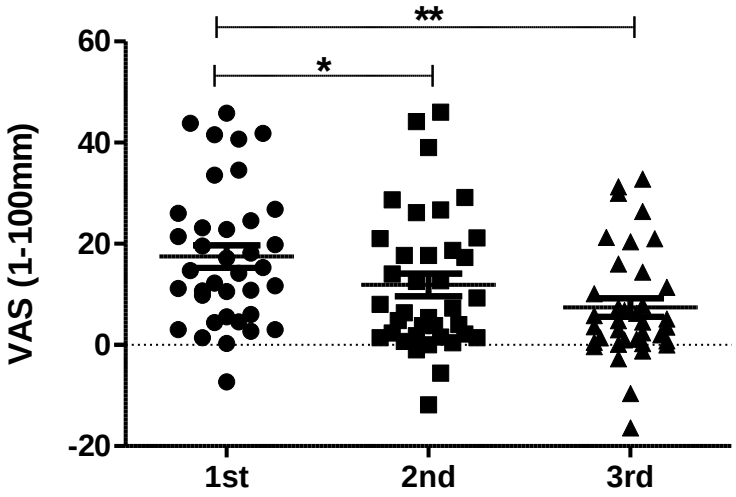

Figure 7.7 Average change in overall symptom severity grouped in order of treatment arm received. The differences were compared by repeated measures ANOVA (*p*=0.001). Differences were also compared between each group by a paired t-test (p*=0.026, ***p*=0.001). VAS, visual analogue scale.**

7.3.4 Effect on fatigue and other symptoms

As shown in Figure 7.8, the low FODMAP run-in period was associated with the lowest D-FIS score, which was significantly less than that in the baseline period. There were no differences in levels of fatigue across the dietary treatment arms (Figure 7.8), but there was a significant increase compared with those in the run-in period for high-gluten (*p*=0.005), low-gluten (*p*=0.0004) and placebo (*p*=0.003, paired t-test). There were no differences in D-FIS scores when only gluten-specific or whey-specific responders were compared with those with apparent non-specific symptomatic responses.

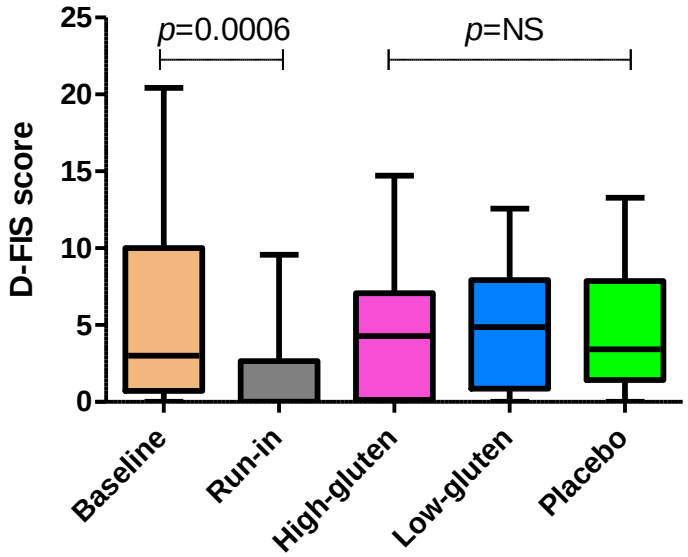

Figure 7.8 Average Daily-Fatigue Impact Scale (D-FIS) score for each study period. Data shown represent the mean and whiskers represent min to max. The differences between baseline and run-in were compared by paired t-test and the differences between the dietary treatment arms were compared by repeated measures ANOVA, in which there were no significant differences. Scores > 10 are consistent with reports of onset of an acute flu-like illness. NS, not significant.

Additional symptoms experienced by the participants not measured on the VAS or D-FIS were recorded by participants and are shown in Table 7.6. There was no specificity of any symptoms to the dietary arm, nor to gluten or whey-specific responders.

Table 7.6 The number (%) of participants reporting non-IBS symptoms. Like-sounding symptoms were grouped; for example, "mood change" refers to descriptions of irritable, grumpy, emotional or jittery.

	High-gluten	Low-gluten	Placebo
Headache/migraine	2 (5%)	3 (7.5%)	0
Musculoskeletal pain	3 (7.5%)	2 (5%)	2 (5)
Heartburn	1 (2.5%)	1 (2.5)	2 (5)
Mood change	2 (5%)	2 (5)	2 (5)
Itchiness/rash	0	2 (5)	0
Forgetfulness (i.e. "foggy brain")	1 (2.5%)	3 (7.5)	1 (2.5)
Other[a]	2 (5%)	4 (10)	5 (12.5)

a acne, reflux, sore throat, fluid retention, cough, dizziness, and sweats

7.3.5 Effect on levels of physical activity and sleep

Table 7.7 shows the results of physical activity and sleep patterns, as measured by the accelerometer, during the dietary periods. Accelerometers were not worn during the low FODMAP run-in period. Data from one participant were excluded due to experiencing a broken wrist during the dietary periods, affecting her normal activity levels (e.g., playing basketball and tennis). Participants spent the majority of time in sedentary (75%) or in light intensity activity (16%), with only 4% spent in moderate to vigorous intensity activity. There was no apparent effect of diet treatment on activity levels. Only one participant spent any time in activities of very vigorous intensity. There were no differences in number or duration of bouts.

There were no differences in any sleep measure including latency (time taken to fall asleep), number or length of awakenings, and total sleep time (see Table 7.7).

Table 7.7 Physical activity and sleep characteristics of study participants (mean ± SEM). There were no significant differences for diet difference on any measure, compared by repeated measures ANOVA.

Characteristic	Baseline	High-gluten	Low-gluten	Placebo
Activity[333]	1732 ± 146	1766 ± 134	1678 ± 121	1640 ± 121
Percentage 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				
Number of bouts	4.4 ± 0.8	4.5 ± 0.7	4.5 ± 0.8	3.8 ± 0.7
Mean time in bout (min)	13 ± 1.2	13 ± 1.3	12 ± 1.3	12 ± 1.3
Sleep characteristics				
Latency (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
Number 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

7.3.6 Effect on cognitive function

Of the 160 SCIT tests originally conducted, there were 33 cases where the SCIT download was faulty or technical difficulties were encountered with the software, resulting in the loss of some participant data.

When subjects were tested on the SCIT at day 6 of each dietary treatment, their pooled data were compared with those from the equivalent baseline condition (Figure 7.9). There were no statistical significant differences in response times, although response time was consistently the longest in the placebo treatment arm than those in the baseline, high- or low-gluten arms (Figure 7.9A; SCIT-RT). There were no differences in error rate (SCIT-E; Figure 7.9B), nor across the treatments for the gluten responders or placebo responders when analysed separately.

Combined data from the four exposure durations at the head of the data curve (11–77 ms; SCIT-E_H, SCIT-RT_H) and for the remaining five exposure durations at the tail of the data curve (99–187 ms; SCIT-E_T, SCIT-RT_T) are shown in Table 7.8. Differences were found for SCIT-E_H, where subjects on baseline (mean 18%) had higher error rates compared with high-gluten, low-gluten and placebo (each mean 12%; each $p<0.02$, paired t-test). Subjects on baseline also had slower head response times compared with high-gluten (536 vs 521 ms; $p=0.019$), but not with low-gluten (526 ms; $p=0.077$) or placebo (569 ms; $p=0.683$).

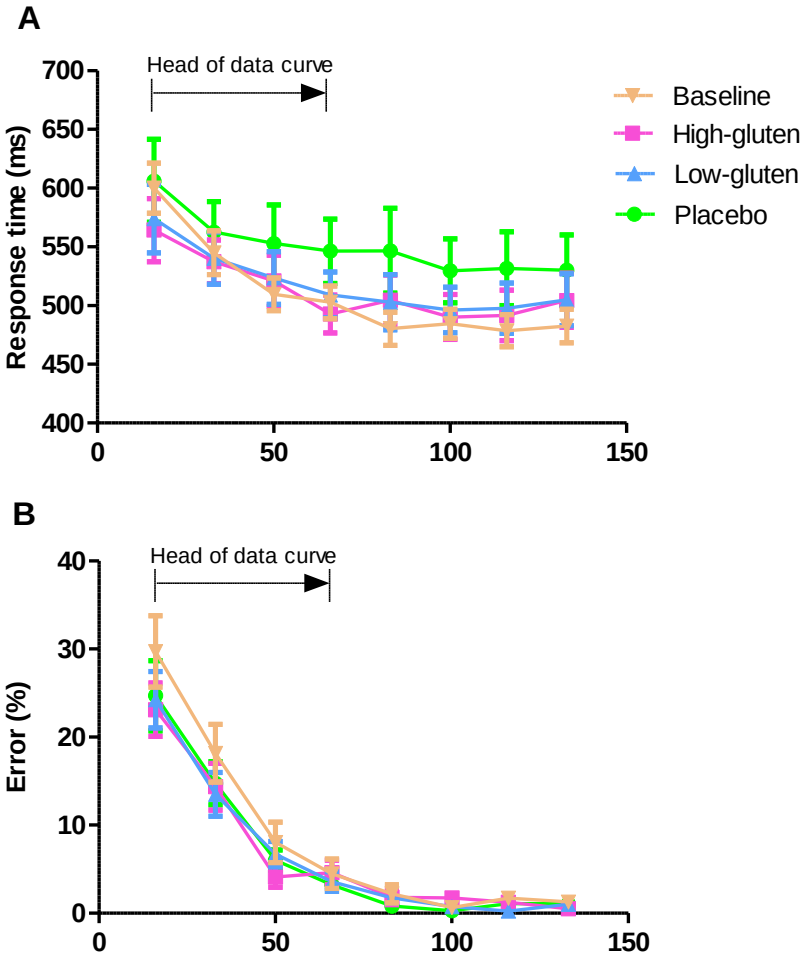

Figure 7.9 Data curves for the Subtle Cognitive Impairment test for each exposure duration (ms): (A) Mean response time (ms); (B) Mean errors (%) (bars represent standard error of the mean).

Table 7.8 Results of critical variables for response times and error rates to the head (exposure durations of 16-64 ms) and tail of the Subtle Cognitive Impairment test distribution (80-128 ms). Data expressed as mean ± SEM.

	Baseline	High-gluten	Low-gluten	Placebo
Error rates (%)				
Head	18 ± 2.5	12 ± 1.7	12 ± 1.7	12 ± 1.7
Tail	1.7 ± 0.5	1.4 ± 0.3	0.9 ± 0.2	0.8 ± 0.2
Response times (ms)				
Head	536 ± 13	521 ± 19	526 ± 19	569 ± 29
Tail	486 ± 12	486 ± 16	490 ± 18	533 ± 29

7.3.7 Effect on gliadin-specific T-cell responses

All subjects responded to one or both of the positive controls (tetanus toxoid and PHA). Only one participant (Subject #38) elicited a positive T-cell response following the high-gluten (16 g/d) challenge, where her day 6 response was more than a three-fold change from day 0 (see Table 7.9 and Figure 7.10A), a response similar to those reported in patients with coeliac disease.[111,112] However, the positive control (PHA) had a diminished response on day 6.

Table 7.9 IFN-γ ELISpot SFU/10^6 PBMC for Subject #38 after high-gluten (16 g/d) treatment.

Peptide	Day 0	Day 6
CT-Gliadin (100 µg/ml)	1	80
Deamidated CT-gliadin (100 µg/ml)	2	63
Gliadin peptide-1 (25 µg/ml)	1	61
Gliadin peptide-1 (50 µg/ml)	0	63
Gliadin peptide-2 (25 µg/ml)	1	20
Gliadin peptide-2 (50 µg/ml)	2	55
Tetanus Toxoid (10 LFU/ml)	1	2
PHA (2.5 µg/ml)	27	10

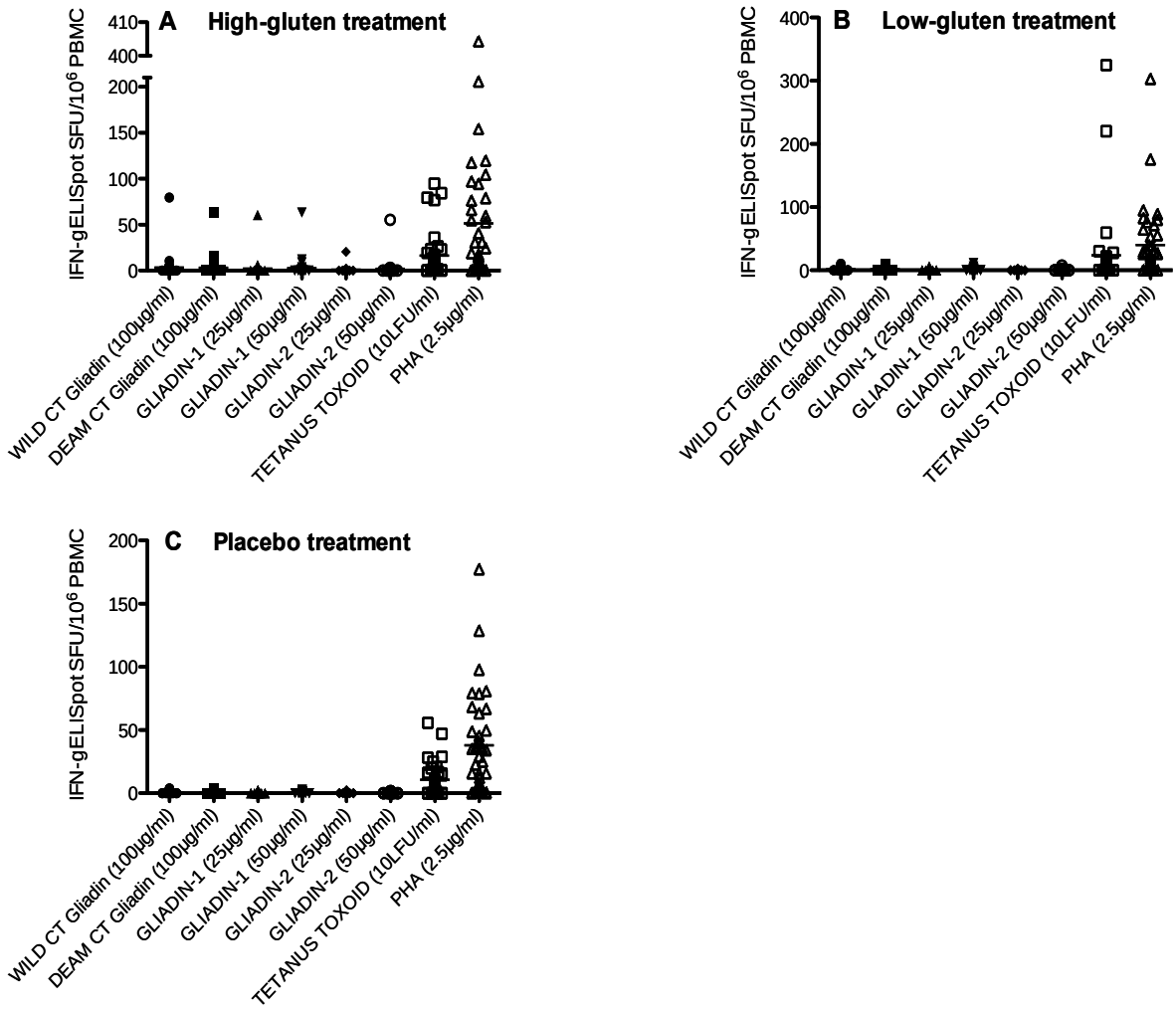

Figure 7.10 Interferon-γ (IFN-γ) ELISpot responses of peripheral blood mononuclear cells (PBMC) from study participants after a gluten-free diet for ≥ 2 weeks in all study participants (n = 37) on Day 6 after commencing a seven-day treatment period in a random order of (A) high-gluten (16 g/d), (B) low-gluten (2 g/d), and (C) placebo (0 g/d). SFU, spot forming units.

7.3.8 Effect on other biomarkers

There were no significant differences across the treatment periods (as shown in Table 7.10) for serological markers, HβD-2, calprotectin or ECP for the whole sample (n = 37), the gluten responders (n = 6) or the placebo responders (n = 11). There were no changes from baseline for serological markers. No correlation existed between average overall symptom score on high-gluten and serology, HβD-2 or ECP. 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 7.10 Coeliac serology and other biomarker results during treatment periods, shown as percentage of patients with elevated scores (mean ± SEM). There were no significant differences for diet difference on any measure (compared by Friedman test).

Biomarker	High-gluten	Low-gluten	Placebo
Coeliac serology (units/mL)			
Whole gliadin[348]	21 % (19 ± 3.6)	13 % (19 ± 3.4)	13 % (17 ± 1.6)
Whole gliadin (IgG)	8 % (11 ± 2.9)	5 % (9.4 ± 1.8)	8 % (11 ± 2.1)
Deamidated gliadin[348]	8 % (16 ± 1.4)	3 % (15 ± 1.4)	5 % (14 ± 1.2)
Deamidated gliadin (IgG)	8 % (8.7 ± 1.5)	11 % (8.8 ± 1.6)	11 % (9.3 ± 1.5)
Human ß-defensin-2 (ng/mL)	21 % (35 ± 4.9)	24 % (33 ± 4.8)	29 % (34 ± 5.6)
Calprotectin (µg/g)	18 % (35 ± 9.1)	18 % (33 ± 7.8)	18 % (43 ± 16)
Eosinophil Cationic Protein (ng/mL)	3 % (3.6 ± 0.6)	3 % (3.5 ± 0.6)	3 % (3.4 ± 0.5)

Healthy reference ranges: Human ß-defensin-2 (<46.4 ng/mL), Calprotectin (<50 µg/g), Eosinophil Cationic Protein (2.45 - 14.12 ng/mL).

7.3.9 Effect on indices of colonic fermentation and faecal gliadin-specific peptides

There were no significant differences between treatment periods (as shown in Table 7.11) for any faecal characteristic (frequency, output and description), faecal pH or faecal ammonia. There were no differences for the whole sample (n = 37), the gluten responders (n = 6) or the placebo responders (n = 11). No correlation existed between average overall symptom score on high-gluten and faecal characteristics, faecal pH or faecal ammonia.

Table 7.11 Faecal characteristics during treatment periods, data are shown as mean ± SEM unless otherwise indicated. There were no significant differences for diet difference on any measure (compared by repeated measures ANOVA or Friedman test as appropriate).

Faecal characteristic	High-gluten	Low-gluten	Placebo
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
Median description, score[#] (range)	4 (2-28)	4 (1-15)	4 (1-21)
Faecal pH	6.9 ± 0.06	6.9 ± 0.07	6.9 ± 0.06
Faecal ammonia (µg/L)	316 ± 23	328 ± 24	336 ± 25

[#] Score is according to Kings Stool Chart which classifies diarrhoea by a daily faecal score of ≥ 15

Mass spectrometric peptide mapping targeted 364 transitions from 22 peptides from the four major proteins in the gluten mixture (gliadin, glutenin, albumin and globulin) using the pepsin/trypsin digestion. All were detected in the standard at varying levels of sensitivity. However, these were not detected in any of the faecal samples.

7.4 Discussion

NCGS is currently defined on the basis of exclusion of coeliac disease and patient-reported benefits on a GFD - all patients participating in the present study fulfilled these criteria. However, gluten-specific induction of GI symptoms in this double-blind, randomised, placebo-controlled, rechallenge, cross-over trial provided little support for the presence of NCGS in more than an extreme minority of the cohort studied. This was in direct contrast to the initial study (Study One – see Chapter 6, page 101). Reasons underlying these apparent disparities results require elucidation.

Key differences listed below were employed in the current study design in an attempt to improve study outcomes, reliability of results and ultimately assist in understanding NCGS. All of these factors may have potentially influenced the results and masked the ability to study gluten-specificity.

- *All food was provided:* All food was low FODMAP and gluten-free provided to reduce background noise and control for changes in participants' usual diet (particularly intake of other potential dietary triggers). This ensured the only difference between the treatments was the protein (gluten or whey). Although some flexibility was given to food preferences (i.e., vegetarian), most participants were provided with a standard diet. However, the 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.

- *Reduced FODMAP intake:* In addition to all food supplied being low FODMAP, all participants were also educated about the low FODMAP approach. This was followed throughout the study period including the run-in and all washout periods. As previously mentioned, FODMAPs are very rapidly fermented and are osmotically active leading to increased gas production and fluid delivery.[195,200] The benefits of a restricted-FODMAP intake in patients with IBS have been previously shown[75] and this was consistent with the

significant improvement in GI symptoms following the reduction of FODMAP intake during the run-in period. Whilst a placebo response is possible, it is also likely that FODMAPs, in particular fructans, are responsible for symptoms in a large number of participants who strongly believe they are gluten-sensitive. While lowering the FODMAP intake also reduces long-chain dietary fibre intake, this is unlikely to be responsible for the significant reduction in symptoms during the run-in period especially given the results of earlier studies in which dietary fibre is higher and only FODMAP intake varies.[350]

- *Crossover design*: A crossover design was employed to reduce the influence of confounders and increase power.[351] Adequate washout and run-in periods were also employed (confirmed with checking of symptom diaries) to avoid carry-over effects and minimise order effects, respectively.[352] Although there has been previous reserved criticism for the use of a crossover design within the IBS population,[353] they have shown to be used successfully in dietary studies with IBS subjects.[196,200] In contrast, an order effect was apparent, where the diet treatment arm first received (regardless of the food component challenged) induced the most symptoms. Additionally, the nocebo effect, a phenomenon opposite to the placebo effect, may have impacted where an expectation of a negative outcome may lead to the worsening of a symptom.[354] The nocebo effect stems from highly active processes in the brain, mediated by psychological mechanisms of anticipatory anxiety and a complex interaction among different neurotransmitter pathways, including the hypothalamus-pituitary-adrenal (HPA) axis and a cholecystokinin (CCK)-ergic pronociceptive system.[355,356] The strong nocebo effect and order effects raise issues about the design and conduct of IBS dietary studies, where a cross-over design may not be suitable in this hypersensitive NCGS group. Psychological influences on the effects of gluten on those without coeliac disease deserve more attention.

- *Shorter exposure to dietary treatments*: Whilst the duration of treatment was reduced from six weeks in Study One to one week in the current study (Study Two), it is unlikely that a

longer time frame of challenge would capture any delayed responses to gluten as the three gluten responders reached their highest symptom level at day 3.

- *Increased complexity of the protocol:* The high participant burden and rigorous demands of the current study included frequent visits to clinic for blood and faecal collection, wearing accelerometers, completion of daily symptom and diet questionnaires and computer-based cognitive tests, all whilst following a restrictive diet. This may have been perceived as stressful and contributed to the positive symptomatic responses across all treatment arms. This has highlighted the need to simplify the protocol in both time and complexity.

- *Whey used as control protein:* Pure whey protein isolate as the control to balance the overall protein content across all treatment arms was chosen for its rapid digestibility[357,358] and minimal effects on the study food's texture and flavour. Whey is a soluble protein, consisting of a complex mix of globular proteins, such as beta-lactoglobulin, alpha-lactoalbumin, bovine albumin, lactoferrin, immunoglobulins. Most reports in the literature relate to the positive effects of dairy proteins, including modulating blood glucose lowering effects,[359] increased muscle protein synthesis,[360] modulation of blood pressure,[361] inflammatory processes,[362] and lipid metabolism,[363] whereas few discuss any negative effects. The whey protein used was lactose-free and, therefore, the unexpected effects of the placebo (whey protein) were surprising. More work is required to understand the potential adverse effects of whey. Indeed sensitivities to whey and/or some other component of the supplied diet may have led to the strong symptomatic responses to the present blinded food challenges. This suggests that whey may not be the best choice of placebo in these types of studies.

- *Wider range of other end-points:* As there are no objective markers for the improvement of IBS, the subjective and objective endpoints chosen in the current study were thought to be the most relevant and had been previously found to play a role in patients with food intolerances. 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. If the complexity of the study design and other factors discussed above did contribute to being able to identify a true NCGS subgroup, this may have had flow-on effects for being able to identify any biomarker-specificity.

- *Assessment of extra-intestinal manifestations:* Study One showed evidence of systemic effects occurring in NCGS with a rapid onset of tiredness in the gluten group. In the current study, fatigue (measured via the D-FIS) and tiredness (measured via the VAS) both improved during the low FODMAP run-in period, and then increased during all treatment periods. However, objective measurements of cognitive impairment or reduced physical function did not differ between the treatment periods. The potential mechanisms of a systemic process and functional gut symptoms remain elusive. Reproducibility is a major problem when subjective symptoms are the primary end-point being measured. Unfortunately there remains no clear, universally accepted guidelines on the choice of a primary objective end-point for IBS clinical trials [364]. It is important, going forward, that better endpoints and a standard challenge protocol for assessing food intolerances in adults are developed.

There were also similarities maintained in the current study to allow comparisons between trials:

- *Same participant eligibility criteria:* The same inclusion/exclusion criteria were applied to both Study One and Two, given the importance of standardised entry criteria has been previously highlighted.[365] Despite recruiting only participants with self-reports of well-controlled GI symptoms prior to enrolment, an average 63% improvement in overall symptoms was observed with reduction of FODMAPs during the run-in period, with 22% experiencing clinical significant improvement in overall symptoms (change >20 mm on VAS). The process of diagnosing NCGS needs to be reviewed; placebo-controlled rechallenges should be incorporated and should not be solely based on self-reporting. How many rechallenges necessitates exploration. More stringent entry criteria and definition of

NCGS is required, involving initial screening for the role of FODMAPs as possible dietary triggers.

- *Same symptom end-point assessment*: The 100 mm VAS to score the severity of gastrointestinal symptoms was used in both studies and classification of symptom response consistent. This was completed weekly for Study One and daily for Study Two.

Whilst the background diet provided was free of lactose, it was not devoid of dairy products. After the study results had started being analysed, participants were asked details on their history of dairy avoidance. Nineteen participants reported avoiding dairy for a mean (SEM) of 5.5 (0.7) years, where the following descriptions were given; "dairy free" (n = 6), "low dairy" (n = 3), "lactose free" (n = 6), "low lactose" (n = 2), "milk free" (n = 1), and "dairy fat free" (n = 1). Almost half of participants had avoided dairy to some extent prior to enrolment, highlighting that these patients have, as a general rule, had unusual and highly restrictive diets. There is, however, little scientific base to some of these food avoidances, being predominantly self-perceived and not clinically diagnosed. Undoubtedly, this patient group have unique characteristics and commonly report additional food intolerances (single in 38% of participants and multiple in 27%) in addition to gluten sensitivity.

For the existence of NCGS to be confirmed, a specific laboratory biomarker must be identified. The patients could not be classified according to changes in faecal or blood markers. While the class II MHC haplotype HLA-DQ2 and HLA-DQ8 are present in almost all coeliac disease patients, these genes were present in 57% of study participants, a percentage still higher compared to the general population.[130,366] Similarly, an association of HLA-DQ2 with NCGS in diarrhoea-predominant IBS has been reported.[146] This study assessed the induction of T-cells, and showed no evidence for the involvement of a MHC dependent, adaptive immune response. The one participant who elicited a positive T-cell response has since undergone follow-up investigations for coeliac disease. She returned after 12 months to repeat an IFN-γ ELISpot assay after a 3-day gluten challenge where there were no gliadin-specific T-cells induced.

A lot of attention has recently been given to the idea of an innate immune response playing a role via, for example, IL15[367,368] or claudin-4.[190] Although such data mostly derive from uncontrolled studies in rat models, they do provide a theory for the clinical question of how gluten acts as a symptom inducer in some IBS patients and suggest an important role of the innate immune system without any involvement of the adaptive immune response.[190] Further investigation is warranted.

Determining the gluten threshold is an essential part of understanding NCGS. However, dose-dependent effects were unable to be examined in this study. Only one participant who responded to the high-gluten arm (16 g/d gluten) also responded to the low-gluten arm (containing 14 g whey and 2 g gluten). Interestingly, this patient also responded to the placebo (16 g whey) and after completion of her participation, discovered she had been pregnant for the duration of the three treatment periods. Not every participant who reacted to the 16 g of whey, also reacted to the 14 g whey. Reproducibility remains the cornerstone of food intolerance diagnostics.

7.5 Conclusions

This double-blind, randomised, placebo-controlled, crossover rechallenge study showed no evidence of specific or dose-dependent effects of gluten, but FODMAP restriction uniformly reduced residual symptoms. Either the patients do not have NCGS as self-reported or the trial design precluded its recognition because of a high nocebo effect. A better understanding is warranted in the diagnosis of NCGS, where self-reporting is probably inaccurate and the need for one or more placebo-controlled rechallenge exists.

Chapter 8 – Study Three: Reproducing the effects of gluten and whey

8.1 Background and aims

Study One (see Chapter 6, page 101) showed results that supported the existence of NCGS. In contrast, Study Two (see Chapter 7, page 118), using a different design, found no positive evidence of specificity in the induction of symptoms by gluten, but rather showed frequent induction of symptoms with whey and/or gluten. The complexity of the study design may have contributed to a strong nocebo effect or indeed sensitivities to whey, gluten and/or some other component of the supplied diet may have led to the strong symptomatic responses to blinded food challenges.

Symptoms of IBS in patients are a complex response to both biological and psychosocial factors.[369,370] The concept of IBS as a disorder of the 'brain-gut axis' with physical and psychological components[369] is gaining acceptance. This concept places emphasis on the 'perception of symptoms' and their impact rather than on the symptoms themselves.[371] This area, therefore, warrants further exploration in relation to the NCGS patient group described in this thesis. For example, it is not known whether gluten ingestion may also contribute to changes in the mental health of these individuals. Changes in mood have been described in coeliac disease[372,373] and in particular, symptoms of anxiety and depression have been shown to be related to symptoms from the gut.[78] Personality and emotional factors including levels of anxiety, anger, depression, and curiosity are major indicators of psychological distress and well-being, and require careful assessment. Dispositional and transitory emotions can be easily measured using the validated State-Trait Personality Inventory (STPI) scale.[374] Determination of cortisol in serum and urine has long been used in the assessment of adrenocortical function and other disturbances in the hypothalamic-pituitary-adrenal axis (HPA) and can therefore be used as an objective

measurement.[375] It also serves in the diagnosis of depressive disorders[376,377] and is a frequently used marker for different kinds of stress-induced reactions.[375,378-381]

Whey protein isolate was used as the placebo in Study Two to balance overall protein levels. Whey protein was chosen for its rapid digestibility in the gut[357,358] and the minimal effects it had on the study food's texture and flavour. The results from Study Two suggested that whey protein itself may have triggered some symptoms in some patients. To date, research into whey has focused mostly on beneficial effects (e.g., modulation of blood pressure,[361] inflammatory processes,[362] and hyperglycaemia),[359] whereas research into adverse effects of whey has focused on milk allergies or lactose intolerance. There have been no published investigations specifically assessing whey protein as inducing IBS-type symptoms. Consequently, a clearer understanding of the effects of whey protein, separate from gluten, is needed including a comparison to an inert placebo.

One difference between the designs of the previous studies was that all food was supplied in the second study. While the FODMPS content of the supplied food was very low, it is possible that other potential dietary triggers may have been present in the food, leading to the apparently non-specific symptomatic responses. Apart from the established effects of carbohydrate malabsorption on GI symptoms,[196,200] there is some limited evidence that naturally-occurring and artificially-added 'food chemicals' can induce IBS and other symptoms via a pharmacological action.[223] The elimination diet for food chemicals includes restriction of salicylates (widely found in fruits, vegetables, herbs, spices, nuts, tea, coffee), amines (chocolate, canned/smoked fish, sauces, stock, nuts, seeds, vinegar, and some fruit and vegetables), monosodium glutamate (MSG: found in strong cheeses, soy sauce, and used as a flavour enhancer) as well as preservatives benzoates, propionate, sulphites, nitrites, sorbic acid, plus added antioxidants and colours.[192,225] Although the low-chemical diet was originally designed for the management of chronic urticaria and eczema,[382] it has also been shown to improve rhinitis, behaviour disorders and migraines[222,383] and anecdotally been said to improve IBS symptoms concurrently.[192] Combining restriction of food chemicals and FODMAPs makes for a highly restrictive diet, but ensures all known potential confounders that may affect visceral sensation are controlled.

The high demand and commitment required of participants during Study Two including frequent visits to clinic, faecal collection, blood-taking and questionnaires may have been perceived as stressful and contributed to the positive symptomatic responses. This highlighted the need to simplify the protocol in both time and complexity. Studies One and Two provided data on the time scale of outcome responses, where the gluten responders from Study Two (see Figure 7.5A) reached their highest symptom level at day 3. Additionally, 3-day gluten challenges are often employed in coeliac disease studies.[87,384]

Thus, there were several aspects that could be addressed to limit potential confounders. For the current study, it was hypothesised that both gluten and whey specifically trigger GI symptoms as well as stress and psychological responses in a proportion of (not necessarily the same) patients who reported having NCGS. The aim of Study Three was to examine these hypotheses by undertaking a controlled feeding trial (randomised, crossover, double-blind, re-challenge design) of three treatment arms – placebo, gluten (16 g/day), whey (16 g/day) – for three days each in patients without coeliac disease, while controlling for FODMAP and food chemical intake in a cohort of patients who participated in Study Two. End-points assessed included changes in overall and individual GI symptoms, fatigue, measures of psychological wellbeing and cortisol secretion, in addition to reproducibility of effects.

8.2 Methods

8.2.1 Subjects

All participants from Study Two were invited to return to complete Study Three. As the time between participation of the two studies varied from 8 to 17 months, participants completed another food and symptom diary to confirm they still met the inclusion/exclusion criteria (see Chapter 3, Section 3.3), including following a GFD (via 3-day food diary) and reporting their symptoms to be well controlled (via 3-day daily gastrointestinal symptom diary).

8.2.2 Study protocol

A randomised, placebo-controlled, double-blinded crossover, re-challenge study design of at least 21 days duration was conducted (Figure 8.1). Eligible volunteers were randomly allocated to receive one of the three dietary treatments (gluten, whey or placebo) for 3 days, followed by a washout period of minimum 3 days (until symptoms induced during the previous dietary challenge resolved), before crossing over to the next diet. The maximum time that a participant could remain on the washout period was two weeks. Participants were asked to follow a low-FODMAP, GFD during the washout periods.

Patients unable to continue the study due to intolerable symptoms were permitted to cease the study food of that particular treatment arm but continue to collect data as per day three (symptom assessment and mouth swab collection) and go onto resume any remaining treatment arms following the allocated washout period. The protocol was approved by Eastern Health Research and Ethics Committee.

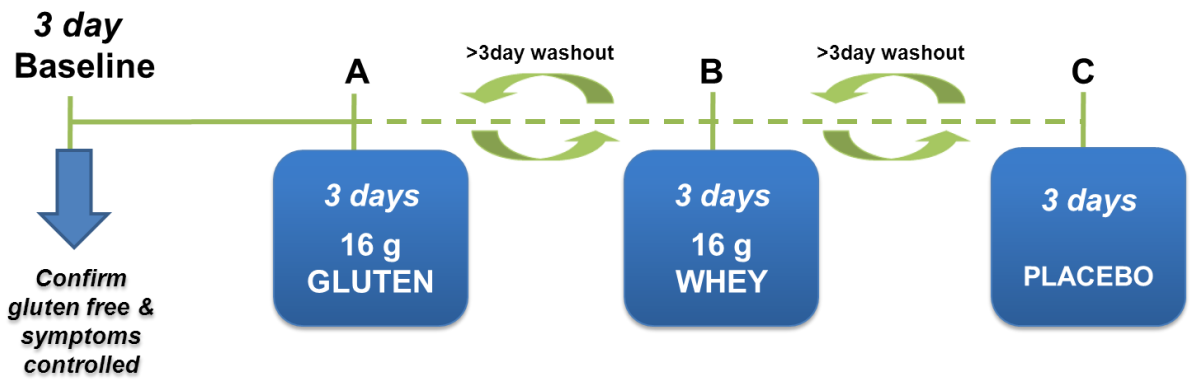

Figure 8.1 Study Three protocol outline

8.2.3 End-points

The primary end-point was the comparison of the change in mean in the overall symptom score measured on the VAS after 3 days of study diet.

The secondary end-points were:

- The proportion of participants demonstrating an increase in the overall symptom score measured on the visual analogue scale of at least 20 mm after 3 days of study diet.
- The proportion of participants demonstrating an increase in individual symptom scores measured on the visual analogue scale of at least 20 mm after 3 days of study diet.
- The change in symptom scores (individual and overall) at 3 days compared with baseline symptom levels.
- Change and comparison over the treatment arms in STPI scores and cortisol levels.
- Reproducibility of gastrointestinal symptom scores between Study Two and Study Three

8.2.4 Study food preparation

The background diet was gluten-free, low in FODMAPs, dairy-free and low in natural food chemicals (see Chapter 3, Section 3.5 for details on diet design). During the three treatment periods, the study diets had the following incorporated:

- Gluten (16 g per day whole wheat gluten); or
- Whey (16 g per day whey protein isolate); or
- Placebo

All main meals and snacks were supplied to the subjects. Participants received information on what foods were appropriate to consume, if and when extra foods were consumed during the treatment periods. A sample meal plan is given in Appendix 5. Details on whey and gluten composition are shown in Chapter 3, Section 3.5.1. The meal plan was adequate in macronutrients, micronutrients and provided 8.5 MJ energy daily (see Appendix 7 for the average daily nutritional breakdown). Volunteers with smaller energy requirements were advised to consume fewer snacks and given smaller portions of the meals, but the same proportions of gluten or whey were added. Volunteers with larger energy requirements were provided with additional low FODMAP, gluten-free, low-chemical, dairy-free meals and snacks.

Meals were similar in texture, taste and appearance across the three diets. All food was prepared by the investigators and Research Chef in the kitchens of Monash University and of the Chef. Meals were packed individually in food-grade foil containers or bags sealed with a cryovac Orved VM-12 vacuum sealer (Orved®, Musile di Piave, Italy) to extend shelf life and quality of the food. All meals were kept frozen and reheating instructions were provided to the volunteers. All food was provided free of charge. Dietary adherence was assessed by entries into a tick-box diary completed during the week and counting returned food.

8.2.5 Measurements

8.2.5.1 Gastrointestinal symptoms

Gastrointestinal symptoms were assessed by the participant completing daily diary cards, which used a 100 mm VAS (see Chapter 3, Section 6.1 and Appendix 8) to score the presence and severity of overall abdominal symptoms, abdominal pain, bloating, wind, lethargy and nausea. Gastrointestinal symptom cards were completed throughout the study (i.e., baseline, three diet treatments periods, washout periods). Clinical significant change of symptoms was arbitrarily defined as a change of at least 20 mm.

8.2.5.2 Fatigue

Severity of fatigue was evaluated by the Daily Fatigue Impact Scale (D-FIS),[284] previously described in Chapter 3, Section 6.2.5.2. Fatigue symptom cards were completed throughout the entirety of the study (i.e., baseline, three diet treatments periods, washout periods).

8.2.5.2 Mental health

Emotional states of anxiety and depression were assessed using the State-Trait Personality Inventory (STPI) subscales. The STPI is a 60-item self-report inventory which consists of six ten-item subscales measuring anxiety, anger, and curiosity rated as a trait (felt generally) and as a state (how participants feel at that moment).[300] The depression subscales were evaluated in separate factor analyses, termed depression-present (dysthymia) and depression-absent (euthymia). The STPI state and trait depression and curiosity scales and subscales are described in more detail in Chapter 3, Section 6.10. The STPI was completed on Day 3 of each treatment and baseline period.

8.2.5.2 Cortisol

Salivary cortisol was used as a biomarker of psychological stress[302] and is described in more detail in Chapter 3, Section 6.11. Clear collection instructions (see Appendix 10) were provided to participants to ensure influential factors were controlled, including sample collection taken at standardised times (in the evening at 2030 h). The Salimetrics Oral Swab (SOS; Salimetrics™, State College, USA) was used to collect saliva samples and stored inside a Swab Storage Tube (clear sterile plastic tube; Salimetrics™, State College, USA) on day 3 of each treatment and baseline period. All saliva samples were transported on ice and frozen at -20 °C until being assayed externally (Stratech Scientific APAC Pty Ltd, Sydney, Australia) by competitive immunoassay using commercially available kits (Salimetrics™, State College, USA).

8.2.6 Statistical analysis

Comparisons of symptom severity scores and measured parameters across treatment periods were assessed by repeated measures ANOVA or Friedman test, as appropriate. Comparisons between two treatments were assessed by paired t-test. The reproducibility between Study Two and Study Three 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.

8.3 Results

8.3.1 Study population

Twenty-two subjects gave written, informed consent, the remaining participants were not able to return for a variety of reasons including pregnant/breast-feeding (n = 3), travel (n = 3), time (n = 8), or did not want to eat gluten (n = 4). One participant had an extended washout period between her second and third diet treatment (11.5 weeks), but was not an outlier and did not affect the result of any analysis. The details of those patients recruited and who completed the study are shown in Table 8.1.

Table 8.1 Study subject characteristics

Characteristics	
Number of patients	22
Gender	5 male
Median age (range) years	48 (24 – 62)
Median BMI (range)	23 (17 – 32)
Predominant bowel habit	
Diarrhoea	36%
Constipation	46%
Alternating	18%
HLA type	
DQ2 or DQ8 positive	55%

BMI, body mass index; HLA, human leukocyte antigen.

8.3.2 Dietary compliance

All volunteers undertook the three diet treatment arms. One patient ceased the whey treatment arm (treatment first received) prematurely because of intolerable symptoms after lunch on day two. Data continued to be collected as per day three (symptom assessment and mouth swab collection).

Nearly all meals (99, 96 and 99%) were consumed in the gluten, whey and placebo groups, respectively. 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 or orange per day), but this did not differ across the treatment arms within participants.

8.3.3 Effect on gastrointestinal symptoms

Data from day three on each treatment period was used for analysis and were corrected for baseline. There were no differences across the dietary treatment arms (gluten, whey and placebo) for overall symptoms (Figure 8.2). As shown in Figure 8.3, changes in individual symptoms (bloating, satisfaction with stool consistency, wind, pain, tiredness and nausea) were similar across the three dietary periods.

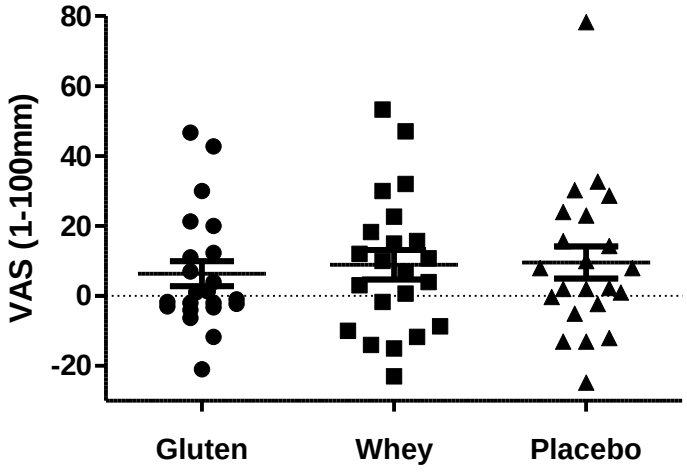

Figure 8.2 Individual changes in mean overall symptom severity score from baseline. Data shown represent the mean and SEM. There were no significant differences across or between treatment periods. VAS, visual analogue scale.

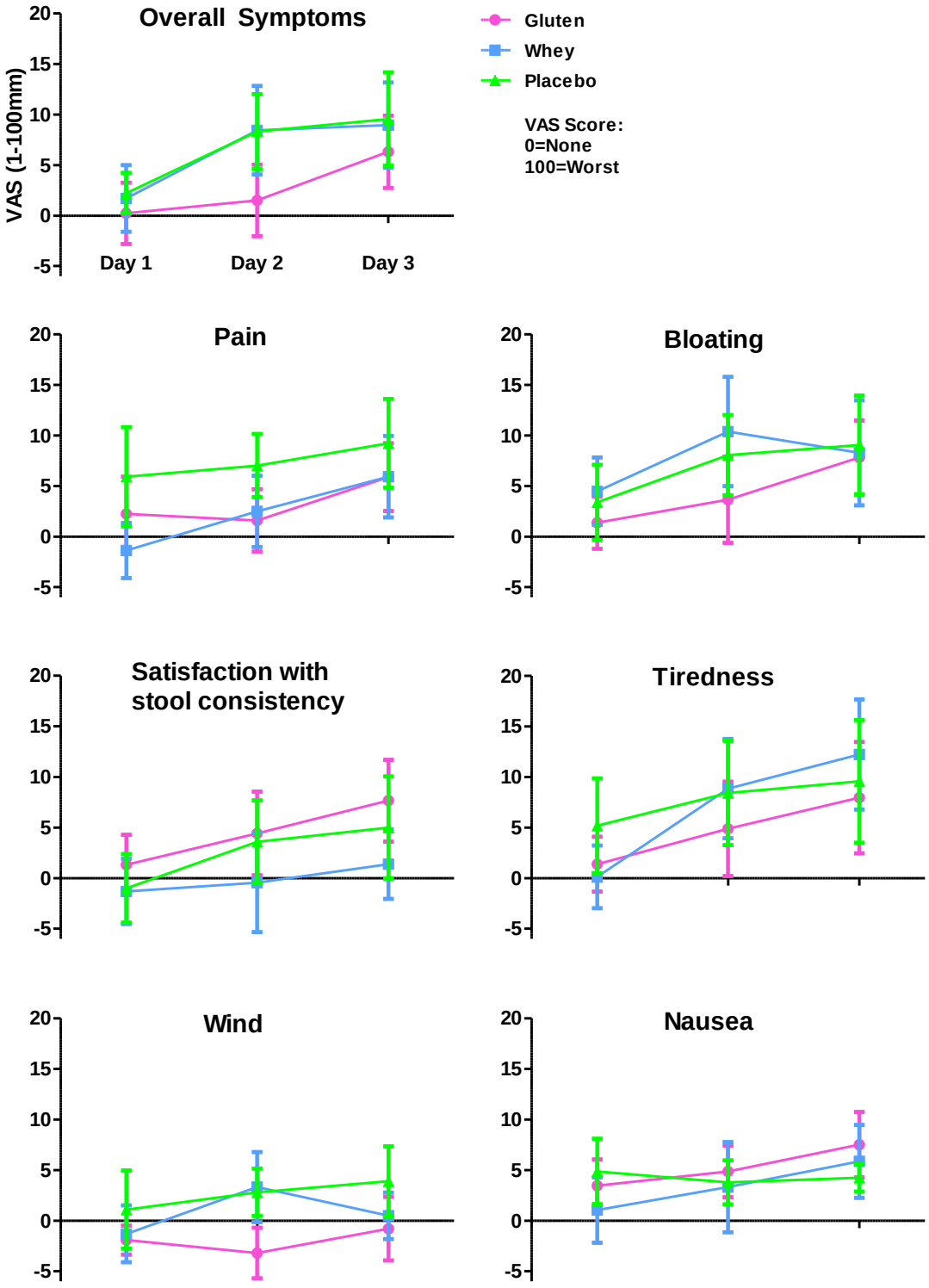

Figure 8.3 Change in symptom severity from baseline for each dietary treatment over 3-day study period. Data shown represent mean and SEM. There were no significant differences across or during treatments periods. VAS, visual analogue scale.

The reproducibility of participants' response to gluten (16 g/d) and whey (16 g/d) between Study Two (7-day challenge) and Study Three (3-day challenge) was evaluated by comparing the change in severity of overall symptoms (Figure 8.4). There were no significant differences (shown in Figure 8.4) and those identified with a positive symptomatic response to gluten and whey differed between the two studies.

- *Gluten-specificity:* The two participants who had an average increase on the VAS for overall abdominal symptoms of more than 20 mm on the gluten (16 g/d) arm in Study Two were not the same two participants who had a positive response to the gluten (16 g/d) arm in Study Three (Figure 8.4A). Thus, gluten-specificity was not reproduced in any subject.

- *Whey-specificity:* Six participants had a positive response in overall symptom severity in the whey (16 g/d) arm in Study Two, one of whom also reacted to the whey (16 g/d) arm in Study Three. Three different participants also had a positive whey response in Study Three (Figure 8.4B). Thus, only one subject reproduced their whey-specific symptomatic response.

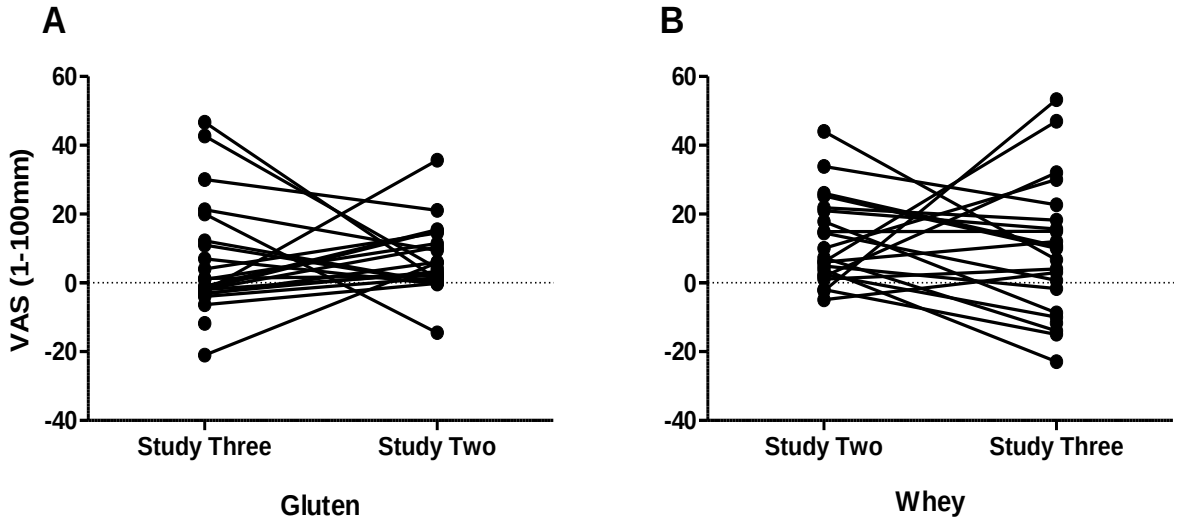

Figure 8.4 Reproducibility in change in overall symptom severity for (A) gluten (16 g/d) and (B) whey (16 g/d) treatment arms. Study Two used average data from the 7-day challenge and Study Three used data from the third day of the 3-day challenge. VAS, visual analogue scale.

Re-test reliability in average change in overall symptom severity score (mm) between Study Two and Study Three showed no correlation between the two studies for either 16 g/d gluten (Pearson r = -0.04, p=0.858) or 16 g/d whey (Pearson r = 0.08, p=0.748) treatment arms.

Several patient-related factors were examined in terms of their association with symptomatic responses to the diets. The predominant bowel habits, BMI, age, sex, duration of GFD and HLA-DQ status did not predict the responses to any of the diets.

The influence of the order of the dietary intervention was also examined. As shown in Figure 8.5, there was a significant difference across the three groups (p=0.044; repeated measures ANOVA). This trend was similar to that seen in Study Two, where the first intervention was associated with greater symptomatic changes (mean 15.5 mm) than the second (mean 5.3 mm) or third (mean 4.0 mm) challenges, regardless of what it contained i.e., gluten, whey or placebo.

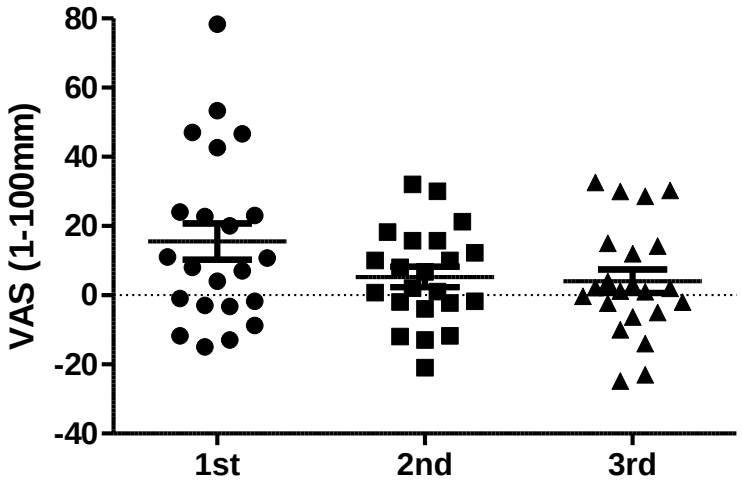

Figure 8.5 Change in overall symptom severity grouped in order of treatment arm received. The differences were compared by repeated measures ANOVA (p=0.044). Differences were also compared between each group by a paired t-test (p=0.066 between 1st and 2nd; p=0.058 between 1st and 3rd; p=0.7077 between 2nd and 3rd). VAS, visual analogue scale.

8.3.4 Effect on fatigue

There were no differences across or during the dietary treatment arms (gluten, whey and placebo) in Daily-Fatigue Impact Scale (D-FIS) scores.

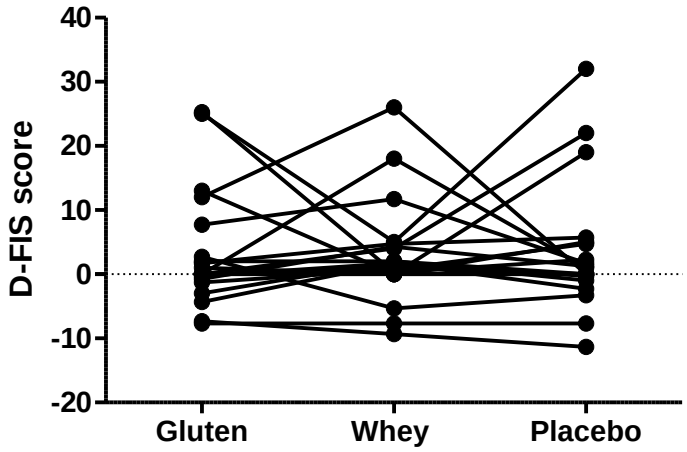

Figure 8.6 Change in Daily-Fatigue Impact Scale (D-FIS) score from baseline for each study period. Data shown represent the mean and whiskers represent min to max. There were no significant differences across the treatment arms (repeated measures ANOVA). Scores >10 are consistent with reports of onset of an acute flu-like illness. NS, not significant.

8.3.5 Effect on mental health

Results from two participants in whom scores for STPI state were gross outliers were removed from analysis. As shown in Table 8.2, the gluten treatment arm was significantly associated with an increased overall STPI state depression score compared to those for whey and placebo. Likewise, gluten (p=0.003) but not whey (p=0.734) or placebo (p=0.383) increased state-depression scores compared with baseline scores. Gluten ingestion tended to be associated with similar or increase in the STPI state depression subscale, dysthymia, and with a decrease in euthymia, but these were not statistically significant (Figure 8.7). No differences were found for other STPI state indices including anxiety, curiosity or anger, or for any of the STPI 'trait' measures.

Table 8.2 Difference in STPI scores following gluten ingestion compared to placebo (paired t-test).

	Placebo		Whey	
	Mean difference (95 % CI)	***P*-value**	**Mean difference (95 % CI)**	***P*-value**
Depression	**-1.65 (-2.99 to -0.32)**	**0.018**	**1.90 (0.40 to 3.40)**	**0.016**
Anxiety	0.88 (-1.91 to 2.21)	0.881	0.85 (-0.80 to 2.50)	0.295
Curiosity	0.60 (-2.23 to 3.43)	0.663	-2.05 (-4.22 to 0.12)	0.063
Anger	-0.25 (-1.18 to 0.68)	0.582	1.05 (-0.43 to 2.53)	0.155

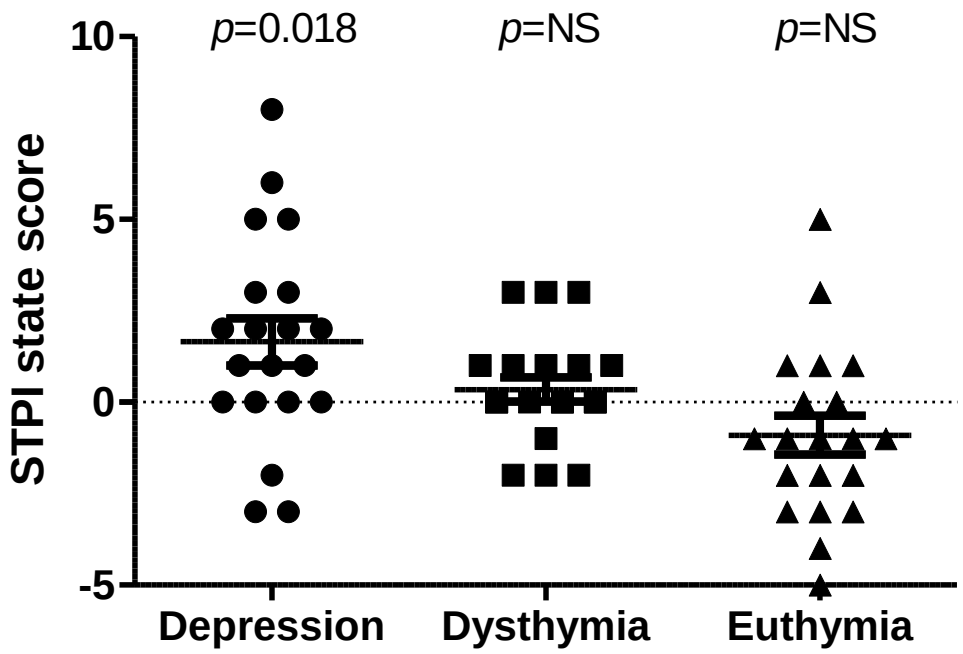

Figure 8.7 Difference in STPI state depression and depression subscales after 3-day gluten treatment arm from placebo. There were no differences in dysthymia (p=0.309) or euthymia (p=0.104) subscales. Differences were compared between each group by a paired t-test).

8.3.6 Effect on levels of cortisol

One participant produced insufficient saliva for analysis. As shown in Table 8.3, there were no differences in salivary cortisol levels between or during the treatment periods.

Table 8.3 Salivary cortisol (µmol/L) during baseline or dietary periods (mean ± SEM)

Baseline	Gluten	Whey	Placebo
0.020 ± 0.005	0.021 ± 0.005	0.021 ± 0.003	0.016 ± 0.001

Salivary cortisol expected values reported for adults is <0.211 µmol/L[192]

8.4 Discussion

The results of this work highlight the complexity of the NCGS entity. This double-blind, randomised, placebo-controlled, repeat re-challenge, cross-over trial in patients who believe they are gluten sensitive does not support the existence of NCGS. Gluten did not specifically induce GI symptoms, nor activate cortisol secretion. The only measure of psychological wellbeing to alter was a significant increase in overall state-STPI depression score after the 3-day gluten challenge arm. There were no differences in the effects induced by whey.

This study was purposely designed to be of a short-duration, highly-controlled and with less participant effort and application. Although combining restriction of food chemicals and FODMAPs did produce a highly restrictive diet, it ensured all potential dietary confounders were controlled.

Irritable bowel syndrome is best regarded as a complex of symptoms without a single cause, including disordered gut motility, visceral hypersensitivity, intestinal inflammation, and genetic and environmental factors.[385,386] The symptoms are an integrated response to a variety of interactions combining biological and psychosocial factors.[369,370] For example, increases in generalised anxiety and depressive symptomology may lead to patients being more concerned about their symptoms.[387] It has also been reported that chronic life stress may predict the intensity of bowel symptoms.[388] Persons suffering from IBS have high rates of 'abnormal' behaviour patterns including anxiety, depression[389,390] and somatisation (conversion of an emotional, mental, or psychosocial problem to a physical complaint).[391,392] There are many tools used for the measurement of anxiety and depression, including the 80-item STPI.[300] In the current study, gluten influenced the current emotional state of NCGS individuals by increasing current feelings of depression, possibly due to a combination of the depression subscales dysthymic (depression-present) and euthymic (depression-absent) effects, but had no influence on depression as a personality trait. Although depressed persons often experience high levels of anxiety and intense anger, there were no differences found in the anxiety, anger or curiosity measures.

If the gluten ingestion is causally related to depression, the mechanism is unclear. One possibility involves serotonin (5-HT). This is a critical signalling molecule in the gut, shown to play a role in IBS symptom genesis.[393,394] Additionally, dysfunctional serotonin neurotransmission has been implicated in depression.[395] As most serotonin is derived from the digestive tract,[396] it may be possible that certain foods, such as gluten-containing ones, influence serotonin production. Alteration in 5-HT signalling has shown to be associated with coeliac disease,[397-399] where excess serotonin production was induced on exposure to a high-gluten, high-carbohydrate meal.[397] Another explanation might implicate gluten "exorphins", opioid peptides derived from partially digested food proteins including gluten; these can cross the blood-brain barrier interfering with central nervous system (CNS) activity, as proposed in studies of autism.[400,401] However, since depression is a complex, multifaceted syndrome with a number of underlying dimensions and can lead to many behavioural and physical symptoms,[301] the clinical significance of these findings requires further research using additional measures of depression.

Stress is known to affect GI symptoms probably by an alteration of visceral sensitivity. Cortisol, the major glucocorticoid produced in the adrenal cortex,[402] acts on the HPA, which is a complex set of interactions between the hypothalamus, the pituitary gland and the adrenal glands. The HPA modulates the immune system and digestion, and plays a key role in the adaptation to stress. Stress of any kind is one of the major stimuli for increased cortisol secretion.[380,403] In addition, cortisol shares a relationship with serotonin in the identification of mood sensors. Cortisol concentrations measured in saliva can provide a feasible, accurate, and practical alternative to blood determinations.[404-406] Levels are known to rise independently of circadian rhythm in response to stress.[407] The observation in the current study that salivary cortisol levels were unaffected by any of the 3-day dietary treatments indicate that high enough levels of stress (enough to elevate salivary cortisol levels) was not occurring.

All of the participants in this study returned from Study Two, enabling the reproducibility of the symptomatic responses to specific proteins to be assessed. Reproducibility is a key feature of any clinical study and diagnostic pathway for food intolerance, since the double-blind placebo-

controlled challenge is the cornerstone for elimination diet methodology.[224] The failure to demonstrate even a hint of reproducibility of symptomatic responses to gluten or whey is of considerable concern. An order effect was apparent, similar to Study Two, where the diet treatment arm first received (regardless of the food component challenged) induced the most symptoms. It shows in this patient group, a strong anticipatory symptomatic response independently on the nature of the challenge protein or 'nocebo' effect. The poor reproducibility can be explained by the subjective nature of the relationship between complaints and food consumption. Such problems have been identified previously. For example, symptom responses to three separate blinded challenges are required in the protocols for investigating some food allergies.[408] An objective assessment would be ideal in patients with IBS to allow accurate assessment of adverse reactions and identification of offending foods. Unfortunately, the only current marker of response to food components in patients with IBS is subjective reporting of symptoms. An objective biomarker is needed.

Placebo rates in IBS trials are known to range typically between 40 and 70%,[409] with the nocebo effect in the current study showing the same trend. Longer study duration and a run-in period may have prevented such an effect.[409] The 3-day treatment duration and limited number of clinic visits was chosen to increase ease of participation and to reduce stress that might have been related to multiple testing. All study materials (food, diaries, swabs and tubes for the collection of saliva) were delivered to the participant and no invasive procedures were undertaken (such as the multiple blood and faecal collections undertaken in Study Two). Palatability and appetite were not measured, but whey has been associated with greater satiety. Whey may have increased fullness, by altering sensations of gastric distension,[410] but this can be discounted as the placebo arm (containing no additive) consistently induced greater symptom severity.

Currently, NCGS is defined according to self-reported improvement of symptoms on the withdrawal of gluten. The findings of Study One suggested that the association of symptoms with gluten ingestion was real. The negative findings of the second cohort subsequently examined in two sequential studies – particularly the relatively low rate of triggering of symptoms with gluten –

might argue that the initial results were a chance phenomenon and that NCGS does not exist. However, there are clues that the methodologies used – very short-term cross-over challenges – may have precluded recognition of any gluten specificity. A very strong and rapid-onset nocebo effect was observed. This has the potential to mask any specific effects and argues against a placebo-controlled rechallenge methodology in making the diagnosis. At the same time, it does not explain the lack of symptom induction following gluten ingestion. Observations of induction of symptoms over a very short period and the at-times severity of those symptoms led to a very short challenge period to avoid excessive drop-outs and prolonged adverse effects on the participants. The shorter challenge periods used may be too short for gluten-specific effects to emerge. Of importance, the finding of acute changes in the current emotional state only in the gluten-specific arm, with no effects on trait indices, provides a clue that the improvement reported by patients may be in the perception of their general well-being rather than in GI symptoms. It was indeed interesting that a high proportion of subjects in the questionnaire study from Chapter 5 felt much improved on a GFD with ongoing GI symptoms of more than mild severity.

Thus, verification of NCGS in an individual is difficult and a single or even double short-term placebo-controlled challenge has doubtful validity. This then compounds defining pathogenic mechanisms, which are needed to develop biomarkers. Perhaps the psychological effects of gluten may be a better target than GI symptoms. Depressive symptoms have been shown to be a feature of coeliac disease[372] and exist in patients with food hypersensitivity,[411] but the mechanisms remain to be determined. Changes in brain-to-gut signalling have been suggested to be associated with prolonged alterations in the autonomic nervous system, which in turn is associated with altered emotional states such as depression.[78] While it should be noted that ‘causality’ cannot be proven by association, further studies are needed to elucidate whether depressive symptoms are the cause or consequence of NCGS, or whether they simply are parallel manifestations of an underlying disorder. More dissection of this aspect is clearly warranted.

8.5 Conclusions

In conclusion, this double-blind, randomised, placebo-controlled repeat-rechallenge study of patients who believe they have NCGS did not show gluten specifically induced changes in GI symptoms, fatigue or cortisol secretion. Gluten did, however, influence current feelings of depression, but had no influence on depression as a personality trait. The clinical significance of these findings requires further research using additional measures of depression. A very high nocebo response was found regardless of all background dietary triggers being controlled and reproducibility of symptom induction to a specific protein was poor. Blinded placebo-controlled dietary challenge may not be a valid way of verifying the existence of NCGS.

Chapter 9 – General Discussion

9.1 Making sense of the demand for gluten-free and wheat-free products

The worldwide demand for gluten-free and wheat-free products has not been supported by good scientific evidence.[160] Aside from the well-defined medical condition, coeliac disease, gluten is also blamed as a trigger of symptoms by 20–45% of adults who self-report food hypersensitivity.[412] With the removal of gluten-containing foods from their diet, patients report marked improvement of their symptoms. We have not clearly understood whether it was the removal of gluten or some other component of wheat that is responsible for these health improvements. The preceding studies (Chapters 4 – 8) have contributed to our understanding about the role the two major wheat constituents (gluten-protein and FODMAP-carbohydrate) have in the genesis of symptoms in individuals without coeliac disease. Moreover, this research sheds some light on why 'avoidance of wheat and gluten' products has some efficacy.

The low FODMAP diet is an evidence-based and well-understood strategy for the management of GI symptoms associated with IBS.[196] The food analysis undertaken in Chapter 4 has expanded existing FODMAP composition tables,[217,218] allowing refinement of food choices included in the low FODMAP approach. This analysis of commonly consumed grain and cereal products showed that wheat- and rye-derived products contain the highest FODMAP content, predominantly fructans and GOS. The products with the lowest FODMAP contents were mostly gluten-free, based on rice, oat, quinoa and corn ingredients. It is likely, therefore, that 'gluten restriction' will automatically reduce a patient's dietary FODMAP intake. The clinical significance of this was confirmed in Chapter 7, where there was significant improvement for overall GI symptoms and tiredness with the initiation of the low FODMAP diet in individuals who had previously reported themselves to be well controlled in their symptoms on a GFD. Additionally, in the survey (Chapter

5) of 132 patients with self-perceived NCGS who responded to advertising, one in four had uncontrolled symptoms despite following a GFD. This also suggests that the low FODMAP diet should be used as an approach to further improve residual IBS symptoms in patients and that patients can often further improve regardless of their current perceptions of their symptom level.

9.1.2 Nutritional concerns for gluten-free and wheat-free diets

In addition to the increasing numbers of the general population who follow a GFD,[7] Chapter 7 showed 65% of participants described avoidance of some other problem food. These food avoidances were predominantly self-perceived and not clinically diagnosed. The health implications of following long-term restrictive diets, particularly avoidance of wheat- and gluten-based products, require investigation. This area is especially significant given the evidence for the important role grain- and cereal-derived long-chain carbohydrates (i.e., dietary fibre) have in relation to bowel health.[38] Chapter 4 discussed fructans being classified as prebiotic-carbohydrates,[24,31,305-307] with benefits including reduced risk of GI infection,[305] improved laxation,[307] increased calcium absorption,[43] maintenance of functional gut mucosal barrier[308] and stimulation of the GI-immune system.[31] There were very low fructan and fibre intakes found in the baseline diets of participants (described in Chapter 7). Indeed, intakes were about half of what is normally found[200,413] due to the reduced intake of the cereal and grain foods.

Assessment and laboratory testing routinely undertaken to recognise clinical nutritional deficiencies were not completed in this research. However, concerns for the long-term nutritional status of patients with coeliac disease living on a GFD[74,252,259,414] include reduced folate, fibre, vitamin B_{12} and calcium.[259,415-417] Gluten-free replacements (such as rice, potato and corn) are not nutritionally comparable to staple gluten-containing grains,[397] especially for energy content, nutrient-density and mandatory fortifications (thiamin, riboflavin or niacin).[260,261,418] Long-term, nutritional inadequacies could play a harmful role in an increased risk of cardiovascular disease (CVD) outcome.[74,414,419,420] For example, a reduced daily intake of B-vitamins has been shown to

account for raised plasma total homocysteine (tHcy) levels,[74] where increased tHcy levels may be an independent risk factor for CVD.[421,422]

The survey showed 45% of self-perceived NCGS patients reported to have self-initiated the GFD without dietetic supervision or education. Regardless, the NCGS patients appear to be well versed in the GFD (Chapters 6 and 7), showing a good overall level of adherence. These self-taught patients do not always understand the fundamentals to successfully identifying nutrient-dense gluten-free foods, especially high fibre options. This further highlights concerns of nutritional inadequacy. Public health agencies and the food industry must not perpetuate public demand and consumer trends for the GFD without sufficient evidence supporting the existence of NCGS.

9.2 The evidence for non-coeliac gluten sensitivity

Evaluation of exclusion diets has previously shown wheat-induced gut symptoms,[171] but, given wheat is also high in fructans (one of the FODMAPs – another trigger for functional gut symptoms), such evidence for NCGS has been inconclusive. Other studies mostly completed in animal models[188] or uncontrolled clinical trials have found some evidence for the efficacy of a GFD. In these published studies, however, either the patients have had coeliac-associated antibodies or intraepithelial lymphocytosis in the duodenum[146,181] and, therefore, have not been convincingly defined as NCGS. Chapters 6, 7 and 8 presented three well-controlled human dietary trials assessing the effects of gluten on GI symptoms in patients with IBS, in whom a GFD had led to an improvement of symptoms and coeliac disease had been definitively excluded. To characterise NCGS, a wide range of other indices were measured across the three trials, including markers of mucosal inflammation and immunological mechanisms**,** measures of cognitive function, measures of fatigue and lethargy, faecal by-products of protein metabolism, measures of psychological wellbeing and cortisol secretion, in addition to reproducibility of symptom effects.

(i) Study One (Chapter 6): Study One was a randomised, double-blind, placebo-controlled trial of a single dose of gluten (16 g/day for 6 weeks) without a controlled background in parallel groups.

The gluten used was FODMAP-free and the muffins and bread (containing gluten or not) fed to the 34 patients were indistinguishable. The results showed gluten specifically induced GI symptoms and fatigue in patients who believe they had NCGS, and had a rapid onset of symptoms within the first week of intervention. A mechanism was not identified in the crude markers assessed, and these initial findings needed to be reproduced before confirming the existence of NCGS.

(ii) Study Two (Chapter 7): Study Two was designed to be better controlled and was a randomised, double-blind, placebo-controlled, dose-finding, crossover trial (of 16 g, 2 g or 0 g per day for 7 days each) in 37 subjects who believed they had NCGS. The effect on symptoms, cognitive function and more detailed potential mechanism(s) of action were evaluated. Potential confounding changes in the subject's background dietary FODMAP intakes were minimised by education on the low FODMAP diet prior to a run-in period and all food was provided during the interventions. The overall protein levels between the three treatment arms were balanced with whey protein. The results suggested some symptoms might be due to FODMAPs with the significant improvement in GI symptoms and tiredness seen during the low FODMAP-run-in period. There was a lack of gluten-specificity, however, and a significant nocebo response and an order effect were found, highlighting a possible function of psychological mechanisms on anticipatory anxiety. No differences were found for any of the biomarkers measured and dose-dependent effects were unable to be assessed because of the low gluten-specificity.

(iii) Study Three (Chapter 8): All participants were invited to return for a rechallenge, which formed Study Three. Twenty-two participants completed the randomised, double-blind, placebo-controlled, crossover, re-challenge study (of 16g gluten, 16g whey or placebo per day for 3 days each). All food was provided, controlling for FODMAPs, dairy and naturally occurring food chemicals. The symptom reproducibility for gluten and whey was very low and a very high nocebo response and an order effect were again found, regardless of the control of all background dietary triggers. Intriguingly, using the well-validated self-administered STPI questionnaire, gluten was shown to influence current feelings of depression, but had no influence on any other emotional state (anxiety, anger or curiosity) or personality trait (including depression).

9.2.1 Concepts of how gluten might induce symptoms

The findings from this thesis provide the best evidence base to date for understanding the 'gluten-sensitivity' puzzle. Clues to the concepts of how gluten might induce symptoms in individuals without coeliac disease can be derived from these findings:

- *Gastrointestinal symptoms:* While the evidence base for non-gluten components of wheat (i.e., fructans) inducing symptoms in many patients with IBS is strong,[37,51,196,423] Study One found gluten induced greater GI symptoms than placebo in NCGS patients. Study Two and Three showed no gluten-specificity and a lack of reproducibility, respectively. Although all of these participants believed themselves to have NCGS, their symptom response induced by gluten appeared to be a random event. There were no differences found in any biomarker assessed to indicate a potential mechanism. Either these patients do not have NCGS as self-reported or the trial design precluded its recognition. In contrast, the likely role FODMAPs have in inducing GI symptoms in NCGS patients is convincing – many self-perceived NCGS patients still had significant symptoms despite a GFD and there was significant improvement of GI symptoms with the low FODMAP diet for all participants (in Study Two). The physiological effects of FODMAPs are well-understood, including osmotic activity[195] and rapid fermentation[200] by virtue of their poor absorption in the small intestine, both inducing luminal distension and consequently GI symptoms.[196]

- *Extra-intestinal symptoms:* Previous studies of food hypersensitivity and coeliac disease have reported prevalence of extra-intestinal symptoms.[160] Non-IBS symptoms experienced by NCGS participants were reported in the survey and during the dietary trials; these included headache/migraine, musculoskeletal pain, heartburn, mood change, itchiness/rash, forgetfulness. Although these multiple unexplained symptoms were self-attributed to gluten by the patients, there was no specificity of any symptoms to the treatment arms found in the dietary trials. The only exception was the rapid onset of tiredness induced in the gluten group from Study One. Importantly, Study Two and Three could not reproduce this effect, nor could we expand on this finding with the use of objective measures (cognitive function, accelerometer). Therefore, it remains to be proven if and how gluten has direct causal effects on extra-intestinal symptoms in patients without coeliac disease.

- *Mental health:* In patients with functional GI disorders, anxiety and depression are present, particularly as a personality trait[424] and may play a role in the genesis and/or the perception of symptoms. Short-term exposure to gluten specifically induced current feelings of depression with no effect on other indices or on emotional disposition (Study Three). The depressive symptoms demonstrated may lead to participants being more concerned about symptoms and more sensitive in relation to visceral sensation. Such findings might explain the basis for patients 'feeling better' on a GFD despite continuation of GI symptoms. It may also give some insight behind why the effect of gluten on GI (and possibly extra-intestinal) symptoms appears to be random, given there are so many influential variables inducing psychological dysfunction (i.e., biological and psychosocial factors).[78] Other possibilities for how gluten may be related to depression include abnormalities of serotonin production or gluten "exorphins" interfering with the CNS, as previously discussed in Chapter 8 (Section 8.4).

9.2.2 Translational research in action and clinical implications

Without convincing results showing effects on inflammatory or immune markers, NCGS should be regarded as a sub-group of IBS and distinct from coeliac disease. A suggested pathway towards diagnosis of NCGS can be seen in Figure 9.1 and is outlined below:

Step 1: Definitive exclusion of coeliac disease is critical as the first step, which can be 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. Disease investigation prior to removal of gluten is essential to reduce the risks and complications if left untreated. Recruitment of NCGS participants for this research was difficult because most individuals had not been formally tested for coeliac disease. This was further emphasised with over 60% of the survey respondents not having had coeliac disease excluded, despite following a gluten-reduced diet.

Step 2: After testing for coeliac disease, other possible dietary triggers should be investigated, importantly FODMAPs,[195,196,200,216,314] which are found in a wide variety of foods,[217,218] including grains and cereals. The low FODMAP diet is an effective intervention in managing symptoms in the majority of patients with IBS.[216] This research also showed the low FODMAP diet reduced symptoms in patients who believe they have NCGS. At present, there is minimal understanding of how to predict who will respond to the reduction of FODMAPs. Consequently, it is worth initiating and trialling the low FODMAP diet for 6 weeks.[223] Skilled dietetic input, particularly from this step onwards is imperative, which will ensure ample FODMAP education and continued nutrition adequacy.

Step 3: If the patient experiences no or partial improvement in their symptom response to the low FODMAP diet, it is then worth considering gluten. Patients should exclude dietary gluten for 4 weeks, which is assumed to be an adequate length of time based on recommendations of other exclusion diets.[425] Given the subjective nature of presenting symptoms, this research has highlighted the importance of recording symptom severity (using a VAS or similar scale), for the reason that some participants were shown to have poor symptom control during the baseline

periods despite having reported otherwise during their recruitment interview. If a daily symptom diary is too cumbersome, then at the very least a weekly dairy should be employed.

Step 4: Provided there is marked improvement in symptoms with the GFD, blinded challenges (that is, monitored reintroduction of gluten) can be subsequently undertaken. Experience in the studies presented in this thesis raise important points of uncertainty about how this should be done.

- The challenges need to be repeated to offset the strong nocebo effect. A minimum of three separate challenges of 16 g gluten per day for 7 days (or until symptoms are intolerable) is suggested. This number of active challenges and the use of 2 to 3 placebo challenges has previously been recommended particularly in the presentation of subjective symptoms.[426,427] There should be at least a 7-day washout period between each challenge (or until symptoms have resolved). This approach offers an oppportunity to offset the nocebo response, characteristic of the NCGS patient group and prevent consequent false positive results.

- The method of challenging needs to be considered. The two options are to use gluten-containing foods or gluten-filled capsules . At least several large capsules would be required to contain 16 g of gluten, although the amount required for the challenge is uncertain (see below). This itself presents other issues including adherence and what is best to use inside the placebo capsule. For example, if glucose were used, the patient would be able to open the capsule and easily determine the difference by sweetness. The use of another protein (such as whey, as done in the Studies Two and Three) opens uncertainty as to whether the pateint may also react specificly to the placebo. Using food (e.g., 4 slices of commercial wheat bread per day) imposes other confounders known to induce GI symptoms (i.e., fructans) and the difficulty of maintaining blinding (sensory differences between gluten-containing and gluten-free bread). The development of a palatable, inert food product, which contains no known confounder (such as FODMAPs, chemicals or dairy-related proteins), but which is still able to adequately hide 16 g of gluten across the day offers an easy and practical alternative.

- The dose of gluten used in the clallenge is not certain. Attmepts to examine a dose-dependent response failed in Study Three due to the lack of gluten-specific responses to the high (16 g) dose. This amount was chosen on the basis of the recommended challenge in coeliac disease, although this recommendation is more expert- than evidence-based. It does seem reasonable, however, to challenge with a large dose in order to minimise false negative responses.

- Accurate interpretation of reactions to challenges is problematic. Complicated models have been developed to estimate the rate of false responses (reaction to the placebo) and the true proportion of sensitised subjects.[426] Certainly, identifying consistency in the patient's symptom response is a priority, which implies that a subject will always experience sensitivity if they are truly sensitive.

Step 5: Following a postive challenge, the amount of gluten tolerated should be established by systematic re-challenges beginning with small amounts of gluten. A suggested approach may be beginning with challenges of 2 g/d of gluten for 7 days, increasing to 8 g/d and finally to 16 g/d, with adequate washout periods between each. This protocol is based upon the rechallenge schedule that is commonly used with individual FODMAPs after succesful institution of the strict low FODMAP diet.[9]

In the clinical setting, this rigorous 5-step approach is fundamentally complex, time-consuming, laborious and rather expensive for the patient (loss of working hours and direct costs). Unfortunately, a more practical approach will only be possible with the development of biomarkers or other clinical predictors.

As for many patients with IBS, additional therapeutic interventions may need to be instituted to optimise patient management. Management options can be made on the basis of the patient's history of symptoms and personal preferences. For example, one option could target the possible depressive effects of gluten (demonstrated in Study Three). Psychological treatments including dynamic psychotherapy, hypnotherapy, relaxation training, biofeedback, cognitive behaviour therapy and assertiveness training have all been suggested for use in the management of IBS.

Although only some have been empirically evaluated,[416-418,428,429] psychosocial treatments have shown some evidence for reductions in generalised anxiety and depressive symptomatology.[387]

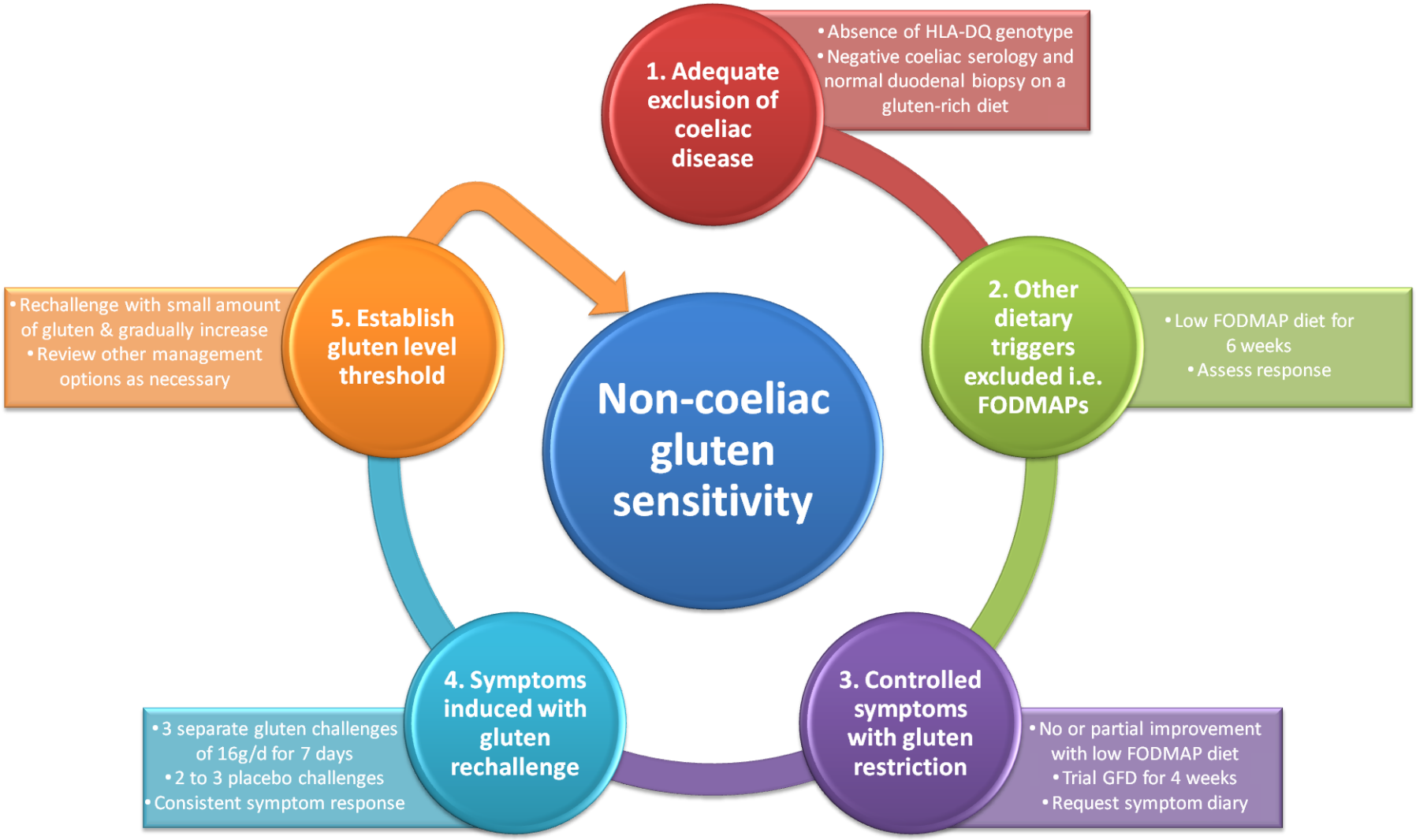

Figure 9.1 Suggested flow chart for defining non-coeliac gluten sensitivity

9.3 Unanswered questions and future research directions

Although this series of studies has provided key evidence into our understanding of NCGS, it has also raised important issues which need to be addressed to allow definition of this entity.

What is the clinical significance of the mental health findings?

The results of gluten influencing current feelings of depression must be reproduced, using both the STPI and additional measures of depression such as the Hospital Anxiety and Depression Scale (HADS)[430] and the IBS-Quality of Life (IBS-QOL).[431,432] Studies must be of longer duration as the gluten-specific effects in Study Three were observed over 3 days and their persistence is not known. Indeed, long-term personality traits would not be expected to change by such a short-term diet. Disease-control populations (i.e., with coeliac disease) should be studied in parallel to compare the magnitude and types of effects. Healthy subjects may also need to be included as normal controls since it is not known what effect gluten specifically has in health on mental function, although it would be anticipated to be minimal. Attempts should also be made to determine if possible pathogenic or biological markers are induced. One example would be measuring anti-ganglioside antibodies, which have been previously detected in coeliac patients with forms of neuropathy[433] and hypothesised to play a role in nerve damage in gluten sensitivity.[434] Until this is achieved, care must be given when discussing these early results, so as not to alarm general consumers from further, unnecessary gluten avoidance.

How to identify NCGS?

Self-reporting is inaccurate and there is likely to be a major difference in perceived versus actual NCGS. Indeed, the survey findings confirmed prior investigations[435-437] finding large discrepancies between perceived and proven food hypersensitivity. A suggested detailed method of identification using repeated blinded placebo-controlled challenges has been outlined above, but the studies described in this thesis clearly indicate that standard single placebo-controlled challenges are not a

valid way of verifying the existence of NCGS, at least in those who already believe they have it. Ideally, identification of biomarker(s) would allow the development of an objective diagnostic test.

What is the prevalence of NCGS?

The patient group recruited in the current studies were highly selected. Much research is still needed to fulfil our understanding of NCGS, importantly the clinical phenotype to allow the accurate prevalence to be defined and understanding whether a broader NCGS group outside of IBS specifically exists. The blinded, placebo-controlled, rechallenge study design is not a methodology that works well with population studies, but is currently best practice. Large population study designs, similar to early prevalence studies of coeliac disease will need to be undertaken. One model could include screening a minimum of 100 IBS patients not previously exposed to gluten-free diets for the diagnosis of NCGS (following a protocol similar to Figure 9.1). The expectation is that nocebo effects on rechallenge in such inexperienced patients would be much less than in the self-perceived NCGS group. This will allow subgroup analysis of patterns of any raised biomarker(s) and common characteristics. This in turn will lead to easier, accurate diagnosis of NCGS patients, permitting better clinical practice and research development.

What is (are) the mechanism(s) of action?

Determining the mechanism is difficult when there is no current way of specifically defining the condition. We have looked at a range of different markers assessing immune reactions (predominantly coeliac-related and adaptive immune pathways), inflammatory responses and poor digestibility of the gluten protein, all of which have found no differences. Future studies must not rule out analysing similar mechanistic concepts, especially at the tissue level. It is of paramount importance that this analysis be undertaken in patients who express positive symptom responses to gluten. If innate immune responses were to be responsible for NCGS, how it induces systemic symptoms requires explanation. A very speculative hypothesis is that gluten may not be directly involved in the triggering of GI symptoms, but rather in the pathogenesis of visceral hypersensitivity. There may be two different effects occurring: first, gluten is sensitising the enteric nervous system especially the mechanoreceptors and, secondly, the poorly absorbed, rapidly

fermentable fructans (and other FODMAPs) are then mainly responsible for inducing GI symptoms. Concomitantly reducing FODMAP intake with the gluten challenges, as done in Studies Two and Three, might have diminished the induction of symptoms specifically in the gluten-challenged group because the genesis of such symptoms are FODMAP-dependent. Analysis of responses of the bowel to distension (by, for example, the rectal barostat) with and without gluten ingestion might provide insight into this hypothesis. We need more sensitive analyses of the enteric nervous system, its receptors and soluble factors such as serotonin levels, and measurement of gluten exorphins released from the partial digestion of the wheat protein gliadin. Defining what differentiates NCGS and gluten-tolerant persons must be fundamental to any future investigations.

Is the gluten-mediated effect all-or-none or a dose-related phenomenon?

This was one of the aims of Study Two, which was unable to be assessed because of the low gluten-specificity in the symptom results. Therefore, this question remains unanswered and is of relevance since a strict GFD may not meet all nutritional requirements and being able to tolerate even just 2 g/day of gluten greatly increases the flexibility of the diet, allowing gluten-derived ingredients (e.g, seasonings, fillers or coatings used in medications or confectionary, wheat starch). This will have implications for the cereal grain industry allowing greater flexibility, better quality and cheaper types of products that may be developed for these individuals. One recommended protocol to establish the lower threshold of gluten that may be tolerated by NCGS individuals is a randomised, double-blind, dose-finding, crossover trial (of 8 g or 2 g or 0 g per day for 7 days each) in NCGS patients.

What part of the gluten is responsible?

Once it has been ascertained that gluten is definitively responsible for the induction of symptoms in individuals without coeliac disease, attention should be given to identifying which component of the gluten is responsible. The complexity and poor solubility of gluten make it a difficult protein to study. Within the gluten complex, there is approximately 46% glutenin, 52% gliadin, 2% albumin/globulin, 2% lipid and 4% ash. Although the most immunodominant T-cell epitope for people with coeliac disease is from α-gliadin,[87] we cannot assume NCGS will be triggered via the

same components that induce coeliac disease. Assessing symptom responses in a randomised placebo-controlled, double-blinded cross-over study of placebo, gluten (16 g/d) and gliadin (8 g/d) for 7 days each in NCGS patients is one suggested protocol. Proteolysis of immunogenic gluten peptides using highly targeted proline- and glutamine-specific endoproteases (glutenases)[21] has been shown to reduce immune responses induced by gluten in patients with coeliac disease.[384] If the effects of NCGS are found to be due to the indigestibility of the gliadin-portion of the gluten protein, then pre-treatment of gluten with glutenases or co-ingestion with an oral glutenase may present an opportunity to lessen symptomatic effects.

What are the concerns raised for the design of dietary trials in IBS?

The conduct of trials for IBS is difficult given this represents a highly heterogeneous population. The gold standard for investigating adverse food reactions remains a double-blind placebo-controlled challenge.[224] In addition to their previously discussed limitations,[438,439] there were several concerns raised from this thesis. The lessons learnt have highlighted the following considerations for conducting future well-designed dietary trials including:

- ***Crossover design*:** The high order effects (found in Study Two and Three) cast doubt on the use of a crossover design. Although there has been previous reserved criticism for the use of a crossover design within the IBS population,[353] they have shown to be used successfully in dietary studies with IBS subjects.[196,200] Longer treatment periods have been suggested as an option to overcome order effects,[351] but the alternative parallel model may be on the whole, better-suited to the NCGS group. Study One produced a positive and specific symptom response to gluten and was indeed a parallel design.
- ***Nocebo effect:*** Consideration should also be given to the large nocebo effects that were common in this NCGS research, but have also been found in other clinical (predominantly drug-based) trials and practice.[440] The self-reported NCGS patients are heterogeneous (e.g., in their range of reported symptoms, clinical histories and characteristics) and are highly suggestible, making a largely difficult patient group to study. For easier interpretation of the data, a larger sample size must be employed. The study design may also have a role in the

genesis of nocebo effects; in a crossover design, patients know they will be certainly receiving the test arm at some point (heighten negative expectations), compared to a parallel design, where the participant has a 50% chance of receiving the treatment or placebo (lessen negative expectations).

- ***Patient selection:*** Adequate patient selection and clear entry criteria must be applied and can only be confirmed with the checking of medical histories and diet/symptom diaries. For example, the beliefs of patients having had coeliac disease ruled out or being adherent to a GFD and their self-judgement of having 'satisfactory symptom control' were not always accurate. An appropriately skilled researcher must thoroughly assess entry criteria and use standard guidelines (for coeliac disease exclusion), validated classifications (for strict GFD adherence) and clear definitions (for 'well-controlled' symptoms i.e., overall symptoms must be less than 20 mm on 100 mm VAS).
- ***Methods of the re-challenge:*** The two main approaches of conducting a food challenge are via foods or capsules. As discussed above, both are associated with their own advantages and disadvantages. Whole foods given in their natural state and administered within the participants' usual eating patterns are ideal. However, supplying individual foods or disguising the test substance in meals or whole diets presents a 'soup' of different constituents that patients may have experienced side effects or prior conditioning to in the past.[441] Using food whilst ensuring each participants' preferences are met seems increasingly difficult, especially given this research also showed self-perceived NCGS patients often report multiple intolerances additional to gluten sensitivity. Indeed, a retrospective cohort study found two distinct groups of wheat-sensitivity; one with wheat-sensitivity alone and another with subjects intolerant to wheat plus many other foods.[442] Perhaps a more convenient method is the use of capsules, given capsules containing the suspected agent are indistinguishable from those containing the placebo and can be administered directly to the patient without any background changes. However, limitations of food capsules include escaping normal salivary enzyme action, thereby exposing the GI tract to less degraded food constituents,[443] and capsules being more resistant to digestion, resulting in delayed absorption.[444] Other disadvantages previously

mentioned include the difficulty of having to use large or multiple capsules needed to administer 16 g of gluten, and the undeniable fact that people do open capsules.

- ***The nature of the placebo:*** The use of a placebo control has always been recommended in IBS trials.[445] However, careful attention must be given to the properties of what is used as the placebo. The placebo used in Study One and Study Three did not contain any additional substance, where as in Study Two the placebo contained a whey protein and induced symptoms in the majority of participants. Although the effects of whey could not be reproduced in Study Three, it raises the question of whether the need for controlling overall dietary protein levels is necessary, especially in short term studies (of less than 7 days). An option may be glucose (a very benign substance), which has been successfully used in past IBS dietary studies, where less than 20% patients responded to this placebo arm,[196,446] however it has produced mixed results in others.[447]
- ***Successful blinding:*** To successfully mask the test substance and maintain blinding, the setting and style of food offered, including the size and portions of meals should be considered. For example in Study One, the muffins and bread from each arm were indistinguishable, as the gluten used had lost its baking functionality. Tasting for sensory testing and proper masking of foods prior to the research study was also undertaken in all studies. The test foods (treatment versus placebo) should be matched identically for taste, texture, appearance and overall nutrition. Participants are likely to be influenced by even the subtlest of differences in study foods; therefore successful masking requires expert cooking advice or experience to enable adaptation or development in recipes. Also, the success of blinding should always be reported.
- ***Control of confounding dietary factors:*** It is important to measure, control and understand a participant's usual intake, including any pre-conceptions of perceived intolerances (as discussed above). Controlling for background noise is essential. Given the intricacies of following a low FODMAP diet, controlling for background FODMAP levels is best done by providing subjects with all of their meals. However, drastically changing the individual's background diet is likely to have other consequences. For example, if also controlling for food

chemicals, complete exclusion is likely to result in withdrawal reactions.[448] A run-in period for at least two weeks is necessary to allow for these withdrawals and also for clearing of residual symptoms.

- ***Endpoints:*** As there are no objective markers for the improvement of IBS, arbitrary rating scales are commonly used,[365] but these must be well-defined particularly when interpreting results. Although there have been IBS severity scoring systems developed (for mild IBS, moderate IBS, severe IBS),[449] there is no recommended tool used in the clinical or research setting for defining cut off points in the determination of a positive or negative response. This research defined a clinical significant change of symptoms as a change of at least 20 mm on the 100 mm VAS from baseline or run-in. It remains unknown whether this cut off should be increased.

Consequently, the most appropriate study design for future analysis of patients who believe they have NCGS may be a randomised, double-blind, placebo-controlled trial in parallel groups. Controlling for background intake of FODMAPs, shown to play a key role in symptom induction in this group, should be undertaken with care. It may not be necessary, provided changes in the intake of FODMAPs are monitored in some way during the baseline and challenge periods. The provision of food should be well received by the participant and any requirements of the participant in the study protocol should be as straightforward and undemanding as possible. Consistently reproducing the symptom effects induced by gluten must be critical to future studies aiming to confirm the existence of NCGS.

It is progressively difficult to overlook the increasing number of people without coeliac disease claiming to be gluten sensitive, not only across Australia but worldwide. This group is largely ambiguous because of the minimal scientific evidence and remains undefined. High quality, well-controlled research in human trials is difficult to carry out and is fraught with its own hurdles (as discussed above). This potential 'NCGS' entity has become a quandary, as patients are powerfully influenced by alternative practitioners, Internet websites and mass media who all proclaim the benefits of avoiding gluten- and wheat-containing foods. Moreover, the food industry has jumped

aboard the public demand, with an explosion of new products in the gluten-free market. Self-diagnosed NCGS patients indisputably report to feel better, which is not surprising given reducing gluten undoubtedly results in the reduction of most, if not all, processed foods, replacing their intake with fresh fruit and vegetables, concomitant reduction in FODMAPs.

The issue of why there is a need to interfere with such people who feel so well must be addressed. The question of why we shouldn't just 'leave them be' is important. What is wrong with being gluten-free? From a medical perspective, failing to diagnose coeliac disease in an individual is hypothetically a potentially big problem. Coeliac disease effectively requires total gluten abstinence to ensure the best outcomes. It is associated with potentially serious risks and complications, such as an increased risk of cancer, osteoporosis and other autoimmune diseases.[92] Some patients with NCGS limit gluten intake rather than remain gluten-free. This practice may have no implications for NCGS except more symptoms, but it can feed tissue injury in coeliac disease and, subsequently, risk of complications. On a practical level, following a GFD is more expensive than a gluten-containing diet[254] and may be more difficult to achieve nutritionally adequacy.[259-261] On a social level, patients following a GFD may feel they are a burden on their family and friends, and the restrictions imposed when eating away from home can lead to social isolation or at least inhibition.[252,253] These issues do argue strongly in favour for the use of the GFD when it is truly needed, rather than for inappropriate scenarios. However, proof of the inappropriateness of GFD in many situations, including IBS awaits strong scientific evidence to negate the emotive arguments.

This research has produced some evidence that NCGS may exist, but probably only in a small number of people. 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. Case finding may be a worthwhile option to pursue our understanding of the phenotype and mechanisms by which gluten can induce symptoms in these patients. With estimates

that NCGS affects one in 15 people, understanding the 'gluten-sensitivity' puzzle is undeniably important for the health of the global community.

9.4 Conclusions

The current thesis has made a significant contribution to our understanding of the 'wheat- and gluten-intolerant' phenomena. Specifically, our understanding about which components of wheat (gluten protein and/or poorly absorbed short-chain carbohydrates) that are most likely responsible for inducing GI symptoms in individuals who do not have coeliac disease has advanced greatly.

The practice of initiation of a GFD without adequate exclusion of coeliac disease appears to be commonplace. Carbohydrate analysis of commonly consumed grains, cereal products and pulses has expanded our understanding about natural food sources of prebiotics (FOS, GOS) and further developed the FODMAP composition tables. This new knowledge has improved our low FODMAP dietary approach for the management of GI symptoms associated with IBS.

One in four individuals who believe they have NCGS still report uncontrolled symptoms despite appearing well versed in the GFD. Given the high frequency of co-existence of gluten and high-FODMAP content in common cereal and grain products, it is likely the majority of people feel some symptom improvement because their 'gluten restriction' automatically reduces their dietary FODMAP intake.

A series of three definitive experiments where the effect of gluten, free from contamination from carbohydrates, was evaluated in patients with IBS where coeliac disease had been definitively excluded and who had reported benefit on a GFD. Study One showed that gluten can trigger GI symptoms and tiredness in parallel groups and without a controlled background. Study Two, using a crossover design showed no evidence of specific or dose-dependent effects of gluten, but FODMAP restriction uniformly reduced residual symptoms. In Study Three, a gluten rechallenge showed poor reproducibility of symptom induction to a specific protein. Gluten did, however, influence current feelings of depression, but had no influence on depression as a personality trait.

Study Two and Three showed a very high nocebo response regardless of all background dietary triggers being controlled. There was no evidence for an underlying mechanism in NCGS.

Either the patients do not have NCGS as self-reported or the trial design precluded its recognition because of a high nocebo effect. However, the low FODMAP diet offers a convincing and efficacious approach to improve residual IBS symptoms in patients. Biomarkers or other clinical predictors must be established to definitively characterise NCGS. How common NCGS is, how it can be reliably identified and what its underlying mechanisms are, warrant further evaluation. There is also a need for standardised guidelines into the design and conduct of IBS dietary studies.

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List of Appendices

Appendix 1. *Food description and sampling details for Quantification of FODMAPs: Chapter 4*

Food	Description	Sampling details
Grains and pasta		
Couscous	Cream coloured pellets, made from approximately 2 parts semolina, 1 part wheat flour and salt and water, then steamed and dried. Subsequently cooked by soaking in boiling water, without addition of fat, oil or salt.	Composite sample of purchases – Bionature Couscous, San Remo Couscous, Divella Couscous
Noodles, rice stick	Noodles made from a paste of water and ground rice only, coiled into blocks and dried before packaging. They are then boiled in unsalted tap water and drained.	Composite sample of purchases – Trident Rice Stick Noodles
Pasta, gluten free	Dried flour based product made from maize. Cooked, by boiling.	Composite sample of purchases – Orgran Rice And Corn Spirals, Orgran Vegetable Rice Pasta, San Remo Pasta Penne Gluten Free
Pasta, gnocchi	Commercially prepared dumplings made from potato and wheat flour, simmered briefly in boiling water.	Composite sample of purchases – You'll Love Coles Potato Gnocchi, Golden Pasta Premium Gnocchi, Ciccarese Gnocchi Con Patate
Pasta, wheat	The dough is made by combining wheat flour or durum semolina with water. Cooked in boiling water, without added salt.	Composite sample of purchases – Zafarelli Spaghetti, San Remo Instant Spaghetti,
Pasta, quinoa	The dough is made by combining quinoa flour with water. Cooked in boiling water, without added salt.	Composite sample of purchases – Olive Green Quinoa And Rice Penne, Orgran Multigrain Pasta With Quinoa
Rice, brown	Rice grain with only the inedible outer husk removed. Cooked by absorption in boiling, unsalted water.	Composite sample of purchases – Sun Rice Medium Grain Rice
Rice, white	A grain that has had its husk, bran and germ removed. Cooked by absorption in boiling, unsalted water.	Composite sample of purchases – Hoyts Aromatic Long Grain Basmati, Riviana White Long Grain Rice
Breads		
Gluten free	Commercially produced bread made predominantly from rice/corn or tapioca flour, with added milk, egg, yeast.	Composite sample of purchases – Country Life Gluten Free White
Rye	Commercially prepared bread made from rye flour, or including a large proportion of rye flour combined with wheat flour.	Composite sample of purchases – Country Life Rye
Rye, dark	Commercially prepared bread made from a large proportion of rye flour.	Composite sample of purchases – Country Life Dark Rye
Rye, Sourdough, light	Commercially prepared bread made from sour dough	Composite sample of purchases – Flinders Bread Light Rye

	process using light rye flour.	Sourdough
Spelt, 100% spelt flour	Commercially prepared bread made from a large proportion of spelt flour.	Composite sample of purchases – Ancient Grains Rustic Loaf Spelt Bread
Spelt, 25% spelt flour	Commercially prepared bread made from a proportion of spelt flour.	Composite sample of purchases – Country Life Wholegrain Spelt Bread
Wheat, Multigrain	Commercially prepared bread made from white flour with kibbled grains added to the mix.	Composite sample of purchases – Noble Rise Multigrain, Coles Bread Multigrain Sliced, Coles Smart Buy Multigrain Bread, Flinders Cobb Multigrain, Helga's Continental Bakehouse Mixed Grain Bread, Mighty Soft Bread Sandwich Multigrain, Tip Top Sunblest Bread Multigrain Sandwich
Wheat, White	Commercially prepared bread made from white, wheaten bread-making flour and other permitted ingredients.	Composite sample of purchases – Coles White 6 Vitamins & Minerals, Buttercup Country Split White, Noble Rise White, Buttercup Country Split White Bread, Coles Smart Buy White Toast Bread, Helga's Bread Traditional White, Mighty Soft Bread Sandwich White, Tip Top Sunblest Bread White Thick, Wonder White Bread +7 Vitamins & Minerals Sandwich
Wheat, Wholegrain	Commercially prepared bread made from whole meal and rye flour with kibbled grains added to the mix.	Composite sample of purchases – Helga's Wholemeal Grain, Noble Rise Wholegrain, Tip Top 9 Grain Wholegrain
Wheat, Wholemeal	Commercially prepared bread made from flour containing all the milled constituents of the wheat grain.	Composite sample of purchases – Sunblest wholemeal, Coles Smart Buy Wholemeal Bread, Noble Rise wholemeal, Coles Bread Wholemeal Sliced, Helga's Bread Traditional Wholemeal, Mighty Soft Bread Sandwich Wholemeal, Tip Top Sunblest Bread Wholemeal Sandwich, Wonder Wholemeal + Iron Bread, Buttercup Country Split Wholemeal Bread
Breakfast cereals		
All-Bran®	Wheat bran pellets made from wheat bran.	Composite sample of purchases – Kellogg's All Bran
Corn flakes	Breakfast cereal made from flakes of corn.	Composite sample of purchases – Coles Smart Buy Corn flakes, Kellogg's Corn flakes
Muesli	Breakfast cereal made from rolled oats, dried fruit and other ingredients including wheat bran, wheat germ, sugar, nuts and/or seeds.	Composite sample of purchases – Lowan Whole Foods Original Harvest Muesli, Carman's Classic Fruit Muesli
Muesli, gluten free	Breakfast cereal formulated using puffed rolled rice, nuts and seeds (linseed, sunflower and almonds), dried fruits, coconut and psyllium.	Composite sample of purchases – Freedom Foods Gluten Free Muesli
Muesli, yeast free	Breakfast cereal formulated using wheat free wholegrain cereals, coconut, seeds and nuts (sunflower, linseed, sesame and almonds).	Composite sample of purchases – Freedom Foods Yeast Free Muesli

Oats, dry	Crushed grain produced by steaming and flattening the oats with rollers. No further cooking. Includes traditional and quick cooking styles.	Composite sample of purchases – Uncle Toby's Quick Oats, Coles Quick Oats, Lowan Whole Foods Quick Oats
Ready-to-eat mixed grain flakes with fruit	Breakfast cereal consisting of flakes of wheat, corn, rice and oats with added dried fruit and nuts.	Composite sample of purchases – Kellogg's Sustain
Rice Bubbles®	Breakfast cereal made from toasted puffed or popped rice grains.	Composite sample of purchases – Coles Rice Puffs, Kellogg's Rice Bubbles
Wholegrain wheat biscuit (Weetbix®)	Breakfast cereal prepared from whole wheat, with added sugar and formed into a biscuit shape.	Composite sample of purchases – Sanatarium Weetbix
Biscuits and snacks		
Biscuit, chocolate chip	Commercially prepared sweet biscuits embedded with chocolate chips	Composite sample of purchases – Paradise Cottage Cookies Chocolate Indulgence, Coles Choc Chip Cookies Premium Quality, Arnott's Premier Chocolate Chip Cookies, Coles Smart Buy Chocolate Chip Cookies
Biscuit, cream filled, chocolate coating	Commercially prepared sweet biscuit, generally chocolate flavoured, sandwiched with sweetened creamy filling and coated in chocolate	Composite sample of purchases – Arnotts Tim Tam, Coles Chocolate Surrenders, Coles Chocolate Mint Supremes, Arnotts Mint Slice
Biscuit, fruit filled	Commercially prepared biscuit filled with dried vine fruit mix	Composite sample of purchases – Arnotts Snack Right Fruit Pillows Wildberry, Arnotts Snack Right Fruit Pillows Apple & Sultana, Arnotts Full O' Fruit
Biscuit, savoury, plain	Commercially prepared, small, savoury biscuits made from white wheat flour and added fat sprinkled with salt	Composite sample of purchases – Arnott's Salada, Kraft Premium Original Crispbread
Biscuit, savoury, wholemeal	Square biscuits made from wholemeal wheat flour, with added fat, sprinkled with salt, commercially prepared.	Composite sample of purchases – Arnott's Wholemeal Salada, Kraft Premium Wholemeal Crispbread
Biscuit, savoury, rye crispbread	A thin, flat rectangle of dry bread made predominantly from rye flour, including lite varieties Commercially prepared; some brands may include a proportion of wheat flour.	Composite sample of purchases – Ryvita Wholegrain Rye Crispbread, Arnotts Rye Cruskits
Biscuit, shortbread	Shortbread-style biscuits, commercially prepared, made using butter. Other ingredients may include wheat flour, sugar and rice flour	Composite sample of purchases – Coles Smartbuy Scoth Finger, Arnotts Scotch Finger, Paradise Butterscotch Shortbread, Walker's Shortbread
Biscuit, sweet, plain	Commercially prepared, plain sweet biscuits made without added flavourings, chocolate, fruit or nuts.	Composite sample of purchases - Arnotts Milk Arrowroot, Arnotts Milk Coffee, Arnotts Nice, Arnotts Teddy Bear, Coles Smartbuy Milk Coffee, Coles Smartbuy Nice, Coles Smartbuy Milk

		Arrowroot
Chips, potato, plain	Snack product that consists of thinly sliced potato, deep fried in vegetable oil, salted & packaged.	Composite sample of purchases - Smiths Thinly Cut, Original, Smiths Crinkle Cut Original, Arnotts Thins Original, Coles Thin Sliced Original, Coles Crinkle Cut Plain
Corn thins, plain	Commercially prepared, round savoury dry cake, approximately 1 cm thick, made from puffed corn. Unsalted	Composite sample of purchases - Real Foods Original Corn Thins
Corn thins, flavoured (sour cream & chives)	Commercially prepared, round savoury dry cake, approximately 1 cm thick, made from puffed corn, flavoured	Composite sample of purchases - Real Foods Sour Cream and Chives Flavoured Corn Thins
Muesli bar, plain with dried fruit	Snack style product whose major ingredients include rolled oats, sugars and vegetable oils, and generally have added dried fruits (typically apricot, sultana and apple).	Composite sample of purchases - Uncle Toby's Body Wise Berry Fusion, You'll Love Coles Oven Baked Fruit bars, Carmans Classic Fruit Muesli Bars, Be Natural Trail Bars 5 Wholegrain Cranberry
Rice cakes, plain	Commercially prepared, round savoury dry cake, approximately 1 cm thick, made from brown puffed rice. Unsalted.	Composite sample of purchases - SunRice Thin Rice Cake
Rice cakes, flavoured (sour cream & chives)	Commercially prepared, round savoury dry cake, approximately 1 cm thick, made from brown puffed rice, flavoured	Composite sample of purchases - SunRice Thin Flavoured Rice Cake Sour Cream and Chives
Pretzels	A baked snack, usually made from wheat flour with yeast. Lightly salted before baking. Includes flavoured varieties. Sold packaged.	Composite sample of purchases - Parkers Lightly Baked Mini Pretzels, Parkers Baked Wheat Pretzel Twists
Pulses		
Beans, mixed, canned	Three or four bean mix comprising red kidney, lima and butter beans and chickpeas. Canned in sweetened brine.	Composite sample of purchases - You'll Love Coles Four Bean Mix , Edgell Four Bean Mix
Borlotti beans, canned	Borlotti beans (also known as roman beans or romano beans) boiled and canned in brine.	Composite sample of purchases – You'll Love Coles Borlotti beans, Annalisa Borlotti beans, Bionature Borlotti beans
Butter beans, canned	Boiled and canned butter beans in brine.	Composite sample of purchases – You'll Love Coles Butter beans, Edgell Butter beans
Chickpeas, canned	Boiled and canned chickpea in brine.	Composite sample of purchases – Woolworths Select Chickpeas, Bionature Organic Chickpeas, Annalisa Chickpeas
Haricot beans, boiled	Dried haricot beans soaked overnight, boiled in unsalted water, and drained.	Composite sample of purchases – Goldfish Haricot beans, McKenzies Haricot beans
Red kidney beans, boiled	Dried kidney beans, soaked overnight, boiled in unsalted water, and drained.	Composite sample of purchases – McKenzie's Red kidney beans, Select Naturals Red kidney beans

Lentils, green, boiled	Dried green lentils soaked, boiled in unsalted water and drained.	Composite sample of purchases – Colonial Fruit Co Green lentils, McKenzies Green lentils
Lentils, red, boiled	Dried red lentils soaked, boiled in unsalted water and drained.	Composite sample of purchases – McKenzies Red lentils
Lentils, canned	Boiled and canned lentils in brine.	Composite sample of purchases – Annalisa Lentils, La Nova Lentils, Edgell Lentils
Lima beans, boiled	Dried lima beans soaked, boiled in unsalted water and drained.	Composite sample of purchases – McKenzies Lima beans, Select naturals Lima beans, Just Deli Lima beans
LSA (Linseed Sunflower Almond mix)	Linseed-Sunflower-Almond mix is a formulated seed-meal supplement	Composite sample of purchases – Nu-Vit LSA Mix, Healthy Life LSA Blend
Soya beans, boiled	Dried soya beans soaked, boiled in unsalted water, drained.	Composite sample of purchases – Goldfish Soya beans, Select Naturals Soya beans
Split peas, boiled	Dried split peas (yellow and green) soaked, boiled in unsalted water, drained.	Composite sample of purchases – McKenzies Yellow Split Peas, Maharajah's Choice Yellow Split Peas, McKenzies Green Split Peas

Appendix 2. *Participant gluten-free diet perception questionnaires: Study One and Study Two*

Please complete the following three pages:

---

1. Subjective Questionnaire

This questionnaire gives a subjective assessment of your gluten intake.
Please indicate which best describes your approach to being on a gluten-free diet.

☐: I do not follow a gluten-free diet, and frequently eat/drink an intake more than once a day

☐: I follow a gluten-free diet most times, but eat/drink a small intake up to once per day

☐: I follow a gluten-free diet most times, but eat/drink a small intake a few times per week

☐: I follow a gluten-free diet most times, but eat/drink a small intake up to a few times per week

☐: I follow a gluten-free diet most times, but eat/drink a small intake up to once per fortnight

☐: I follow a gluten-free diet, in that I never deliberately eat gluten, but maybe have accidents (no more than once per fortnight)

☐: I follow a gluten-free diet totally

2. Compliance Questionnaire

	Yes	No
Do you believe you follow a gluten free diet?	☐	☐
Do you knowingly eat gluten?	☐	☐

If yes, how frequently do you eat foods containing gluten?

Daily	☐	Weekly	☐
Monthly	☐	Every 3-4 months	☐
Two or three times per year	☐	Once per year	☐
Less than once per year	☐		

What do you typically eat when you "break the diet"?_____

Why do you "break the diet"?_____

Do you ask about how foods are made when prepared by your friends or family?

☐ ☐

How often do others prepare meals for you? _____

Do you believe they understand your gluten free requirements? Y / N

Do you check for gluten in a dish before ordering it at a restaurant or take-away?

☐ ☐

How often do you eat at a restaurant or takeaway?_____

Do you have accidental gluten intake? ☐ ☐

How often would you estimate this occurs? _____

When would you say this most often occurs? _____

If you are uncertain if a food is gluten free, but suspect it is probably safe, would you eat it? ☐ ☐

Do you check for crumbs (from regular wheat bread or biscuits) and avoid them in butter/margarine, jam and other spreads, dips, etc? ☐ ☐

3. Knowledge Questionnaire

Are the following gluten-free?	Yes	No
Milo	☐	☐
Soy Sauce	☐	☐
Thickened Cream	☐	☐
Kellogg's cornflakes	☐	☐
Maize cornflour	☐	☐
Tamari soy sauce	☐	☐
Grated tasty cheese	☐	☐
Plain chocolate	☐	☐
Hot chips from a Fish and Chip shop	☐	☐
"Roast of the Day" at a restaurant, covered with gravy, but you scrape away as much of the gravy as you can.	☐	☐
Hamburger (100% beef pattie, lettuce, tomato, egg, onion, tomato sauce, wheat bun), but you remove the bun	☐	☐
A stuffed roast chicken, but stuffing and skin has been removed	☐	☐
Oven Fries: (Ingredients): Potato, Canola Oil, Dextrose (from wheat), salt.	☐	☐
Passionfruit Sauce (Ingredients): Sugar, Passionfruit Pulp, Passionfruit Juice, Water, Thickener (1442), Food Acid (330), Salt.	☐	☐
Salt and vinegar chips (Ingredients): Potato, Vegetable oil, wheat starch, vinegar, salt.	☐	☐
Soft Jellies (ingredients): cane sugar, glucose syrup from wheat, gelatine, fruit juice concentrate.	☐	☐
Low Fat vanilla icecream (ingredients): water, skim milk concentrate, sugar, maltodextrin, cream, egg yolk, vanilla essence.	☐	☐

Thank you. Please also take the time to complete the following:

1. Have you seen a dietitian for explanation of the gluten free diet?	☐	☐
2. Are you a member of the coeliac society?	☐	☐

3. Where have you obtained information about the gluten free diet? Please tick all relevant boxes

☐ internet ☐ friend/family ☐ dietitian ☐ coeliac society
☐ books ☐ magazines ☐ information from doctor ☐ other_____

Appendix 3. *Food diary sheets: Study One, Study Two and Study Three*

FOOD RECORD – DAY 1 – DATE: ___/___/2010

Time	Name of Food or Drink	Amount Consumed	Brand and cooking method
Before Breakfast			
Breakfast			
Morning Tea			
Lunch			
Afternoon Tea			
Before Dinner			
Dinner			
Supper			

RECIPE DETAILS – DAY 1

Name of Recipe: **Number of serves:**

Name of Ingredient	Brand or Cooking Method	Amount Used

Comments:

_____
_____
_____
_____

Appendix 4. *Meal plan for the low FODMAP, gluten-free diet: Study Two*

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
BREAKFAST						
French toast Berry sauce	Muesli with lactose free yoghurt Fruit (banana)	Corn flakes with lactose free milk Toast with topping (vege spread) Fruit (banana)	French toast Berry sauce	Muesli with lactose free yoghurt Fruit (banana)	Corn flakes with lactose free milk Toast topping (vege spread) Fruit (cantaloupe)	Pancakes with topping (lemon & sugar) Fruit (strawberries)
LUNCH						
Spinach salmon roulade & salad	Sausage roll & salad	Corn fritters with quinoa tabouli	Minestrone	Capsicum dip with gluten free bread (toasted or as sandwich with rocket leaves)	Mini pizza	Quiche & salad
DINNER						
Ratatouille with gluten free pasta	Chicken risotto	Shepherd's pie & vegetables (steamed carrots, beans)	Soy and ginger fish with vegetables	Chicken stirfry with rice	Vegetable pastie & vegetables (steamed carrots, beans)	Braised lamb shanks with vegetables & gravy
SNACKS (MORNING TEA, AFTERNOON TEA, SUPPER)						
Chocolate fudge brownie Carrot and celery sticks Fruit (orange)	Chocolate royal Rice crackers Fruit (cantaloupe)	Berry crumble Buckwheat crispbread with tomato and cheddar cheese Fruit (grapes)	Carrot cake Corn cruskits with cheddar cheese Fruit (pineapple)	Lemon curd tart Rice crackers Fruit (grapes)	Chocolate royal Buckwheat crispbread with tomato and cheddar cheese Fruit (orange)	Bread & Butter Pudding Corn cruskits with cheddar cheese Fruit (pineapple)

Appendix 5. *Meal plan for the low FODMAP, gluten-free, dairy-free, low chemical diet: Study Three*

Day 1	**Day 2**	**Day 3**
BREAKFAST		
Pumpkin muffin	Toast	Waffles
LUNCH		
Sandwich (e.g. with chicken and salad)	Vegetable noodle soup with bread	Sandwich (e.g. with beef and salad)
DINNER		
Shepherd's pie	Thai fish cakes (with salad)	Roast chicken
SNACKS (MORNING TEA, AFTERNOON TEA, SUPPER)		
Rhubarb crumble Rice cakes	Shortbread Rice cakes	Carrot cake Rice cakes

Appendix 6. *FoodWorks analysis of the gluten free, low FODMAP diet: Study Two*

	Average/Day
Weight (g)	1769.63
Energy [333]	8004.36
Protein (g)	78.94
Total fat (g)	74.28
Saturated fat (g)	30.53
Polyunsaturated fat (g)	6.87
Monounsaturated fat (g)	28.24
Cholesterol (mg)	393.79
Carbohydrate-available (g)	223.55
Sugars (g)	100.75
Starch (g)	135.17
Water (g)	1313.16
Alcohol (g)	0
Dietary fibre (g)	27.01
Sodium (mg)	7043.47
Fat as mono (%)	41.55%
Fat as poly (%)	10.87%
Fat as saturated (%)	47.59%

Appendix 7. *FoodWorks analysis of the gluten free, low FODMAP, low chemical, dairy free diet: Study Three*

	Average/Day
Weight (g)	1384.78
Energy [333]	8406.76
Protein (g)	64.96
Total fat (g)	68.11
Saturated fat (g)	14.04
Polyunsaturated fat (g)	22.79
Monounsaturated fat (g)	24.73
Cholesterol (mg)	443.54
Carbohydrate-available (g)	277.12
Sugars (g)	78.07
Starch (g)	199.2
Water (g)	912.67
Alcohol (g)	0
Dietary fibre (g)	20.63
Sodium (mg)	4439.92
Fat as mono (%)	40.18%
Fat as poly (%)	37.01%
Fat as saturated (%)	22.81%

Appendix 8. *Visual Analogue Scale: Gastrointestinal symptom questionnaire: Study One, Study Two and Study Three*

EXAMPLE GASTROINTESTINAL SYMPTOM QUESTIONNAIRE

Please fill out this questionnaire EVERY evening

Over the day, please rate how your abdominal symptoms were:
Please draw a vertical line where you would rate your symptoms

Overall gastrointestinal symptoms:

Excellent, none at all — Really awful, the worst it has been

Abdominal pain/discomfort:

Excellent, none at all — Really awful, the worst it has been

Abdominal bloating/distention:

Excellent, none at all — Really awful, the worst it has been

Passage of wind: (ie. *flatulence*)

Excellent, none at all — Really awful, the worst it has been

Satisfaction with stool consistency:

Excellent, none at all — Really awful, the worst it has been

Tiredness and lethargy:

Excellent, none at all — Really awful, the worst it has been

Nausea:

Excellent, none at all — Really awful, the worst it has been

FEMALES: Did you have your period this day? YES NO

Appendix 9. *Accelerometer log book: Study Two*

Accelerometer Log Book:

INSTRUCTIONS:

1. Wear the Accelerometer firmly around your trunk on the right hip. It should be snug against your body but should not cause any discomfort
2. Wear the Accelerometer for seven days continuously (including five weekdays and two weekend days) - do not wear the Accelerometer during showering, bathing or water sports and you may remove the monitor during contact sports for safety concerns (you decide when to remove the monitor)
3. During any non-wearing period, provide details on your activity duration, type and intensity in the table below
4. Provide the 'sleep time' and 'awake time' in the table below. Record the time you **begin trying to fall asleep**, for example if you were to go to bed and read or watch TV, record the time stopped reading/watching TV and decided to actually go to sleep as your sleep time

DATE: ***Eg DAY 1 - MON*** *__ __ / __ __ / 09*	*SLEEP TIME:* ***Eg 10:30PM***	*AWAKE TIME:* ***Eg 10:30AM***	*Activity undertaken whilst not wearing the Actigraph:* ***Include duration, type, intensity***
DAY 1 - __ __ / __ __ / 09			
DAY 2 - __ __ / __ __ / 09			
DAY 3 - __ __ / __ __ / 09			
DAY 4 - __ __ / __ __ / 09			
DAY 5 - __ __ / __ __ / 09			
DAY 6 - __ __ / __ __ / 09			
DAY 7 - __ __ / __ __ / 09			

Appendix 10. *Saliva collection instruction sheet: Study Three*

SALIVA COLLECTION – DAY 1

Please follow the following instructions when providing a saliva sample for the determination of salivary cortisol.

1. MORNING:

The time that you wake up on the days that you are required to give your saliva sample is very important.

- YOU MUST wake up at the same time each day that you give your saliva sample i.e. if you wake up at 7.30am on the day that you give your first saliva sample (baseline sample) you must also wake up at 7.30am on the other days that you give a saliva samples – these will be day 3 of treatments A, B and C..

2. EVENING of Day 1:

- Dinner should be consumed by NO LATER THAN 7.00 PM
- Rinse mouth with water at 8.20PM
- Saliva collection should be performed at 8.30 PM SHARP

INSTRUCTIONS FOR SALIVA COLLECTION

1. Remove the swab from the suspended insert.

2. Gently chew on the swab for 1 minute – or until you feel that you can no longer prevent yourself from swallowing the saliva produced.

3. Replace the swab in the suspended insert (do not use your hands).

4. Use the stopper to firmly close the Salivette.

5. Please place the Salivette into your freezer IMMEDIATELY.

6. The Salivette will be collected at the end of the dietary challenge.

Appendix 11. Survey questionnaire: Chapter 5

Please take the time to complete the following important information to better understand gluten intolerance in people without coeliac disease.

Personal Details

Date: **Name:**

Gender: **Date of birth:**

Phone: **Email:**

Address:

Symptom Details

Describe your main symptoms?

Do you currently feel in control of your symptoms?

Have you had a hydrogen breath test? If yes, what were the results (please circle)?

Fructose: pos/neg **Lactose: positive/negative**

Diet Details

Do you follow a strict gluten free diet?

How long have you been following a gluten free diet?

Where did you find out about a gluten free diet (eg: family, dietitian, internet)?

When was the last time you consumed gluten?

Coeliac Disease Investigation

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?

If yes, 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?

When did you have the gastroscopy done?

Study interest

How did you find out about this study? E.g You saw an ad where?

Are you interested in participating in this research trial looking at the effects of gluten in people without coeliac disease based at Box Hill Hospital? (Include reasons for not wanting and/or being able to participate e.g travel)

Thank you.