The Gut-Bone Axis in Coeliac Disease

A thesis presented in partial fulfilment of the
requirements for the degree of

Doctoral of Philosophy

in

Nutritional Science

at Massey University, Manawatū,
New Zealand.

Katie Elizabeth Coad Pedley Schraders

2022

DEDICATION

To my parents, who inspired me to start this

and

to my husband, for giving me the motivation I needed to get it finished

ABSTRACT

Coeliac Disease (CD) is a lifelong autoimmune disease, highly prevalent in New Zealand, triggered by ingestion of gluten in genetically susceptible individuals. It leads to villous atrophy and nutrient malabsorption which often compromises bone mineral density (BMD). Although a gluten-free diet (GFD) usually improves symptoms, BMD is often not fully resolved. Persistent low BMD may be due to ongoing gut inflammation and malabsorption, or the uncoupling of bone formation and resorption. Previous research indicates that individuals with CD and low BMD may have altered bone signalling pathways, particularly in the expression of receptor activator of NF-κβ ligand (RANKL) and osteoprotegerin.

RANKL is implicated in both the initiation of the immune response and persistent low BMD because it has a role in bone resorption, via the differentiation of osteoclasts, and a possible role in translocating gluten across the gut, via the expression of microfold cells.

The objective of this PhD was to examine underlying mechanisms underpinning low BMD in individuals with CD using a small intestinal organoid model which allows for investigation of otherwise inaccessible gut cells and signalling pathways implicated in the gut-bone axis. A further study, *Close to the Bone,* investigated BMD in premenopausal women with CD compared to healthy controls. A third online *A Gut Feeling* study investigated dietary advice that individuals consuming a GFD in New Zealand receive, focusing on bone health.

Although the organoid research was interrupted due to the SARS-CoV-2 pandemic, the model was established using murine tissue and future research opportunities were identified. The results of the *Close to the Bone* study demonstrated no differences in BMD between the coeliac group and healthy controls but identified further research into bone density in people with CD in New Zealand was warranted. This study raised concerns about iodine intake

and status in people with CD. The *A Gut Feeling* study found inconsistencies in advice given to individuals diagnosed with CD.

The research identified that the organoid model offers potential for future study of the gut-bone axis and that individuals with CD are at risk of nutritional deficiencies but often are not advised well or referred for a bone-scan.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

4DDD	4-day diet diary
ADHB	Auckland district health board
AITD	Autoimmune thyroid disorder
Anti-TPO	Anti-thyroid peroxidase antibodies
APC	Antigen presenting cell
ATI	Amylase trypsin inhibitor
BB	Blocking buffer
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BUA	Broadband ultrasound attenuation
CD	Coeliac disease
CDAT	Celiac Disease Adherence Test
CMDHB	Counties Manukau district health board
CTX	C-terminal telopeptide of type I collagen
DcytB	Duodenal cytochrome B
DGP	Deamidated gluten peptides
DHB	District health board
DXA	Dual x-ray absorptiometry
EMA	Endomysial antibodies
ESPGHAN	European Society for Pediatric Gastroenterology, Hepatology and Nutrition
FAE	follicle-associated epithelium
FFQ	food frequency questionnaire
FN	Femoral Neck
GALT	Gut associated lymphoid tissue

GD	Graves' Disease
GF	Gluten-free
GFD	Gluten-free diet
GIT	Gastrointestinal tract
GP	General Practitioner
GP2	Glycoprotein 2
GPAQ	Global physical activity questionnaire
GRAS	Generally recognized as safe
HLA	Human leukocyte antigen
hsCRP	High sensitivity c-reactive protein
HT	Hashimoto thyroiditis
IDA	Iron deficiency anaemia
IDD	Iodine deficiency disorder
IEL/IEC	Intraepithelial lymphocytes
IFN	Interferon
IL	Interleukin
LS	Lumbar spine
M-cell	Microfold cell
M-CSF	Macrophage stimulating factor
MET	Metabolic Equivalent of Task
MHC	Major histocompatibility
mTG	Microbial transglutaminase
NCGS	Non-coeliac gluten sensitivity
NCWS	Non-coeliac wheat sensitivity
NHANES	National health and nutrition examination surveys
NPV	Negative predictive value
NRCD	Non-responsive coeliac disease
OPG	Osteoprogerin

PBM	Peak bone mass
PBS	Phosphate buffer solution
PMT	Photomultiplier tube
ppm	parts per million
PTH	Parathyroid hormone
QUS	Quantitative ultrasound
RANK	Receptor Activator of Nuclear Factor-kappa β
RANKL	Receptor Activator of Nuclear Factor-kappa β ligand
RCD	Refractory coeliac disease
RT	Room temperature
SI	Stiffness index
SOS	Speed of sound
T_3	Triiodothyronine
T_4	Thyroxine
TEER	Transepithelial electrical resistance
Tg	Thyroglobulin
TJ	Tight junctions
TNF	Tumour necrosis factor
TSH	Thyroid stimulating hormone
tTG	Tissue transglutaminase
UIC	Urinary iodine concentration
UIE	Urinary Iodine excretion
WA	Wheat allergy
WB	Whole body
WHILA	Women's Health in the Lund Area
WHO	World Health Organization
ZO-1	Zonula occludens-1

ACKNOWLEDGEMENTS

This PhD would not have been possible without the invaluable guidance of my wonderful supervisors, Marlena Kruger, Jane Coad, Janet Weber and Louise Brough. Thank you for your support, expertise and patience over the last 4 years and for not letting me quit when it got too hard, for helping with my never-ending battles for funding and for reading more drafts than I (and you) ever believed possible. A special thanks to Professor Marlena Kruger, for your ceaseless support over the past 4 years, ridiculously fast replies to my queries, for your emoji-filled supportive text messages and for fighting all the battles that got thrown at me along the way. And to Professor Jane Coad, for attending and helping with almost all my participant visits and being my sounding board while I worked through problems aloud. You invested so much time and energy and so very many early starts. In addition, I would like to thank Kevin Pedley, for your help and guidance of the laboratory work that underpinned the whole PhD. Thank you for the time you put into supporting and teaching me and for your patience and determination when experiments went wrong.

This work would also not have been possible without the help of my phlebotomy team, Chris Kendrick and Toby Mundell, who were amazing and kept my participants happy and calm and never made a fuss about the early morning starts.

I would also like to acknowledge the College of Sciences and Massey University for awarding me the Vice-Chancellor's scholarship for my PhD studies. In addition, the Palmerston North Medical Research Fund and the School of Food and Advanced Technology, Massey University for the financial support I received to fund this research.

In addition, I would like to thank Dr James Irwin and Dr Kamran Rostami for discussions about the organoid work and their clinical insights.

Thank you to my team of supporting postgrad students, Ciara, Hannah, Lise, Ella (Pickles), and Sophia. Thank you for your shoulders to cry on, for the walks round

campus and the chocolate runs, and for preserving (what was left of) my sanity. A special thanks to Hannah Morton who generously shared her time and experiences.

A massive thank you to my wonderful family. My husband, Sebastian for your unwavering support, patience, the never-ending supply of chocolate and caffeine and for never doubting that I would eventually finish. Never did I imagine we would take on so much during my PhD, but I wouldn't change it for the world. Thank you for making it all worth it.

My beloved parents because no other teacher has ever inspired me the way you have. I hope I have made you half as proud as I am of both of you. Papa, you were the original reason I wanted to do a PhD. Thank you for your endless love and support. OB, my biggest supporter, thank you for your patience and for believing in me even when I didn't believe in myself. To my brother Rob and the marvellous Schraders family, thank you for cheering me on, celebrating the little triumphs along the way and sympathising when things didn't go to plan.

A massive thank you also to my study participants for your commitment, time, kindness, your experiences and of course your blood.

Lastly, to the late Anne Broomfield, who taught me the art of DXA scanning and how to analyse and interpret the scans. I will forever see Oscar the Grouch in lumbar spine scans. You gave me endless help and kindness and made even the most tedious of tasks a little more fun.

CHAPTER 1

Introduction

1.1 The inspiration behind the research

In 2015, after completing my BSc in Human Nutrition and Physiology, I was diagnosed with coeliac disease (CD). I had struggled with iron deficiency anaemia since I was a teenager, but it was not until the year leading up to my diagnosis that I started to experience more classical gastrointestinal symptoms of CD. After repeat blood tests over about a year, my diagnosis was confirmed by a biopsy that suggested, although it was in its early stages, I did indeed have CD. Despite having learnt about the disease as part of my undergraduate study, it was a disease that was for the most part new to me. Although I had skills like being able to understand food labels, I still felt very out of my depth and joined Facebook support groups to help with this challenging lifestyle change. It was here that I learnt many things that helped me along the way; however, it was the same place where week by week I heard stories of other people's experiences and how they were misinformed by friends and family, hospitality workers, internet sources, other people in support groups and in the worst instances, health professionals like their general practitioners (GP) and dietitians.

Skip forward a couple of years and, as part of my BSc honours research project, I was doing DXA scans when I had the opportunity to volunteer to have a DXA scan myself. After the DXA scan, the lab technician quietly recommended that although the results are for research purposes only, I should speak to my GP and get a referral for a diagnostic DXA scan. At the age of 24 I already had a z-score of -2.1 in my spine, below what was expected for my age group, and a bone density that in a postmenopausal woman would indicate osteopenia. The technician who performed my diagnostic DXA scan mentioned that I should not

be disheartened as low bone density was common in people with CD. These results and the related reading inspired my PhD, as although numerous international studies demonstrated low bone density in people with CD, there appeared to be no such research to support this in New Zealand.

As well as developing an interest in the relationship between CD and low bone density I was also interested, as a physiology major, in the mechanisms involved. I was familiar from my studies with research being done in the physiology group at Massey University using a novel *in vitro* model of intestinal epithelium called intestinal organoids and it was clear that these presented a potentially powerful and possibly unique approach to study the mechanisms involved in this relationship using the minute amounts of tissue which could be rarely available from a small number of biopsies used to diagnose patients. Also, the study of a minor cell component of intestinal epithelium (microfold or M-cells) would be greatly simplified by the ability to increase their expression *in vitro* using the organoid model.

1.2 Background

Coeliac disease (CD) is a lifelong autoimmune disease resulting in an innate immune response to the consumption of gluten, from a variety of grains including wheat, rye, and barley. Globally, CD is estimated to affect 1% of the population (Matthias et al., 2011), with regional differences in prevalence, in part due to variations in genetic predisposition (Lebwohl et al., 2018). New Zealand has one of the highest prevalence, with 1.1% of people believed to have the disease (Burkhardt et al., 2018). In individuals with CD, activation of the immune system in response to consuming gluten causes both villous atrophy and hypertrophy of intestinal crypts leading to impaired nutrient absorption (Kaur et al., 2017). In addition to symptoms caused by inflammation and active damage, ongoing malabsorption can result in deficiency conditions (Kreutz et al., 2020). One of the major

comorbidities believed to result from nutrient malabsorption is low bone mineral density (BMD). CD commonly impacts absorption of nutrients required for optimal BMD including vitamin D, calcium, iodine and magnesium (Cardo et al., 2021). It is estimated that at the time of diagnosis, up to 75% of adults have low bone density (Bianchi & Bardella, 2008; Galli et al., 2018), with low bone density also observed in children (Lerner & Matthias, 2016). Although strict adherence to a gluten-free diet (GFD) results in improvements in BMD, persistent low BMD is commonly observed (Bathrellou et al., 2018).

CD is more common in women, with a reported ratio of 2.1:1 in New Zealand (Cook et al., 2004). In addition to the increased risk of low BMD experienced by women (Guerrini & Takayanagi, 2014), the higher prevalence of CD in women can result in an exaggerated impact on bone density in this group and a heightened risk of osteopenia or osteoporosis.

Although international studies have reported low BMD observed at diagnosis, which persists after adherence to a GFD (Lerner & Matthias, 2016), there has been limited research in New Zealand to investigate this (Bolland et al., 2016), despite nutrient deficiencies that affect BMD, such as iodine and vitamin D, being common in the general population (Brough & Skeaff, 2020; Nowson et al., 2012). In addition, in spite of New Zealand having one of the greatest prevalences of CD in the world, assessment of nutrient intake in people with CD in New Zealand has not been investigated.

Although malabsorption related to villous atrophy has been identified as the primary cause of low BMD in this population group, it does not explain why low BMD persists after diagnosis and adherence to a GFD in many individuals with CD. Research indicates that the GFD may be nutritionally inadequate and lack nutrients which are needed for optimal bone metabolism (Melini & Melini, 2019). Inclusion of individuals who consume a strict GFD for reasons other than CD, such

as non-coeliac gluten sensitivity (NCGS), allows for investigation of how a GFD in the absence of CD and the associated inflammation, impacts BMD.

In addition, recent studies have proposed alternative explanations for persistent low BMD in patients with CD, including ongoing inflammation and uncoupling of bone formation and resorption (Kotze et al., 2016). The RANKL/RANK/OPG pathway has been identified as an area of interest as RANKL plays a role in the activation of osteoclasts responsible for bone resorption (Martin & Sims, 2015), as well as in the differentiation of microfold cells (M-cells) which translocate luminal antigens across the gut epithelium (Williams & Owen, 2015). The OPG:RANKL ratio has been found to be lower in patients with CD who have low BMD (Fiore et al., 2006), this theory offers a promising pathway to explain both the immune response in CD and the presence of low BMD. In addition, as women are more at risk of low BMD later in life, due the effects of oestrogen on bone mass (Chew & Clarke, 2018), assessment of BMD in young women who have reached peak bone mass (PBM) allows for consideration of a potentially high-risk group and how the disease may impact achievement of PBM. This PhD research therefore aimed to investigate BMD in young women with CD, while also exploring the role of microfold cells in the translocation of gluten across the gut epithelium using the novel small intestinal organoid model.

1.3 Research questions

- What is the relationship between bone health and gut inflammation in young (18-40y) women with coeliac disease compared to healthy women?
- Is RANKL-mediated increased expression of antigen-transporting M-cells in the intestinal epithelium integral to gut-bone cross talk?

1.4 Study objectives

- To assess bone health and bone metabolism in young women with coeliac disease compared to healthy controls.

- To determine dietary intake and nutritional status related to requirements for optimal bone health in young women with coeliac disease.
- To investigate the possible role of the RANKL/RANK/OPG pathway in bone-gut interactions.
- To investigate the characteristics of M-cells in biopsy tissue fragments from patients with and without coeliac disease.
- To develop an intestinal organoid model of coeliac disease to examine responses to dietary factors known to trigger adverse reactions in patients with coeliac disease.
- To investigate where intestinal organoids could be used to explore the effects of exposing the tissue, particularly M-cells, to cellular signalling components (e.g., RANKL) involved in regulating bone turnover and cocktails of inflammatory cytokines characteristic of gut inflammation.
- To identify differences in nutritional intake and nutritional status between premenopausal women with CD, healthy controls and those with NCGS
- * To identify how and why the participants make changes to their diets.
- * To identify where people with coeliac disease seek advice about the GFD
- * To identify sources of misleading/contradictory advice

* Objectives added during the SARS-CoV-2 lockdown as part of the *A Gut Feeling* study to make the planned research "covid-resilient".

1.5 Hypotheses

- Bone health, as indicated by markers of bone turnover and assessment of bone density, will be compromised in individuals with a positive diagnosis of CD compared to individuals with NCGS and healthy controls.
- Bone density will be associated with markers of inflammation.

- Intake of micronutrients associated with optimal bone health will be inadequate (and less than healthy controls) in individuals with CD
- The gut-bone axis becomes dysfunctional in coeliac disease and that bone tissue continues to produce signals which not only affect bone turnover but also cause the M-cells in the gut to become hypersensitive to dietary components.
- Sources of advice, specifically advice about the need for assessment of bone density will vary depending on age, gender and region

1.6 Outline of the thesis

This thesis aimed to investigate the relationship between CD and BMD, while also assessing the quality of the diet in these individuals and the underlying mechanisms behind the commonly observed low BMD. The research was split into 3 primary areas, with the chapters and what they include described below:

Chapter two provides an extensive review of literature regarding CD and the association with low BMD. In addition, this includes a discussion of the GFD in terms of nutritional quality and adherence, as well as the use of organoid models to assess intestinal tissue, as was done in our approach.

Chapter three describes our underlying hypothesis and investigates the role of M-cells and RANKL in the dysregulation of the gut-bone axis in individuals with CD. This research was cut short due to the SARS-CoV-2 pandemic which affected the availability of tissue and the ability to undertake laboratory work. As such, this chapter highlights the work done using animal models and describes the experimental plans which were unable to continue since the required daily access to the laboratory to maintain and passage organoid cultures was not allowed during lockdown.

Chapter four, five and six describe the *Close to the Bone* study which investigated differences between premenopausal women with CD and age-

matched healthy controls. Chapter four describes the original protocol, which includes a third group of women with NCGS; the latter group was removed due to recruitment issues because of restrictions put in place due to SARS-CoV-2. Chapter five describes findings of the *Close to the Bone* study as they relate to BMD, biomarkers for bone health and intake of nutrients essential for healthy bones. Chapter six focuses on iodine and thyroid function and the differences observed in iodine status between the two groups.

Chapter seven describes the *A Gut Feeling* study which investigated nutrition advice and sources of advice for people living with CD in New Zealand. This was a preliminary study that was designed during the lockdown caused by SARS-CoV-2 when it was unclear if the *Close to the Bone* study would be able to resume as only two participants had taken part so far.

Chapter eight concludes the findings of the research and describes possible approaches and areas of interest for future research.

1.7 References

Bathrellou, E., Kontogianni, M. D., & Panagiotakos, D. B. (2018). Celiac disease and non-celiac gluten or wheat sensitivity and health in later life: A review. *Maturitas*, *112*(June), 29–33. https://doi.org/10.1016/j.maturitas.2018.03.014

Bianchi, M. L., & Bardella, M. T. (2008). Bone in celiac disease. *Osteoporosis International*, *19*(12), 1705–1716. https://doi.org/10.1007/s00198-008-0624-0

Bolland, M. J., Grey, A., & Rowbotham, D. S. (2016). Outcomes of bone density measurements in coeliac disease. *New Zealand Medical Journal*, *129*(1429), 40–44.

Brough, L., & Skeaff, S. (2020). Iodine status in New Zealand. In P. A. C. Richards, *Iodophor: Tamed Iodine: The Status of Iodine in Tasmania*. Gatekeeper Press

Burkhardt, J. G., Chapa-Rodriguez, A., & Bahna, S. L. (2018). Gluten sensitivities and the allergist: Threshing the grain from the husks. *Allergy: European*

Journal of Allergy and Clinical Immunology, *73*(7), 1359–1368. https://doi.org/10.1111/all.13354

Cardo, A., Churruca, I., Lasa, A., Navarro, V., Vázquez-Polo, M., Perez-Junkera, G., & Larretxi, I. (2021). Nutritional imbalances in adult celiac patients following a gluten-free diet. *Nutrients*, *13*(8), 1–18. https://doi.org/10.3390/nu13082877

Chew, C. K., & Clarke, B. L. (2018). Causes of low peak bone mass in women. *Maturitas*, *111*(September 2017), 61–68. https://doi.org/10.1016/j.maturitas.2017.12.010

Cook, B., Oxner, R., Chapman, B., Whitehead, M., & Burt, M. (2004). A thirty-year (1970–1999) study of coeliac disease in the Canterbury region of New Zealand. *The New Zealand Medical Journal*, *117*(1189), 8.

Fiore, C. E., Pennisi, P., Ferro, G., Ximenes, B., Privitelli, L., Mangiafico, R. A., Santoro, F., Parisi, N., & Lombardo, T. (2006). Altered Osteoprotegerin/ RANKL Ratio and Low Bone Mineral Density in Celiac Patients on Long-term Treatment with Gluten-free Diet. *Horm Metab Res*, *38*(6), 417–422. https://doi.org/10.1055/s-2006-944548

Galli, G., Lahner, E., Conti, L., Esposito, G., Sacchi, M. C., & Annibale, B. (2018). Risk factors associated with osteoporosis in a cohort of prospectively diagnosed adult coeliac patients. *United European Gastroenterology Journal*, *6*(8), 1161–1168. https://doi.org/10.1177/2050640618784340

Guerrini, M. M., & Takayanagi, H. (2014). The immune system, bone and RANKL. *Archives of Biochemistry and Biophysics*, *561*, 118–123. https://doi.org/10.1016/j.abb.2014.06.003

Kaur, A., Shimoni, O., & Wallach, M. (2017). Celiac disease: from etiological factors to evolving diagnostic approaches. *Journal of Gastroenterology*, *52*(9), 1001–1012. https://doi.org/10.1007/s00535-017-1357-7

Kotze, L. M. S., Skare, T., Vinholi, A., Jurkonis, L., & Nisihara, R. (2016). Impact of a gluten-free diet on bone mineral density in celiac patients. *Revista Espanola de*

Enfermedades Digestivas, *108*(2), 84–88. https://doi.org/10.17235/reed.2015.3953/2015

Kreutz, J. M., Adriaanse, M. P. M., van der Ploeg, E. M. C., & Vreugdenhil, A. C. E. (2020). Narrative review: Nutrient deficiencies in adults and children with treated and untreated celiac disease. *Nutrients*, *12*(2). https://doi.org/10.3390/nu12020500

Lebwohl, B., Sanders, D. S., & Green, P. H. R. (2018). Coeliac disease. *The Lancet*, *391*, 70–81. https://doi.org/10.1016/S0140-6736(17)31796-8

Lerner, A., & Matthias, T. (2016). Gut-bone Cross Talks and Implications in Celiac Disease. *International Journal of Celiac Disease*, *4*(1), 19–23. https://doi.org/10.12691/ijcd-4-1-4

Martin, T. J., & Sims, N. A. (2015). RANKL/OPG; Critical role in bone physiology. *Reviews in Endocrine and Metabolic Disorders*, *16*(2), 131–139. https://doi.org/10.1007/s11154-014-9308-6

Matthias, T., Neidhöfer, S., Pfeiffer, S., Prager, K., Reuter, S., & Gershwin, M. E. (2011). Novel trends in celiac disease. *Cellular & Molecular Immunology*, *8*(2), 121–125. https://doi.org/10.1038/cmi.2010.68

Melini, V., & Melini, F. (2019). Gluten-Free Diet: Gaps and Needs for a Healthier Diet. *Nutrients*, *11*(1), 170. https://doi.org/10.3390/nu11010170

Nowson, C. A., McGrath, J. J., Ebeling, P. R., Haikerwal, A., Daly, R. M., Sanders, K. M., Seibel, M. J., & Mason, R. S. (2012). Vitamin D and health in adults in Australia and New Zealand: A position statement. *Medical Journal of Australia*, *196*(11), 686–687. https://doi.org/10.5694/mja11.10301

Williams, I. R., & Owen, R. L. (2015). M Cells: Specialized Antigen Sampling Cells in the Follicle-Associated Epithelium. *Mucosal Immunology: Fourth Edition*, 211–229. https://doi.org/10.1016/B978-0-12-415847-4.00013-6

CHAPTER 2

Literature Review

2.1 Introduction:

Coeliac disease (CD) is an incurable autoimmune disease, triggered by exposure to gluten in genetically predisposed individuals (Singh et al., 2018). Globally the prevalence is estimated at 1%, making it one of the most common food intolerances in the world (Matthias et al., 2011). CD affects about 1.1% of the New Zealand population, a prevalence which is one of the highest in the world (Burkhardt et al., 2018) and is increasing, for reasons which are not fully understood. CD causes small intestinal inflammation and villous atrophy which compromises absorption of nutrients. Individuals with untreated CD usually have poor calcium and vitamin D absorption (Lerner & Matthias, 2016a) and compromised bone density is a frequent finding at the time of diagnosis (Laszkowska et al., 2018). Although strict adherence to a gluten-free diet (GFD) dramatically improves both gut inflammation and nutrient absorption, it is common for bone density to not fully recover. Possible mechanisms underlying low bone density in CD have included malabsorption, inflammation and secondary hyperparathyroidism (Kotze et al., 2016). However, these do not explain the lack of resolution of low bone density on a GFD and apparent persistent imbalance between bone resorption and bone formation. Worldwide, it is estimated that osteoporosis affects more than 200 million women (International Osteoporosis Foundation, 2017); with an ageing population this prevalence will continue to rise. Bone loss leads to bone fragility and microarchitectural deterioration of bone tissue, resulting in increased risk of low-trauma fracture (Briot et al., 2017) and accounts for a significant increase in both morbidity and premature mortality and a decreased quality of life (El Miedany, 2022). In New Zealand, the prevalence of osteoporosis is increasing, presenting a huge burden to the national economy and

health care systems (Brown et al., 2011). With the increasing prevalence of CD and our ageing population, it is imperative that we understand the pathophysiology underlying low bone density in CD to identify therapeutic targets and improve understanding of the disease and quality of life.

2.2 Coeliac Disease

2.2.1 Pathophysiology of Coeliac Disease

CD is a T-cell-mediated autoimmune enteropathy which is triggered by the consumption of gluten proteins by genetically susceptible people. Gluten is comprised of a group of proteins, termed prolamins, which are found in wheat, barley, and rye. The prolamins include gliadins and glutenins in wheat, secalins in barley and hordeins in rye. Some of these peptides are resistant to proteolytic degradation in the gut due to the large number of proline residues, which results in long peptides remaining in the lumen of the small intestine (Aboulaghras et al., 2022; Lindfors et al., 2019).

The most important genomic risk factors for CD are the major histocompatibility (MHC) protein class II genes, HLA-DQ2 and HLA-DQ8. Approximately 90% of CD patients express HLA-DQ2.5, whereas almost all the other people with CD express either HLA-DQ2.2 or HLA-DQ8 (C. Catassi et al., 2022). Over 95% of people with CD are HLA-DQ2-positive with the remainder being HLA-DQ8-positive. Individuals who are homozygous for HLA-DQ2 or carry two copies of the DQB1*02 allele, have an increased risk of developing CD (Kuja-Halkola et al., 2016). However, approximately 40% of the general population are DQ2- or DQ8-positive, and only 2–3% of these will develop CD making it clear that other genes and/or environmental factors are also involved.

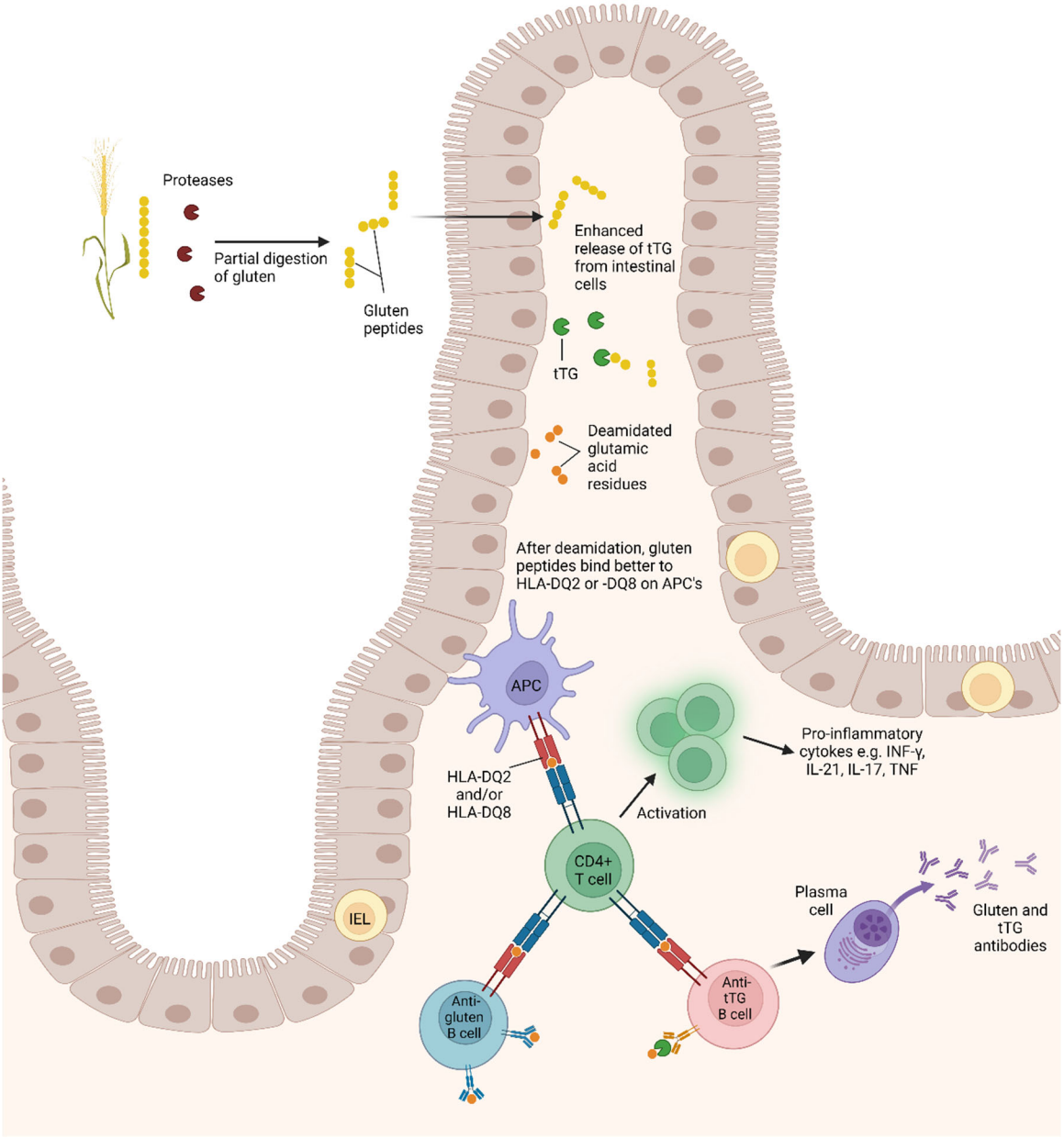

Figure 2.1 Immune system activation in response to the consumption of gluten in Coeliac Disease. Incompletely digested gliadin peptides are translocated across the gut barrier. In the lumina propria they are deamidated by tissue transglutaminase (tTG) to deamidated glutamic acid residues, increasing their binding to human leukocyte antigen (HLA)-DQ2 and DQ8 on antigen presenting cells (APCs) including dendritic and B-cells. APCs present these residues to T-cells, triggering the immune response (Created with BioRender.com)

The epithelial barrier is compromised in CD, allowing gluten peptides to cross into the lamina propria via either transepithelial or paracellular routes (Lebwohl et al., 2018). In the lamina propria, the peptides are deamidated by the intestinal enzyme tissue transglutaminase (tTG), which is secreted into the extracellular matrix, converting glutamine residues in gluten proteins to glutamic acid. Deamidation alters the binding of the peptides and increases their binding affinity to the major histocompatibility proteins HLA-DQ2 and DQ8 on the surface of antigen presenting cells (APCs). APCs include dendritic cells, macrophages and lymphocytes, including tTG-specific and gluten-specific B-lymphocytes (Lindfors et al., 2019). B-lymphocytes recognise and internalise glutamic acid residues and present these peptide sequences to gliadin-reactive CD4+ T (T-helper) -lymphocytes. Once activated, CD4+ T-lymphocytes proliferate, secreting pro-inflammatory cytokines such as interferon-gamma (IFN-γ), interleukins IL-21 and IL-17, and tumour necrosis factor. These pro-inflammatory cytokines are toxic, causing damage to the epithelium. Activated B-lymphocytes also differentiate into plasma cells which secrete antibodies against tTG, endomysial proteins and deamidated gluten peptides (DGP). Pro-inflammatory cytokines increase tissue destruction and intestinal atrophy. This damage results in mucosal inflammation, crypt hyperplasia and eventually leads to villous atrophy (Lindfors et al., 2019); the extent of the damage can be measured using the Marsh-Oberhuber grading scale (described in table 2.1, pg. 21).

2.2.2 Aetiology of Coeliac Disease

Although the pathophysiology of CD is well described, it is still not fully understood, and the aetiology of the disease remains unclear. The prevalence of CD has increased more than 4-fold in the United States of America (USA) during the past 50 years, (Rubio-Tapia et al., 2009), with a similar increase reported in Europe (Lohi et al., 2007). However, only a small percentage of individuals who are

genetically predisposed as carriers of HLA-DQ2 or HLA-DQ8, and also consume gluten, actually develop CD. Differences in prevalence between genetically similar populations demonstrate that genetics alone are not responsible for the onset of CD and suggest that environmental factors play a role in the pathogenesis of the disease. For example, the Finnish Karelia population has a prevalence of >2%, while the neighbouring and genetically similar Russian Republic of Karelia has a prevalence of 0.2% (Cernat & Furman, 2018). This is further supported by a longitudinal study in the USA that found the incidence of CD doubled over a 15-year period in the same participant group, indicating later onset of the disease and a loss of gluten tolerance over time (Catassi et al., 2010). However, currently, although there are many theories behind possible triggers, there is no conclusive evidence of what they are.

Possible Environmental triggers

Possible triggers include infections, proinflammatory stimuli and the increasing use of food additives by commercial food producers including microbial transglutaminase, emulsifiers and other components that impair gut barrier function. Microbial transglutaminase (mTG), used to enhance food quality and extend shelf-life, has been shown to increase intestinal permeability and the uptake of gluten peptides and can itself undergo transepithelial movement into the lamina propria (Stricker et al., 2019). Given this finding, it is surprising that this food additive is still classified by the US Food and Drug Administration as GRAS (Generally Recognized as Safe).

Gut microbiota

The human gastrointestinal tract is home to the gut microbiota comprised of approximately 10^{14} microorganisms which have an enormous impact on human health and disease (Fan & Pedersen, 2021). The microbiome play essential roles in

intestinal homeostasis, contributing to nutrient availability via carbohydrate fermentation, competing with ingested pathogens for nutrients and 'educating' the immune responses of the gut-associated lymphoid tissue to protect the host from infection (Hou et al., 2022). The gut microbiome also has an important role in the maintenance of epithelial barrier function which is vital to health. Not surprisingly, dysbiosis of the gut microbiome has been implicated as a potential contributor to CD.

CD has been associated with changes in the composition of gut microbiota in both paediatric and adult CD (Girbovan et al., 2017). Both duodenal and faecal samples have been reported to show an increased abundance of Proteobacteria (Verdu et al., 2015) and Bacteroidetes (Collado et al., 2009) in active CD patients whereas Lactobacillus and Bifidobacterium were decreased when compared with controls (Di Cagno et al., 2011). The reported changes in the microbiome were also associated with changes in the generation of metabolites (Di Cagno et al., 2011) and with CD disease gastrointestinal symptoms (Wacklin et al., 2013).

It is essential, however, to consider the correlation of changes in microbiome composition, and its impact on metabolite production and gut homeostasis, with the stage of CD, whether changes occur before CD pathophysiology or after its establishment, i.e., the 'cause or effect'. Although the studies mentioned above reported changes in the acute phase of CD, changes in gut microbiota may impact factors such as tolerance of gluten. A study by Sellito et al. (2012) followed infants genetically at risk of CD until 2 years old and monitored changes in the microbiota and bacteria-derived metabolites in these infants compared with controls. These and other studies suggest that dysbiosis of the gut microbiota and their metabolic products may contribute to the pathogenesis of CD. A similar role in the development of CD in adults is clearly a possibility with behavioural and environmentally induced changes in microbiota composition. Key

factors which can change the composition of the gut microflora during adulthood include nutrition, infections, antibiotic use and stress (Bajinka et al., 2020).

Changes in the proteolytic activity of gut microbiota may also be involved in the development of CD. Caminero et al. (2016) reported that *Pseudomonas aeruginosa*, an opportunistic pathogen found in CD patients, exhibited elastase activity which cleaves gliadin peptides increasing their ability to cross the intestinal barrier. These modified gluten peptides also activated gluten-specific T-cells from CD patients. Conversely, *Lactobacillus,* from the duodenum of non-CD controls, degraded gluten peptides produced by human and *P. aeruginosa* proteases, reducing their immunogenicity. These findings indicate that changes in the composition of the intestinal microflora and their impact on gluten peptide toxicity may prove an important aspect of CD and a potential target for intervention.

Hygiene Hypothesis

The hygiene hypothesis is based on the proposition that a reduction in the frequency of infections in early childhood blunts the normal development of the immune system and contributes directly to the increase in the frequency of allergic and autoimmune disease (Bach, 2018). This hypothesis fits with the observed increases in the incidence of autoimmune diseases such as CD, compared with the overall decrease in the incidence of most infectious diseases.

Studies of stored serum showing that the prevalence of CD has increased 4-fold over the past 50 years may be in part explained by the hygiene hypothesis, with an underlying decrease in exposure to microorganisms and an additional decrease in infection due to antibiotic use (Rubio-Tapia et al., 2009). However, as understanding of the pathophysiology of CD has improved, so have the methods of diagnosis. Numerous undiagnosed patients, particularly in poorer countries, remain untreated and at risk of morbidity from intestinal and extra-intestinal

symptoms together with the increased risks of cancer. The 'hygiene hypothesis' may explain, in part, the increasing prevalence of CD but other underlying factors must contribute to its increasing prevalence in both developed and developing countries.

Introduction of gluten

Higher gluten intake during the first 5 years of life has been associated with increased risk of CD in genetically predisposed children (Aronsson et al., 2019). Although the reasons for the 4-fold increased prevalence of CD over the last 50 years are unknown, the impact of environmental challenges on human genetics is too slow to take effect in that time course so it is more likely to be due to environmental changes. Major changes in wheat genetics and food processing have occurred in the past 40 years (Cabrera-Chávez et al., 2008; Cronin & Shanahan, 2001) which may have contributed significantly to the increasing incidence of CD.

2.2.3 Symptoms of Coeliac Disease

CD was originally identified as a disease which primarily affected children, with the first known report about the disease published in 1552 (García-Nieto, 2014). Although the cause was unknown at this time, it was clear that the disease affected the gut resulting in impaired absorption, as children commonly presented with severe malnutrition.

> "*Upon first sight the child appears to have a great pallor… he gives the impression of a balloon held up by two sticks*"
>
> (Recalde Cuestas & Travella, 1935, as cited in García-Nieto, 2014)

Over the past 50 years, understanding and recognition of the disease have changed, resulting in an increased identification of the condition (King et al., 2020). The disease is now more commonly diagnosed in adults, with the median

age of diagnosis reported as 40 years (Tye-Din, 2018). Originally thought to be only associated with gastrointestinal symptoms, the presentation of the disease is also now understood to vary due to its systemic nature; it is increasingly common for patients to present with extra-intestinal symptoms as well or sometimes independently of intestinal symptoms (Dos Santos & Lioté, 2017).

Classical CD is associated with gastrointestinal symptoms including changes in bowel habits, bloating, heart burn, steatorrhoea (fat malabsorption resulting in change in stool), abdominal pain and constipation (Bianchi & Bardella, 2008; Castillo et al., 2015).

Extra-intestinal symptoms are more common in adults and are often quite non-specific. They include tiredness, infertility, depression, anaemia, low bone density, epilepsy, cardiac and dental abnormalities, along with even less specific symptoms such as headaches, mood swings and joint pain (Khaleghi et al., 2016; Kotze et al., 2016; Lerner & Matthias, 2016b; Raiteri et al., 2022).

Many of these extra-intestinal symptoms are believed to be a result of nutrient malabsorption caused by intestinal damage (Holtmeier & Caspary, 2006), with the degree of damage correlated to the severity of some nutrient deficiencies (Kreutz et al., 2020). Common nutrient deficiencies at the time of diagnosis include iron, zinc, calcium, magnesium, vitamin D, vitamin B12 and folic acid. All these nutrients, with the exception of vitamin B12, are predominantly absorbed in the proximal small intestine, the region of the gut most compromised in CD. These nutrient deficiencies are discussed further in 2.4.4 Nutritional Implications of a GFD.

2.2.4 Diagnosis of Coeliac Disease

Diagnosis of CD requires a combination of tests including serology and biopsy sampling. During testing and for a period of at least 6 weeks prior to testing, patients are required to consume gluten, as removing gluten from the diet

would result in negative serology. Historically, it has been recommended patients consume at least 10g/day for 6 weeks or more. However, the recommended minimum intake varies with newer studies reporting that more than 3g per day for at least 2 weeks is sufficient (Lebwohl et al., 2018). The lower recommendations suggest one slice of bread per day for 2 weeks would be a sufficient intake; one slice of bread contains roughly 4g of gluten (Biesiekierski, 2017). In New Zealand patients are advised to consume a minimum of 4 slices of gluten-containing bread per day for 4-8 weeks in adults (or the equivalent) (Coeliac New Zealand, n.d.-a). This can present a high burden on individuals with symptomatic CD, with symptoms significantly impacting their lives over this period.

Genetic Testing

CD occurs in people who are genetically predisposed, with genetics accounting for roughly 40% of the genetic risk for developing CD (Lebwohl et al., 2018). The most recognised of these genetic traits is the possession of specific human leukocyte antigen (HLA) class II genes, DQA1 and DQB1. These genes encode the α and β chains of the heterodimers DQ2 (DQA1*05-DQb1*02) and DQ8 (DQA1*03-DQB1*0302) (Cernat & Furman, 2018) which are expressed on the surface of APCs (Lebwohl et al., 2018). It is estimated that more than 98% of patients with CD are positive for DQ2 or DQ8 (Horan et al., 2018); with DQ2 seen more commonly (90% patients) than DQ8 (Lebwohl et al., 2018). However, although DQ2/DQ8 are found in most patients with CD, these alleles are also present in people without CD; roughly 30-40% people of European descent possess these alleles (Cernat & Furman, 2018; Lebwohl et al., 2018). HLA typing is therefore not a good positive predictive marker of CD but has a good negative predictive value (NPV) (Horan et al., 2018).

Serology

Serological testing for CD includes measuring tissue transglutaminase (tTG) antibodies and endomysial antibodies (EMA) (Lerner & Matthias, 2016b); with the IgA isotypes having the greatest diagnostic accuracy (Cernat & Furman, 2018). IgA-tTG is recommended as the first test due to its high sensitivity, negative predictive value and lower cost (compared with EMA) (Lebwohl et al., 2018). Blood samples are also screened for IgA deficiency to rule out false negatives; if a patient is IgA deficient, IgG isotypes can be used. IgA-tTG is calculated based on reference values, with results communicated as arbitrary units (Cernat & Furman, 2018).

If the patient's IgA-tTG value is only slightly raised, suggesting a weak positive result, EMA is also tested; this is because IgA-tTG is raised in other autoimmune conditions such as liver disease, cancer and some infectious diseases (Cernat & Furman, 2018). EMA is highly specific to CD, but testing is both labour intensive and expensive (Lebwohl et al., 2018). It is also highly subjective and interpretation of results can vary significantly when viewed by different observers (Cernat & Furman, 2018)

Biopsy

Upper intestinal biopsy is regarded as the gold standard to confirm diagnosis of CD (Reilly et al., 2018), with multiple biopsy samples collected from the duodenum, which is the section of the small intestine affected in most patients (Zammit et al., 2020).

Assessment of biopsy samples examines the crypt to villous ratio to confirm villous atrophy and crypt hyperplasia; comparing the height of the villous to the adjacent crypt, which in healthy intestinal tissue in adults should be between 3:1 to 5:1 (Owen & Owen, 2018). In addition, the number of intraepithelial lymphocytes (IEL) are also assessed. There is a lack of consensus about the exact cut-off for IEL that indicates intestinal damage, however the most recent diagnostic cut-off

suggests 25 IELs per 100 enterocytes or greater is likely to be associated with intestinal damage (Charlesworth, 2020).

Classically, these intestinal damage indicators can be considered together through the use of the Marsh-Oberhuber classification (table 1) in order to confirm diagnosis (Adelman et al., 2018).

Table 2.1 Marsh-Oberhuber classification

Marsh Score	Lesion type	Villi	Crypts	IELS	Diagnostic of CD
I	Infiltration	Normal	Normal	>25/100 enterocytes	No
II	Infiltration-hyperplastic	Normal	Hyperplasia		
III					Yes
IIIA	Flat destructive	Mild VA	Hyperplasia		
IIIB		Moderate VA	Hyperplasia		
IIIC		Total VA	Hyperplasia		
IV	Atrophic-hypoplastic	Total VA	Hyperplasia		

IEL, intraepithelial lymphocyte

Although Marsh scores are not used by all clinicians, with some favouring other classification systems (Peña, 2015), villous atrophy, crypt hyperplasia and

lymphocyte infiltration remain the key indicators of active CD in all methods (Adelman et al., 2018), and it is common for clinicians to not confirm a diagnosis of CD until the biopsy tissue has been assessed. However, medical literature currently suggests that 5-13% of patients experience delays in diagnosis due to lack of or delayed biopsy referral or poor biopsy testing (Lebwohl et al., 2018). There is evidence these delays were further exacerbated by the lockdowns that stemmed from the SARS-CoV-2 pandemic (Catassi et al., 2020).

As patients must continue to consume gluten prior to biopsy, delays in referral can result in unnecessary discomfort to patients. Delays, along with the procedure being invasive and requiring anaesthesia can result in patients choosing to avoid biopsy.

A proposed alternative to the upper intestinal biopsy, is the use of capsule endoscopy. Capsule endoscopy has been demonstrated to be effective in diagnosis of CD (Branchi et al., 2020; Zammit et al., 2018). This approach covers more of the intestinal tract, allowing for detection of villous atrophy even when the lesions are interspersed with patches of healthy, unaffected tissue, with high magnification mimicking that seen in microscopy of biopsy samples (Lewis & Semrad, 2019). Capsule endoscopy has shown to be beneficial in clinical practice for patients who are unable or unwilling to undergo an endoscopic biopsy (Akin & Ersoy, 2012), however, the technology currently used in capsule endoscopy lacks the ability to harvest biopsy samples and so it is unlikely to be used for general clinical practice.

Biopsies also have additional roles in assessing tissue repair and recovery on a GFD which is particularly important in patients who have negative serology (seronegative patients) and those with non-responsive CD (discussed below). If patients have positive serology but the biopsy is clear, it suggests a recent onset of CD and patients are recommended to be tested again at a later date; however, if serology is negative but signs of CD are seen in the biopsy then the patient is considered seronegative (Caio & Volta, 2012).

ESPGHAN recommendations

The European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) guidelines (Husby et al., 2020) advise that children and adolescents with a tTG antibody level greater than 10x the upper limit of normal (ULN), the likelihood they also have Marsh III intestinal damage (villous atrophy) is very high (Husby et al., 2012). In these individuals it has been decided that biopsy is not necessary, with testing for tTG antibodies, EMA and presence of HLA DQ2/DQ8 accepted as sufficient for diagnosis; this is known as the "triple test".

In a study assessing the "triple test" for diagnosis in adults, 59 patients were identified as having CD using these criteria; these patients then received a biopsy to confirm diagnosis. Of the 59 patients, 56 were confirmed with the initial biopsy. The remaining three patients displayed only Marsh I lesions but were confirmed to have CD after a further year on a GFD, presenting with Marsh III lesions at a second biopsy. These findings suggest that it may be possible to diagnose patients with CD without the inclusion of biopsy (Fuchs et al., 2019)

However, previous studies have found that using serology only leads to unreliable diagnosis; with some research suggesting that patients may be incorrectly diagnosed (as many as 2/3 of cases reported) and others suggesting this may lead to underdiagnosis (Grober et al., 2014). The absence of positive serology or genetic predisposition in someone who is symptomatic and consuming a gluten rich diet strongly suggests that they do not have CD, however, it is not possible for the condition to be completely excluded without biopsy (Husby et al., 2012).

Non-Responsive Coeliac Disease (NRCD) and Refractory Coeliac Disease (RCD)

Currently the only effective treatment of CD is the strict lifelong exclusion of gluten from the diet (Plugis & Khosla, 2015), however a proportion of patients with CD do not response to the GFD. This is known as non-responsive CD (NRCD)

which is defined by ongoing symptoms and serology consistent with the consumption of gluten despite apparent strict adherence to the GFD for 6-12 months (Baggus et al., 2020). NRCD can lead to villous atrophy and associated malabsorption, as well as presence of abnormal intraepithelial lymphocytes (IEL) (Abadie & Jabri, 2014; van Beurden et al., 2016). If no alternative cause of symptoms can be identified, patients may be diagnosed with refractory CD (RCD) if symptoms continue after 12 months on a strict GFD. RCD does not improve when gluten is eliminated from the diet and is estimated to affect between 0.3-4% of patients with CD (Penny et al., 2020).

Non-Coeliac Gluten Sensitivity (NCGS)

Non-Coeliac Gluten Sensitivity (NCGS) is a condition usually diagnosed in patients who have previously undergone testing for CD and wheat allergies but do not fit the diagnostic criteria for either condition. The patients generally suffer from intestinal and/or extra-intestinal symptoms which are resolved upon removal of gluten from the diet (Bathrellou et al., 2018).

Although the first reports of NCGS date back to 1978, it has only recently begun being recognised as a standalone condition (Fasano et al., 2015). There is limited research on the prevalence of NCGS, however it is suggested that prevalence may be around 6% in western populations (Graziano & Rossi, 2018; Scherf et al., 2016), with some studies reporting the prevalence as high as 50% in some population groups (Bathrellou et al., 2018). Research conducted in Wellington and Christchurch between 1997 and 2001, found that 5% of children excluded gluten despite not being diagnosed with CD (Tanpowpong et al., 2012). The authors noted that this was more common in the children from Christchurch and although these children were not defined as having NCGS the authors reported that many had negative tests for CD but had improved abdominal symptoms upon removal of gluten. These findings suggest that prevalence or

recognition of NCGS may depend on location, while also supporting other research findings that prevalence of NCGS is greater than CD.

That many people with NCGS suffer from extra-intestinal symptoms indicates that there may be people who remain unaware that their symptoms may be caused by gluten (as they assume gluten only causes intestinal symptoms). This supports suggestions that prevalence of NCGS may be greater than currently reported (Fasano et al., 2015). However, as it is common for patients to self-diagnose and remove gluten from their diet without medical advice, prevalence data which include self-diagnosis may be misleading (Czaja-Bulsa, 2015). It is likely that many participants who have self-diagnosed have not sufficiently excluded the presence of other conditions such as CD, with a study by Biesiekierski et al. (2014) reporting that only 28% of respondents fit the criteria for diagnosis of NCGS. This trend to self-diagnose makes it difficult for researchers to differentiate between patients with NCGS and those following a GFD for other reasons (Schuppan et al., 2015).

Medical diagnosis of NCGS is difficult as the condition has no specific serological or laboratory markers (Czaja-Bulsa, 2015). Diagnosis therefore requires eliminating the possibility of symptoms being caused by other conditions such as CD and wheat allergy (WA). In 2014, the Salerno Criteria were established at the 3rd International Expert Meeting on Gluten Related Disorders in Italy (Catassi et al., 2015). Through initial assessment of symptoms whilst consuming and excluding gluten, followed by a double-blind placebo-controlled challenge with crossover, this approach allows for a definitive diagnosis of NCGS. Unfortunately, the criteria have not been widely adopted, as it requires significant time commitment by clinicians which may not be feasible in a clinical setting (Casella et al., 2018).

The pathophysiology of NCGS remains unclear, however it has been suggested that the immune system may play an important role in the condition. This is supported by reports of increased expression of mucosal Toll-like receptor 2

and elevated numbers of CD3+ intraepithelial T-cells in individuals with NCGS compared with healthy controls (Graziano & Rossi, 2018). However, unlike CD, NCGS does not lead to autoimmune enteropathy (crypt hyperplasia and villous atrophy) or the development of autoantibodies to tTG (Fasano et al., 2015). There is also limited association with the HLA DQ2/DQ8 genotypes (Skodje et al., 2018) suggesting that the role of the immune system in NCGS is an innate immune mechanism rather than adaptive.

Some researchers believe that it is not specifically gluten which causes this condition which may instead be due to another component of wheat, rye and barley; this has led to the alternative name, non-coeliac wheat sensitivity (NCWS) (Schuppan et al., 2015). Non gluten components of wheat thought to be responsible for the pathogenesis of NCGS include wheat amylase trypsin inhibitors (ATIs) (Czaja-Bulsa, 2015) (although this has currently only been demonstrated in animal studies). In addition it has been proposed that NCGS may be linked to irritable bowel syndrome because low FODMAP diets which remove fermentable carbohydrates may improve symptoms in some individuals (Gibson et al., 2017).

Regardless of the actual dietary cause of NCGS, this condition presents an interesting research opportunity to investigate the link between a strict GFD and comorbidities such as low BMD. As nutrient deficiencies are not a symptom of NCGS (because there is no villous atrophy) (Graziano & Rossi, 2018), this condition also allows researchers to assess the nutritional adequacy of the GFD in the absence of malabsorption (Bathrellou et al., 2018).

2.2.5 Prevalence of Coeliac Disease

It has been estimated that CD currently affects at least 1% of people globally (Kumar et al., 2017; Lebwohl et al., 2018). Prevalence in European countries is considered to be highest, however, this may be at least partly explained by the distribution of screening studies, with most taking place in Europe (Lindfors et al.,

2019). A recent meta-analysis by Singh and colleagues (2018) split countries into quartiles to assess prevalence of CD diagnosed via biopsy. Those countries in the top quartile included Argentina, Egypt, Hungary, India, Finland, New Zealand and Sweden which had a prevalence of between 0.9 and 2.4%; whereas the bottom quartile identified a prevalence of 0.2-0.4% across Brazil, Republic of San Marino, Germany, Tunisia and Russia. Their findings also showed a greater prevalence identified through serology (1.4%) than biopsy (0.7%).

In more recent years, non-western countries including poorer regions like the Middle East and North Africa, where prevalence was previously thought to be low, have seen a rise in prevalence (Aboulaghras et al., 2022). Seroprevalence studies in China indicate a prevalence of 1.27% in populations assessed in both Southern and Northwest regions (Chen et al., 2020; Zhou et al., 2020). In addition, seroprevalence studies in Northern India indicate an estimated prevalence of 1.44% (Makharia et al., 2011). It is estimated that up to 10 million people with CD are yet to be diagnosed in India, with figures appearing to be similar in China (Castillo et al., 2015).

Factors such as ethnicity may play a role, probably related to differences in genetic susceptibility between ethnicities (Makharia et al., 2022). In addition, differences in reported prevalence between countries and regions may be related to possible aetiology of the disease with potential triggers being more common in certain regions or countries (section 1.2.2).

It is therefore difficult to determine accurate global prevalence of the disease, with estimates varying significantly depending on the method of diagnosis and geographical location. Assessment of prevalence based on biopsy-based diagnosis is likely to significantly underestimate prevalence, with many individuals opting out of confirmation through biopsy due to requirements to continue consuming gluten prior to testing which can prolong symptoms and impact quality of life (Lebwohl & Rubio-Tapia, 2021). In comparison, serology-based

prevalence is believed to overestimate prevalence. A recent study which followed 1339 genetically susceptible children (positive for DQ2 or DQ8) over 20 years found an incidence closer to 3.1% in the general population (Liu et al., 2017). The study, which was based in Denver, Colorado, raised some concerns regarding reliance on serological testing alone, with 3.4% of participants demonstrating raised tTG for a period of 3 months or more without going on to develop CD (no detection of Marsh II or greater damage or continued high levels of tTG). The authors note that upwards of 5% of children may experience periods of raised tTG without developing CD, further supporting beliefs that seroprevalence studies may overestimate actual prevalence of the disease.

In addition, the recent rise in popularity of the GFD in people not diagnosed with CD may result in inaccuracies in prevalence screening (Tye-Din et al., 2018). In the USA, the National Health and Nutrition Examination Surveys (NHANES) indicated the percentage of non-coeliac individuals consuming a GFD increased from 0.5% in the 2009-2010 survey to 1.7% in the 2013-2014 survey (Choung et al., 2017). In comparison, an Australia survey found 7.3% of respondents reported consuming a GFD for reasons other than CD (Golley et al., 2015). As gluten needs to be consumed for activation of the immune system in CD, detection of the disease through serological prevalence studies may result in an under-estimation of the actual prevalence in populations where the GFD is popular in those without CD.

Regardless of actual prevalence (Lebwohl et al., 2018), the trend in serum studies still suggests an overall increase in prevalence of CD. Evidence from the USA indicates prevalence has increased 4-4.5-fold over the past 50 years, and in Finland prevalence appears to have doubled between 1978 and 2001 (Lebwohl et al., 2018). However, although these findings, along with those of screening studies, suggest the prevalence of CD is increasing, diagnosis of these patients appears to be increasing more slowly (Lebwohl et al., 2018) and there still appear

to be many people who remain undiagnosed (Parzanese et al., 2017). This is often described using the iceberg model (Admou et al., 2012) with symptomatic CD, which is more easily diagnosed, being only the tip of the iceberg (Figure 2.2).

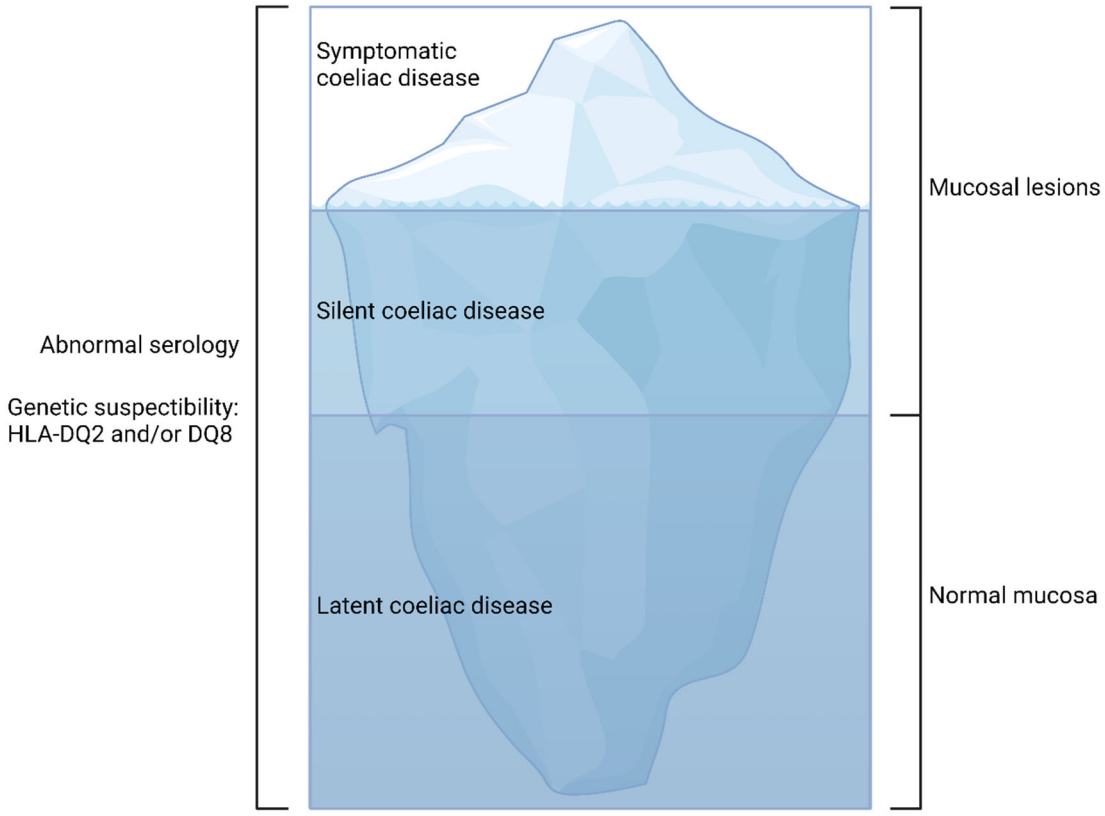

Figure 2.2 The coeliac iceberg model which describes the symptomatic patients as those 'above sea level', easily diagnosed due to fitting with clinical characteristics. Whereas those represented by the iceberg 'under sea level' consist of those with coeliac disease who do not fit with these previously recognised characteristics (Created with BioRender.com)

Gender differences

CD has long been purported to have a higher prevalence in women than men (Jansson-Knodell et al., 2019), however, research from screening studies investigating prevalence of the disease in the general population has reported equal seroprevalence in men and women (Kvamme et al., 2022), suggesting that the observed differences in prevalence between men and women may not be as

vast as initially thought. Differences in diagnosis rates, potentially as a result of practitioners' assumptions that males are at a lower risk of CD, may be partially to blame, with physicians reported to perform less biopsies in men (Lebwohl et al., 2012).

Prevalence in New Zealand

New Zealand is reported to have one of the highest biopsy determined prevalence of CD in the world at 1.1% (Burkhardt et al., 2018). This is based on a study by Cook et al. (2004) which assessed diagnosis rates over a 30-year period in the Canterbury region. In addition, this study also reported a ratio of females to males of 2.1:1. Unfortunately, no further research has been undertaken in New Zealand to investigate prevalence in other regions or whether this has risen further in the 21st century. However Coeliac New Zealand currently estimates that 1 in 60-70 New Zealanders (1.4-1.7%) have the disease, but up to 80% have not yet been diagnosed (Coeliac New Zealand, 2017).

In addition to gender differences observed in New Zealand, research by Edinur et al. (2013) investigating HLA polymorphisms in Māori and Pacific peoples found the DQ2 haplotype was less common in these populations, potentially indicating a lower risk of CD in these ethnicities.

2.2.6 Morbidity

As well as improving symptoms, adherence to a strict GFD also reduces risk of morbidity and mortality in patients with CD. If left untreated, CD increases risk of enteropathy-associated T-cell lymphoma (Lebwohl et al., 2018) and other malignancies (Pelizzaro et al., 2021), with studies reporting a 3-6-fold increase in non-Hodgkin lymphoma in undiagnosed CD patients (Bathrellou et al., 2018). This increased risk is also found in patients with refractory CD and those who do not strictly adhere to the GFD. However, researchers report that the risk of malignancy

is not as great as previously estimated; likely as a result of earlier diagnosis in patients, resulting in a reduction in accumulative intestinal damage (Collin et al., 2018).

In addition, patients with CD commonly experience comorbidities including conditions such as osteoporosis (section 1.5) and other autoimmune disease. The latter of these is referred to as polyautoimmunity (PA), which occurs when an individual diagnosed with an autoimmune disease develops more than one (Aaron et al., 2019). A variety of factors are implicated in the development of autoimmune diseases including a genetic predisposition (Jonkers & Wijmenga, 2017), the breakdown of immune tolerance, immune system exposure to previously hidden self-antigens, exposure to antigens which antigenically mimic self-antigens (molecular mimicry), exposure to non-self-cells (microchimerism) and changes in hormone levels. Of the 80+ different autoimmune diseases, several are seen to cluster in families, though not necessarily the same disease, and a number of them are much more common in females than males (Aaron et al., 2019). A number of studies have investigated the incidence of other autoimmune diseases in patients with CD and the underlying pathophysiology; patients with CD are at greater risk of developing systemic lupus erythematosus (SLE), autoimmune thyroiditis, type 1 diabetes, inflammatory bowel disease (IBD; both Crohn's disease and ulcerative colitis) and rheumatoid arthritis (Emilsson et al., 2015).

2.2.7 Approaches to investigate signalling pathways and cellular mechanisms in Coeliac Disease

Research investigating CD is difficult, in part because the disease only affects humans, with no accurate animal model available (Valitutti & Fasano, 2019). A variety of models have been used to study the mechanisms of CD both *in vivo* and *in vitro*.

Experimental models of CD

In vivo models*:*

In vivo studies to investigate the pathophysiological mechanisms underlying CD have been conducted over the last several decades. These have included *in vivo* challenges which demonstrated rapid damage of enterocytes following infusion of gluten fractions into the small intestine. In early studies, histological damage, including deterioration in the villus architecture, was reported in biopsies taken at hourly intervals after gluten administration (Freedman et al., 1987). Exposure to intraduodenal infusions of different doses of either unfractionated gliadin, or α, β, γ or Ω -gliadin subfractions induced damage in the mucosa of jejunal biopsies taken 2-6 h later (Ciclitira et al., 1984). Similarly, exposure to high molecular weight glutenin subunits in volunteers with CD decreased the villus height: crypt depth ratio; the expression of IL-15 in the small intestine was also increased within 2 hours (Dewar et al., 2006). Although these *in vivo* studies detected rapid changes in enterocyte morphology, administration of gluten *in vivo* does not allow an in-depth identification of the cellular mechanisms which underly the pathophysiological changes. For this reason, other models have been developed.

A number of different transgenic and knockout mice have been generated in studies designed to identify the mechanisms involved in the development of celiac disease (Stoven et al., 2013). These studies have demonstrated the importance of HLA-DQ8 and DQ2 in the inflammatory T-cell response against gliadin but have also made it clear that other factors are necessary for gluten to trigger enteropathy such as villous atrophy. Recent research conducted at the University of Chicago has led to development of the first transgenic mouse model of CD which not only has the genetic predisposition but also manifests the immune system defects found in CD patients. These HLA-DQ8 transgenic mice were the first transgenic mouse model which develops villous atrophy in the small intestine following

gluten consumption which is ameliorated when they are returned to a GFD (Abadie et al., 2020), reflecting the situation in humans. Animal models like this will provide the opportunity to develop new drugs for treatment of CD, and hopefully prevention of the development of CD, in people genetically at risk.

In vitro models:

An alternative to *in vivo* studies is the use of *in vitro* preparations of intestinal tissue which offers the potential to more easily manipulate their exposure to molecular triggers and to monitor responses at the cellular level. One of the most commonly used cell lines to address many aspects of intestinal cell function are Caco-2 cells. Studies using these and other intestinal cell lines such as IEC-6, have reported cytoskeletal changes, tight junction disruption and other effects of gluten peptides which undermine epithelial barrier integrity in confluent monolayers of these cells. It has also been reported that the α-gliadin peptide p31–p43, which stimulates the innate immune response in CD, triggers production of the inflammatory cytokine IL-15 which, in turn, stimulates proliferation of Caco-2 cells, as it does with enterocytes in CD biopsies (Barone et al., 2011). The p31–43 gliadin peptide also tiggers the expression of tissue transglutaminase 2 by Caco-2 cells (Caputo et al., 2012).

A major shortcoming of cell lines is that they are comprised of a single cell type whereas the intestinal epithelium is comprised of seven major cell types which mutually interact and communicate as a crucial part of normal epithelial function. Although Caco-2 cells have been extensively used as a model for the small intestinal epithelium, they are not only a single cell type, analogous to none of the cell types present in the small intestine, but they are actually derived from colon cancer (Sun et al., 2008) and not cells from the small intestine.

The ideal source of tissue for *in vitro* studies is from intestinal biopsies from CD patients, which can then be compared with non-CD controls. Intact pieces of

biopsy material from coeliac patients have been studied in organ culture. Using this approach, tissue is maintained in an oxygen-rich environment in contact with culture medium but, unlike normal tissue culture, the tissue is not submerged (Cooper et al, 2015). Using this model, addition of gluten-derived proteins has been shown to trigger rapid changes in enterocyte morphology, including a reduction in enterocyte height (Aksnes & Fluge, 1981). Other studies have also reported an increased level of cell death by apoptosis in enterocytes (Mazzarella et al., 2008). Organ culture studies have also reported that a peptic/tryptic digest of gluten resulted in disruption of the enterocyte cytoskeleton via effects on microtubules, microfilaments and intermediate filaments detectable after 4 hours of exposure but increasing by 24 hours (Dunne et al., 2020).

Although organ culture of biopsy material has been informative, its limitations are restrictive. Valuable and infrequently available biopsy material can only be maintained in culture for around 24 hours and access to individual cells and membranes within the tissue is extremely limited (Stoven et al., 2013). The ability to grow, expand and maintain authentic intestinal tissue for weeks in tissue culture, without loss of function, would bypass the 'use once' and restricted lifetime of the rarely available tissue biopsies and provide access to the basolateral membrane of the epithelium in intact intestinal epithelium. The ability to grow and maintain tissue in culture for several months without functional deterioration, and to convert some of this tissue into confluent single-cell thick confluent monolayers, would provide immediate access to both apical and basolateral cell membranes to examine transport processes and allow cell junction, cytoskeletal and transepithelial transport to be assessed in ways that no other model system permits.

The introduction of small intestinal organoid models in recent years has provided a novel approach for the investigation of disease. These models mimic the small intestine, demonstrating matching histology and allowing researchers to

study aspects of disease that were previously not possible (Kim et al., 2020). The use of organoid models in CD has been surprisingly slow, with the first paper published in May 2019 (Freire et al., 2019). The model allowed the authors to isolate duodenal crypt stem cells from biopsy samples collected during routine endoscopy in CD patients and healthy controls. These crypt stem cells were then used to establish intestinal organoid monolayers, allowing the authors to investigate cell differentiation, intestinal permeability, and cytokine activity, comparing these between healthy and CD organoids. The intestinal organoid model provides a powerful approach to assessing CD pathogenesis by comparing biopsy-derived organoids from CD and control patients with the ability to monitor cell function directly, maintain and grow these organoids in culture and produce from them single cell monolayers with access to both the apical and basolateral surfaces and the ability to dynamically monitor transepithelial transport and cell junction integrity.

2.3 The gluten-free diet

2.3.1 Definition

Gluten is found in wheat (gliadin), barley (hordein) and rye (secalin) (Lebwohl et al., 2018). All of these forms of gluten must be removed from the diet in people with CD, with even a small level of cross contamination having the potential to trigger an immune response (Wieser et al., 2021a). Internationally the Codex Alimentarius requirement for a food to be considered GF is that contains less than 20 parts per million (ppm); 20 milligrams of gluten per kilogram of product (Codex Alimentarius Commission, 2008). However, both New Zealand and Australia have much stricter cut-offs, with a food required to have less than 3ppm to be considered GF (Coeliac New Zealand, n.d.-b). These stricter requirements can make it difficult for manufacturers if food is prepared in the

same facility as food containing gluten, so many GF food manufacturers have separate facilities for such preparation.

2.3.2 Oats

In addition to the grains mentioned above, which are always excluded in a GFD, there is also controversy over whether oats should also be omitted. Although oats do not contain gluten, they contain a similar proline-rich protein called avenin (Sharma et al., 2020). In addition, the production of oats often results in an increased risk of cross contamination with gluten; with oats commonly sown, cultivated, harvested, milled and processed in close proximity to wheat, barley and rye. A recent meta-analysis by Guennouni et al, (2022) identified oats as the food most commonly contaminated with gluten.

In New Zealand, adherence to a GFD includes exclusion of all oats, including those which are free from contamination (presence of gliadin, hordein and secalin) (Food Standards Australia New Zealand, 2022). Coeliac New Zealand supports this recommendation based on the research of Dr Robert Anderson, a patron of Coeliac New Zealand, who reported that a small portion of people with CD (20%) have a similar response to the avenin in oats, as they do to other gluten-containing foods. However, it is interesting to note that in Dr Anderson's more recent publications only 8% of his patients responded to avenin in an oats challenge of 100g dry weight oats per day (Hardy et al., 2015). This study found that the amount of oats in a recommended daily serving is not sufficient to cause symptoms in individuals with CD (Hardy et al., 2015). The contradictory nature of these findings is consistent with international research which report low quality RCTs and uncertainty regarding the role of oats in CD (De Souza et al., 2016; Pinto-Sánchez et al., 2017).

2.3.3 Adherence to the gluten-free diet

Strict adherence to a GFD is important; a recent review suggested tolerable limits vary between individuals, indicating intakes of greater than 10-50mg gluten per day are sufficient to result in mucosal damage (Cohen et al., 2019). In addition, intake of gluten, even from minor contamination, can trigger symptoms resulting in discomfort (Wieser et al., 2021b). However, assessment of adherence is difficult and variation in methods results in significantly different adherence outcomes (Hall et al., 2009). The most accurate assessment of adherence involves longitudinal studies of biopsies to detect any evidence of ongoing gastrointestinal damage (Rodrigo et al., 2018). Yet, this method is invasive, costly and not considered a practical assessment of adherence in most circumstances. Alternative methods for assessing adherence include consideration of serology, such as raised antibodies to tTG and EMA, which can indicate ongoing damage. However, after starting a GFD, levels of both antibodies can be slow to return to normal: a study found antibody levels took more than 2 years to normalise in 1/3 of children (Gidrewicz et al., 2017). In addition, serology assessment requires participants to have a venous blood sample taken which may not be feasible in larger studies. It is therefore more common for research studies to assess adherence through questionnaires, either alone or in combination with serological testing (Gładyś et al., 2020). Adherence questionnaires can indicate the extent that an individual is adhering to the diet and how often they are accidentally or intentionally consuming gluten (Wieser et al., 2021b). Although several adherence questionnaires are commonly used, these generally rely on participants' recall and honesty, with self-reported responses to questions possibly being associated with participant bias (Gładyś et al., 2020).

These difficulties make it hard to estimate adherence to the diet. This is highlighted in a systematic review by Hall et al. (2009) which identified an adherence rate of between 42 and 91% depending on the method used for

assessment. Despite the difficulties faced, assessing adherence to the dietary recommendations is important because although it is presented as a simple solution for a complex disease, the GFD is known to be difficult for patients to follow, because of contamination risk, additional costs, and poor quality of food (Abu-Janb & Jaana, 2020). Misidentification of GF foods can result in unintentional gluten intake, leading to ongoing mucosal damage (Paganizza et al., 2019) and increased risk of morbidity.

There is confusion among both consumers and food producers regarding labelling of GF products and cross contamination risk, which often results in poor identification of foods that are considered safe (Gutowski et al., 2020; Lerner et al., 2019). Although New Zealand has extensive laws regarding labelling of GF products, this does not limit cross contamination risk of those foods which naturally contain no gluten and so do not need to claim to be GF. A recent meta-analysis (Guennouni et al., 2022), investigated contamination of both naturally GF foods, processed foods labelled as GF and meals provided by food services which were said to be GF. Overall, 15.2% of foods analysed were found to contain more than 20mg/kg of gluten, a level sufficient to prevent these foods from being considered GF. The most contaminated of these sources were foods considered to be naturally GF, with 28.32% of samples contaminated with gluten. Although most studies in this analysis were carried out in Europe and America, as no comparable studies have taken place in New Zealand, it is impossible to rule out a similar level of contamination in the GFD in New Zealand. Testing of products in New Zealand is only required for those marketed and labelled as 'gluten-free'; contamination levels of naturally GF products and meals provided by food services are currently impossible to estimate.

Due to concerns about cross contamination, eating out is a common challenge raised by people with CD (Ciacci & Zingone, 2015), with a Canadian study reporting that 48% of patients with CD avoided dining out at restaurants

(Zarkadas et al., 2006). Dimidi et al. (2021) reported that individuals with CD had greater difficulty in finding suitable foods when eating out, resulting in reduced dietary adherence. However, despite this, a third of participants in a questionnaire of New Zealanders and Australians with CD reported they did not ask about cross contamination risks when dining out (Sainsbury et al., 2018), potentially putting themselves at increased risk.

Another barrier for adherence to the GFD is the additional cost of GF products, with GF alternatives to foods which usually contain gluten, costing significantly more (Capacci et al., 2018). Estimates of how much more the GFD costs vary depending on the products assessed and the country these studies are carried out. However, research suggests GF products are generally at least 113% more expensive than their gluten-containing counterparts and that for some products, such as flour and flour mixes, the additional cost is greater than 400% (Myhrstad et al., 2021). These higher costs are supported by recent data from the USA assessing the economic burden of the GFD, which indicated that although prices have begun to decline and move towards the cost of comparable products containing gluten, these are still on average 183% of gluten-containing products (Lee et al., 2019). In New Zealand, where GF products are less readily available and options are more limited, these GF products have been reported to be 1.7 to 5 times more expensive than gluten-containing equivalents (Edmonds, 2016).

Despite these barriers, numerous studies report better adherence to a GFD is associated with an increased quality of life (QoL) (Al-Sunaid et al., 2021; Casellas et al., 2015; Choung et al., 2020; Enaud et al., 2022; Rustagi et al., 2020). However, evidence indicates hypervigilance of the GFD can be associated with reduced QoL, possibly as a result of increased anxiety (Wolf et al., 2018). In addition, anxiety regarding cross contamination risk and how to identify safe foods has been associated with increased food restriction, with participants reportedly reducing their overall food intake (Satherley et al., 2017). These findings indicate that

although adherence to the GFD can result in significant benefits to the wellbeing of people with CD, lack of understanding or education regarding safe foods can result in additional stressors that impact QoL. This highlights the importance of education and support in these individuals.

Follow up

International guidelines advise that patients with CD are regularly followed up (Al-Toma et al., 2019), including repeated serology testing to ensure that they are adequately adhering to the diet. Furthermore, it has been suggested that dietary advice from a nutritionist or dietitian can promote understanding of the GFD and food labels (Gładyś et al., 2021; Moore et al., 2018). The research findings suggest individuals with greater knowledge about the GFD have better adherence (Halmos et al., 2018). Education programmes about cooking and culinary skills related to the GFD result in improved attitudes and self-efficacy in newly diagnosed CD patients (Hasan et al., 2019), suggesting additional guidance in this area could support individuals who struggle with the move to the GFD.

Reliance on other sources for advice and information may lead to newly diagnosed patients sourcing inaccurate information and making poor dietary choices. An investigation of worldwide websites designed to provide advice to people with CD, including those run by coeliac organisations, hospitals, universities and 'alternative' health practitioners, found only 33% of sites scored greater than 50% on an assessment of accuracy of information provided, with only 3.2% scoring greater than 75% accuracy (England & Nicholls, 2004). Although, no similar studies have been undertaken in New Zealand, interpretation of the accuracy of information on the internet is made additionally difficult because of the different recommendations compared to other countries (Cohen et al., 2019).

2.3.4 Nutritional implications of a gluten-free diet

Nutritional deficiencies are a common consequence of CD; at diagnosis, the most commonly identified deficiencies are of iron, vitamin D, calcium, vitamin B12, folic acid and zinc (Kreutz et al., 2020) though deficiencies in other nutrients are likely but usually not assessed. A recent review (Raiteri et al., 2022) compared the guidelines for management of CD compared from 7 reputable clinical and scientific organisations (American College of Gastroenterology 2013, British Society of Gastroenterology 2014, UK National Institute for Health & Care Excellence 2015, Central Research Institute of Gastroenterology Russia 2016, World Gastroenterology Organization 2017, European Society for the Study of Coeliac Disease 2019 and the European Society Paediatric Gastroenterology, Hepatology and Nutrition 2020). The guidelines were highly concordant and all of them recommended that patients should be tested for micronutrient malabsorption at both diagnosis and follow-up; nutrients identified to be of concern were iron, folate, vitamin B12, calcium, phosphate and vitamin D.

After diagnosis, once a GFD has been adopted, the absorptive surface of the small intestine is expected to recover and nutrient absorption to return to a normal healthy level so nutrient deficiencies should be resolved. There are two significant issues. The first is time taken to make a complete histological recovery which may be lengthy, particularly in adults when time to diagnosis could be long and associated with more extensive mucosal damage (Hære et al., 2016). Many research studies report on improvements in micronutrient levels after one year of removing gluten from the diet (Kreutz et al., 2020). However, unintentional gluten consumption may complicate the healing process and delay recovery. The second issue is whether the GFD is as nutritionally adequate as the one it replaced (Miranda et al., 2014). Research suggests that GF products are higher in both total and saturated fat (Vici et al., 2016) and very few products are fortified with micronutrients compared with their gluten-containing counterparts (Bascuñán et

al., 2017). It has also been reported that a GFD is more likely to be insufficient in B12, folate, and iodine (Bituh et al., 2011; Mijatov & Mičetić-Turk, 2016).

In addition to nutrients which are commonly found to be consumed in low quantities because the diet excludes foods containing gluten, there is evidence that some nutrients continue to be poorly absorbed regardless of intake, because of continued malabsorption. Due to the difficulties in assessing adherence to the GFD, it is unclear if this malabsorption is a result of gut damage due to ongoing unintentional consumption of gluten or whether malabsorption continues due to irreparable damage, irreversible changes to the small intestinal mucosa or persistent inflammation (Kreutz et al., 2020).

Iron

Iron deficiency is a common presenting symptom of CD; diagnosis of unexplained iron deficiency anaemia (IDA) often leads to referral for testing for CD (Oxentenko & Murray, 2015). Recognition of IDA as a presenting feature of CD has tended to result in much earlier diagnosis of the condition (Montoro-Huguet et al., 2021). Enterocytes, located in the small intestine are the site of iron absorption, so villous atrophy reduces both the number of these cells, and consequently the area available for nutrient absorption and the number of divalent metal transporters (Talarico et al., 2021) which transport iron into the enterocyte. This is further exacerbated by damage to the brush border which reduces the expression of duodenal cytochrome B (DcytB), a ferrireductase located on the brush border of the enterocyte which reduces ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), the form that can be transported by the divalent metal transporters across the gut (Martín-Masot et al., 2019). Iron deficiency is estimated to affect 6-82% of adults with treated CD (Kreutz et al., 2020).

Despite adherence to a GFD, many adults with CD continue to have impaired iron absorption (Stefanelli et al., 2020) after diagnosis. This may be due

to repletion of iron stores being slow so biomarkers indicating iron repletion are not in the normal range when the assessment of iron status is undertaken after a certain period on a GFD (usually 12 months after diagnosis of CD). In the case of iron, persistent inflammation may affect iron absorption. Inflammatory states stimulate hepatic production of the hormone, hepcidin. Hepcidin binds to ferroportin, the iron exporter located on the basal side of the enterocytes, causing it to be internalised and degraded so newly absorbed iron remains sequestered within the enterocyte. Hepcidin similarly blocks ferroportin exporting recycled iron from the macrophages involved in the phagocytosis of effete red blood cells. The trapping of circulating iron in the reticuloendothelial system is part of the acute inflammatory response, important in host defence, which can lead to anaemia of inflammation (anaemia of chronic disease) if inflammation persists (Pagani et al., 2019). The effect of a chronic inflammatory state in individuals with CD, which may persist after gluten has been excluded from the diet, could have a dramatic effect on iron status, not only affecting iron absorption and recycling of red blood cells but also being refractory to treatment by diet or iron supplements.

Zinc

Zinc deficiency is also common at the time of diagnosis, with low levels of zinc reported in 67% of adult patients (Kreutz et al., 2020). Zinc is absorbed in the duodenum and jejunum, with villous atrophy likely to be the primary reason for this observed deficiency. Although research suggests that zinc deficiency is usually alleviated after a year of adhering to a GFD (Caruso et al., 2013), it persists in up to 20% of patients after 2 years on a GFD (Kreutz et al., 2020) perhaps as a result of some of the GF alternative products being low in zinc.

Calcium and Vitamin D

At time of diagnosis, the prevalence of hypocalcaemia and low vitamin D levels in adults were reported to be 0-26% and 5-88% respectively (Kreutz et al., 2020) and to improve after the adoption of a GFD. Absorption of both nutrients occurs in regions of the proximal small intestine which are most likely to be affected by CD. Usually, calcium homeostasis is tightly regulated so despite poor absorption (or intake), serum calcium might be expected to be buffered by reduced calcium excretion and calcium released from bone resorption (Wawrzyniak & Suliburska, 2021). Dietary vitamin D is often not a significant contributor to vitamin D status because endogenous synthesis in skin exposed to ultraviolet light is frequently the main source until older life. However, several of the studies also reported vitamin D insufficiency in the control (non-CD) population.

The hormone precursor vitamin D3, synthesised in the skin, is biologically inactive and requires two hydroxylation steps for the active form (calcitriol) to be produced (Bikle & Christakos, 2020). The first hydroxylation occurs in the liver to produce 25(OH) vitamin D. Vitamin D status is usually assessed by measuring the level of 25(OH)vitamin D which is 100-1000 times the level of the biologically active form, 1,25$(OH)_2$vitamin D, which is produced after the second hydroxylation step in the kidney or intracellularly. This second hydroxylation is carried out by 1α-hydroxylase (CYP27B1) which is simulated by parathyroid hormone (PTH).

Although 25(OH)vitamin D levels are lower in CD patients than controls, a meta-analysis (Zingone & Ciacci, 2018) of the studies that also measured 1,25$(OH)_2$vitamin D found that levels of the unbound active metabolite were higher in CD patients prior to treatment compared to those on a GFD and controls. The authors interpreted these findings to mean that calcium malabsorption in CD is not a result of vitamin D deficiency but may be due to decreased calcium-binding proteins produced by the enterocytes.

Vitamin D is implicated in CD (Vici et al., 2020) because it affects both regulation of the immune system and the integrity of the gut barrier function. It has been postulated that vitamin D deficiency is associated with the development of CD in children and likely to be responsible for the association between season of birth and incidence of CD (Tanpowpong & Camargo, 2014). However, Bittker (2015) controversially proposed that high doses of vitamin D supplementation were a risk factor for inducing allergic disorders including CD in children, identifying oral vitamin D drops given to infants for longer than 3 months as a possible environmental trigger. Bittker supported this hypothesis by suggesting that the "Swedish Epidemic" of CD in 1984-1996 was linked to the mandatory fortification of milk and margarine with vitamin D in Sweden from 1983 (Bittker, 2020). The possible mechanisms include increased activity of Th2 cytokines which upregulate the sensitivity of the immune system to external stimuli. However, this hypothesis is not supported by other studies (Rewers et al., 2018) and alternative explanations have been proposed for the "Swedish Epidemic" (King et al., 2020). Once a GFD is adopted, calcium levels appear to return to normal healthy levels (Kreutz et al., 2020) whereas vitamin D status tends to be similar to the status of the broader population.

B vitamins

Wheat-based foods are rich in most B vitamins, except vitamin B12. A recent narrative review indicated that folate deficiency was present in 11-75% of adults with CD at the time of diagnosis (Kreutz et al., 2020). Adoption of a GFD, which permits healing of the mucosal lining of the gut, tends to be associated with normalisation of vitamin status because absorption increases. However, folate deficiency continues to be more common on a GFD than in healthy control groups (Cardo et al., 2021), particularly in women (Vici et al., 2016). In many countries, inadequate folate intake has been targeted through the introduction of mandatory

fortification of folic acid, with bread usually the selected staple-food vehicle for delivery. Although implementation of this is underway in New Zealand, with manufacturers required to fortify bread with folic acid by mid-2023, GF products are exempt from the mandatory requirement (Ministry for Primary Industries, 2021) and as such individuals with CD may not benefit from this fortification programme and could continue to be at risk of folate deficiency.

Vitamin B12 deficiency is reported in 5-19% of patients with CD prior to treatment (Kreutz et al., 2020). Interestingly there is currently no consensus for the cause of low vitamin B12 at the time of diagnosis in individuals with CD. Vitamin B12 is derived from foods of animal origin, including milk, so vegans are at risk of deficiency. The vitamin is released from the food matrix by hydrochloric acid and pepsin in the stomach where it binds to haptocorrin (R-binder) produced by salivary glands or intrinsic factor (IF) produced by the parietal cells of the stomach (Sobczyńska-Malefora et al., 2021). In the acidic environment of the stomach, the binding affinity of haptocorrin for vitamin B12 is greater but pancreatic proteases in the duodenum release B12 from haptocorrin and it then binds to intrinsic factor. The vitamin B12-IF complex reaches the terminal ileum and binds to cubam receptors on the enterocytes which then endocytose it.

It is thought that the stomach is not affected by CD so comprised production of IF is not a likely explanation for B12 deficiency at diagnosis. Intestinal damage in the small intestine in CD was thought to primarily affect the proximal duodenum, with the terminal ileum, where B12 absorption takes place, generally not impacted by villous atrophy (Sobczyńska-Malefora et al., 2021). Inspection of inflammatory damage to the entire length of the small intestine has been limited using standard flexible endoscopy and most investigation of suspected CD uses upper gastrointestinal tract (GIT) endoscopy, so it is limited to the duodenum and excludes the terminal ileum which must be investigated by colonoscopy. However, in studies which have also investigated the terminal ileum

in suspected CD, a proportion of patients had raised intraepithelial lymphocytes associated with the tissue, suggesting that the damage was more extensive and not restricted to the upper regions of the small intestine (Hopper et al., 2006).

Iodine

A study based in Slovenia by Mijatov et al. (2016) reported low intake of iodine in coeliac participants who had been adhering to a GFD for a minimum of a year, with intake estimated to be 53-64% of daily requirements. Similar findings were described in an Italian study by Churruca et al. (2015) which reported inadequate iodine intake in 94% of premenopausal women with CD who had been consuming a GFD for a year or more. In addition, suboptimal iodine intake was reported in a Norwegian study in adults consuming a GFD for 6 months or more due to diagnosis of NCGS (Skodje et al., 2019). These findings suggest that the GFD may be contributing insufficient levels of iodine.

Concerns regarding absorption of iodine in people with CD have also been raised by a recent longitudinal pilot study by Delvecchio et al. (2021) which investigated iodine absorption in Italian children. This study reported that iodine absorption was impaired in children with CD, with no significant improvements in iodine status after adherence to a GFD for a year. Although the study had a small sample size and only assessed iodine status in children, these findings have implications for those with CD in New Zealand, where iodine deficiency is a known issue due to low levels in the food supply (Brough & Skeaff, 2020). This concern is exacerbated further by evidence suggesting that GF breads may have a lower level of iodine supplementation compared to gluten-containing breads (New Zealand Institute of Plant and Food Research, 2013), indicating that those on a GFD may not be benefiting to the same extent.

2.3.5 Fibre

The gluten-containing grains, predominantly wheat, are consumed widely and provide about a quarter to a half of the energy intake in both industrialised and developing countries (Raiteri et al., 2022). These foods are also significant contributors to the intake of fibre, protein, B vitamin, calcium, magnesium, phosphorus, potassium, zinc and iron (Sabença et al., 2021). However, the composition of wheat varies depending on variety, for instance, there are over 25,000 varieties of Triticum aestivum L (de Sousa et al., 2021) which is the main wheat used to produce bread flour, and the composition is affected by the region where it is grown and growing conditions. The nutritional composition can also be deliberately manipulated to improve its profile; examples include using soil and foliar fertilisers containing sodium selenate to biofortify wheat with selenium (Radawiec et al., 2021) and producing transgenic (genetically modified) varieties of wheat which have increased bioavailability of iron and zinc (Balk et al., 2019). Wheat flours used in bread making may also be mandatory or voluntary fortified with minerals and vitamins (de Sousa et al., 2021). The type of bread or wheat products commonly consumed depends on culture (Lockyer & Spiro, 2020) and, in many countries, food based dietary guidelines promote fibre intake, but consumers may preferentially chose wheat-based foods made from less refined grains.

This variability makes it difficult to generalise about the typical nutrient composition of wheat-based foods and to compare GF products with their gluten-containing counterparts. Wheat confers unique viscoelasticity to foods and there is no single substitute that can replace it. The formulation of GF foods tends to be driven by economics and replicating the organoleptic properties rather than considering the nutritional contribution. However, research studies which have compared the nutritional adequacy of GF foods with the best-matched gluten-containing counterpart have identified that most GF foods have lower protein and

higher fat content than gluten-containing products (Aguiar et al., 2021). Fibre content tends to be variable, and many products have a high glycaemic index because rice flour and starch are frequently used in the formulations (Giuberti & Gallo, 2018). It is suggested that the observation that the GFD is associated with increased risk of cardiovascular disease and type 2 diabetes is because individuals consuming the GFD have a lower fibre intake (Wünsche et al., 2018).

2.4 Bone

2.4.1 Prevalence of osteoporosis

Worldwide, it is estimated that osteoporosis affects more than 200 million women (International Osteoporosis Foundation, 2017). In 2007 there were 84,000 osteoporotic fractures reported in New Zealand (International Osteoporosis Foundation, 2017). The total direct medical cost of the disease, in New Zealand, was predicted to be over $458 million/year by 2020 (Brown et al., 2011). Osteoporosis is characterised by a reduction in bone mass and deterioration in the micro-architecture of bone tissue which together result in an increased risk of fracture (Correa-Rodríguez et al., 2016; Høiberg et al., 2016). Osteoporotic fractures mainly occur in the vertebrae, distal radius or hip (Iseme et al., 2017). With an ageing population and increased prevalence of osteoporosis it is imperative to gain greater understanding of the risk factors and implications to minimise risks, maintain mobility and quality of life, and reduce the economic burden placed on healthcare.

One major determinant of osteoporosis and fracture risk is the development of peak bone mass (PBM). PBM is the maximum bone density achieved in an individual's lifetime (Heaney et al., 2000). PBM is determined by genetic factors (accounting for up to 80% of variability) as well as environmental factors and health status such as diet and physical activity (Rozenberg et al., 2020;

Weaver et al., 2016). There is no consensus on the exact age that PBM is achieved, with timing of accrual dependent on the skeletal site and gender. However, it is usually reported as being achieved in the second or third decade, with later and greater accrual in men compared with women (Rozenberg et al., 2020). In addition, there is gender variation in the rate of loss with a faster and earlier loss of bone density observed in women. This loss of bone density is markedly increased in women at menopause due to the rapid decline in oestrogen production (Chew & Clarke, 2018). With PBM initially lower and a faster rate of deterioration, achieving an optimal PBM is particularly important in women in order to reduce the risk of osteoporosis and fracture later in life.

2.4.2 Diagnosis of osteoporosis

The current "gold standard" for measuring bone mineral density (BMD) and diagnosing osteoporosis is Dual X-ray absorptiometry (DXA) (Hammad, 2016; Whittle et al., 2012). DXA uses the principle of differential absorption of two X-ray beams of different energies in order to quantify calcium levels in tissue (Kotze et al., 2016). The method is precise and easily reproduced (Ensrud et al., 2018). However, it is costly, clinically based, invasive (involving ionizing radiation) and requires a highly trained operator. It therefore cannot easily be used for screening purposes (Pisani et al., 2017) and must be avoided in certain population groups such as children and pregnant women.

The World Health Organization (WHO) defines osteoporosis as BMD more than 2.5 standard deviations (SD) below the average BMD for a healthy young adult, i.e., a t-score of less than -2.5 (Robertson et al., 2017). Whereas having bone density between 1-2.5 standard deviations below a healthy young adult (a T score between -1 to -2.5) is considered to put a person at a greater risk of developing osteoporosis and is defined as osteopenia (Iseme et al., 2017). In subsets of the population, the z-score is more appropriate because the comparison is to the BMD

of healthy age-matched individuals (Carey & Delaney, 2010; Shenoy et al., 2014). This includes premenopausal women, for whom diagnosis of osteopenia and/or osteoporosis are not recommended, with patients instead classified as being "below the expected range for age" if their z-score is lower than -2.0 and "within the expected range" if above -2.0 (Shenoy et al., 2014; International Society for Clinical Densitometry, 2019). The risk of fracture is reported to double with each standard deviation lost; a person with a t-score of -1.2 would be estimated to have double the risk of fracture as person with a t-score of -0.2 (Iseme et al., 2017).

Other methods of assessing osteoporosis risk include the use of Quantitative Ultrasound (QUS). QUS measures calcaneus bone quality by measuring the attenuation and velocity of ultrasound waves passing through the bone (Park et al., 2015). Using QUS data, osteoporosis is defined as a t-score of less than -2.5, osteopenia as a t-score of -1 to -2.5, with anything above -1 considered as neither osteopenic nor osteoporotic (Hammad, 2013). QUS is becoming increasingly popular due to being cost effective and portable (Li et al., 2022). It is also an ideal method for measuring young women as it is non-invasive, non-ionizing (Hammad, 2016) and is therefore still safe in pregnancy. Research in post-menopausal women suggests that data from QUS correlate well with that from DXA (Chin & Ima-Nirwana, 2013; Høiberg et al., 2016; Trimpou et al., 2010), however a study by Iida (2010) found a strong correlation but reported that QUS data cannot be used to accurately diagnose or predict osteoporosis. Few studies have investigated the relationship between QUS and DXA in young women (Hammad, 2013, 2016), however, one New Zealand study in young women found similar outcomes, demonstrating good correlation but poor predictive value for risk assessment (Schraders et al., 2019).

2.4.3 Cell physiology of bone

Bone is constantly remodelled to maintain mineral homeostasis; to ensure normal bone composition is maintained, the formation and resorption of bone must remain tightly coupled (Briot et al., 2017). The activity of bone cells and the signalling pathways that control their differentiation are therefore important to both ensure that optimal BMD is accrued (at peak bone mass) and that bone mass is maintained to reduce fracture risk.

Bone cells

During the activation phase, quiescent osteoblasts are stimulated to release osteoclast differentiation factors, such as macrophage stimulating factor (M-CSF) and receptor activator of nuclear factor-κβ (RANK) ligand (RANKL), which trigger pre-osteoclasts. Derived from mononuclear cells of macrophage/monocyte lineage, these pre-osteoclasts fuse to the bone and are differentiated into multinucleated osteoclasts (Iseme et al., 2017).

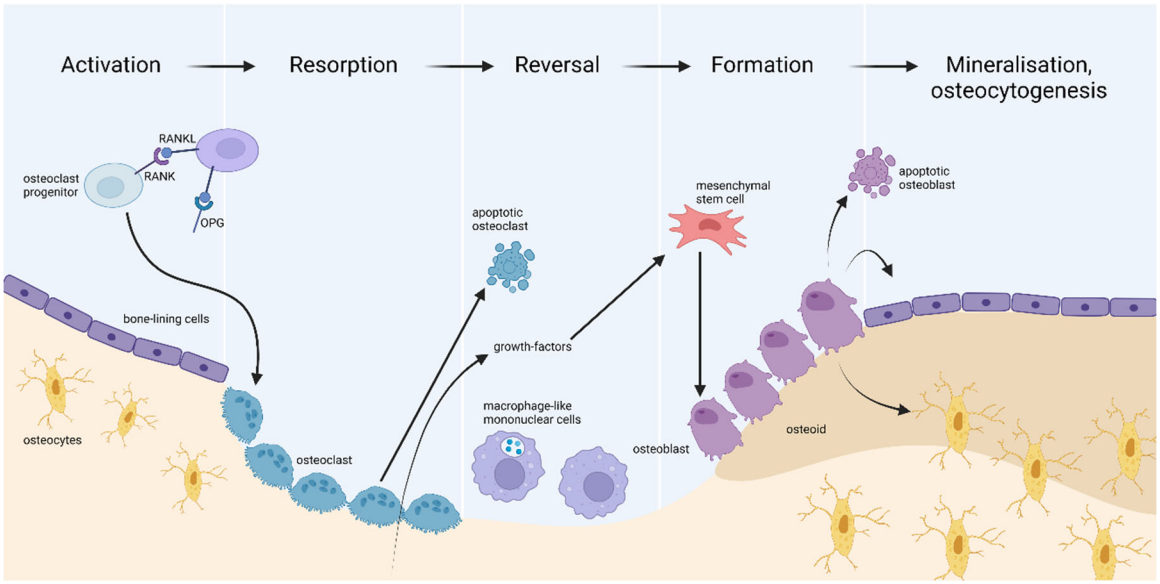

Figure 2.3. The stages of bone remodelling. Osteoclast progenitors are stimulated by RANKL to differentiate to osteoclasts which resorb old or damaged bone. New bone is then laid down by osteoblasts which are then apoptosed,

become bone-lining cells or are incorporated into the bone matrix as osteocytes (Created with BioRender.com)

Osteoclasts are bone-resorbing cells and during the resorption phase they adhere to the surface of the bone and dissolve it. The debris created by this degradation of the bone matrix is cleared by macrophage-like mononuclear cells in the reversal stage (Gu et al., 2017; Iseme et al., 2017). This leads to the release of growth factors stored in the bone matrix in the final phase. These growth factors stimulate bone formation through the recruitment of osteoblasts (Iseme et al., 2017). Differentiated from pluripotent mesenchymal stem cells, osteoblasts secrete organic bone matrix (osteoid), primarily type I collagen, and induce its mineralisation to rebuild the resorbed bone.

After bone formation, osteoblasts are incorporated into the mineralised bone matrix as osteocytes or remain on the bone surface (bone-lining cells) to be apoptosed. Osteocytes constitute over 90% of the bone cells found in the matrix (Amarasekara et al., 2015a) and act as mechanosensory cells and are the primary coordinators of osteoblast and osteoclast activity (Amarasekara et al., 2015b; Loi et al., 2016).

Bone remodelling

The RANK pathway is one of the central signalling pathways regulating bone loss (Iseme et al., 2017). Receptor activator of nuclear factor-κβ (RANK) is a receptor expressed by B and T-cells, fibroblasts, dendritic cells and both osteoclast progenitors and mature osteoclasts (Liu & Zhang, 2015). RANK is responsible for the initiation of signalling needed for osteoclast differentiation, through the binding of its ligand RANKL (RANK ligand), expressed by osteoblasts, on osteoclast precursors (Martin & Sims, 2015). This binding can be blocked by the soluble decoy receptor osteoprotegerin (OPG) also expressed by osteoblasts, which binds to RANKL and prevents the binding of RANK thereby inhibiting the

activation of osteoclasts, which in turn reduces bone loss. RANKL has been found in greater levels in postmenopausal women with osteoporosis suggesting that it may be a possible therapeutic target to reduce bone loss (Martin & Sims, 2015).

2.5 Coeliac Disease and bone

2.5.1 Relationship between Coeliac Disease and osteoporosis

In recent years there has been growing interest in the relationship between CD and bone, bought to light by epidemiological studies (Bianchi & Bardella, 2008); with low bone density presenting as fractures, falls, osteopenia and osteoporosis (Lerner & Matthias, 2016a). It is estimated that as many as 75% of patients with CD may suffer from reduced bone mass (osteopenia or osteoporosis) at diagnosis (Bianchi & Bardella, 2008; Galli et al., 2018). The low bone density has been demonstrated across age groups; when CD is diagnosed in childhood it is estimated that 1/3 of children have bone mass suggestive of osteopenia, with another 1/3 with bone mass low enough to indicate osteoporosis (Lerner & Matthias, 2016a). The greatest difference in BMD compared with healthy controls has been found in the axial skeleton; with the lumbar spine identified as the worst affected region and therefore findings from this site being of greatest interest to researchers (Kirsaçlioğlu et al., 2016).

The presence of low BMD in CD varies in studies depending on the method for assessing prevalence. Research findings tend to report either the prevalence of CD in osteoporosis or the prevalence of osteoporosis in CD. Overall, the former approach appears to report a lower prevalence of CD when assessing patients with osteoporosis; suggesting that bone density is not a concern in this population. However, the overwhelming majority of studies which have assessed the prevalence of osteoporosis in patients with diagnosed CD, have found it to be significantly greater than the prevalence in the general population.

2.5.2 Prevalence of Coeliac Disease in osteoporosis

A recent systematic review by Laszkowska et al. (2018) investigated the prevalence of biopsy confirmed CD in patients diagnosed with osteoporosis across 8 studies from 2015 and earlier. From this study a weighted pooled prevalence of 1.6% was determined. Although higher than reported prevalence in the general population, the authors concluded this was not sufficiently greater to justify screening of CD in patients with osteoporosis. Overall prevalence was greater in women at 2.3% compared with 1.5% in studies including men. Although the authors note that this difference was not significant, as no studies were completed exclusively in men (with only studies including men compared), it is probable this difference in prevalence may be higher than reported. In addition, there were insufficient data to allow for consideration of menopausal status of women or severity of osteoporosis.

In addition, a more recent study by de Bruin et al. (2020) investigated CD prevalence in women who had a recent fracture evaluated in a clinic in the Netherlands. Of 1042 women who participated in the study, only 4 had biopsy confirmed CD, giving a prevalence of 0.38%. The authors noted that although lower than the overall prevalence for western populations, this was equivalent to the prevalence previously found in a healthy Dutch population.

Researchers in New Zealand have also assessed the prevalence of CD in people with osteoporosis using DXA results collected from the Auckland District Health Board (ADHB) and Counties Manukau DHB (CMDHB) between 2008-2012 and 2009-2013 respectively (Bolland et al., 2016). They were able to ascertain the prevalence of CD in people who had received a DXA scan in this period and identified 137 adults who were referred for a DXA due to diagnosis of CD (61 from ADHB and 76 from CMDHB). The authors found that BMD on average was within the normal range (88-93% of z-scores were within normal range), albeit lower than expected. There were 16/137 adults identified as having BMD below the normal

range. However, the authors noted that this relationship appears to be associated with other bone risk factors independent of CD; had they excluded people with low body mass index (BMI) (<20kg/m^2) as well as those over 50 years old, 11/16 people identified as having below the normal range would still have been identified.

One concern regarding this method of estimating prevalence, is that patients are generally only referred for DXA if low BMD is expected, for example they have suffered a stress fracture, are postmenopausal or have a high family history of low BMD. This means only patients with CD who have experienced a fracture or are post-menopausal are likely to be identified and therefore does not accurately represent the prevalence of osteoporosis in the Coeliac population in New Zealand.

The findings of Bolland et al. (2016) have been contradicted by international research using the same method of screening people with osteoporosis; with results of one study identifying a frequency of CD 10 times greater than expected in the general population (Lucendo & García-Manzanares, 2013).

In addition, the variation in prevalence between studies may be in part due to the method of identifying CD in these patients. Most studies discussed have determined diagnosis of CD through biopsy, however many of these studies indicate greater prevalence through serology (de Bruin et al., 2020; Laszkowska et al., 2018). With studies reporting that some participants were reticent to undergo biopsy for further investigation, this may help to explain the low prevalence determined through this approach.

2.5.3 Prevalence of osteoporosis in Coeliac Disease

The prevalence of osteoporosis can also be assessed in patients already diagnosed with CD. A study in Rome, Italy, assessed the bone density in 214 patients (71.5% female) newly diagnosed with CD before they began a GFD;

identifying 60.3% with low BMD (Galli et al., 2018). Of the patients with low BMD, 42.5% were diagnosed with osteopenia (n=91) and 17.8% with osteoporosis (n=38). This study is unusual as it assessed bone density in both male and female patients with CD, finding that osteoporosis was more common in males with CD in this group. Unfortunately, this study did not assess vitamin D and calcium status and no follow-up was done to see if there was any improvement on a GFD.

The Women's Health in the Lund Area (WHILA) study screened 6917 women for raised tTG levels indicative of CD between 1995 and 2000 (Agardh et al., 2009). Of the total 117 women with detectable tTG levels, 59 had levels of 17U/mL or above indicating a positive result for CD. These women had both lower BMD and t-scores compared with women with tTG levels of <16.9; 0.41g/cm^2 vs 0.44g/cm^2 and -1.40 ± 1.28 vs -0.9 ± 1.40 respectively. Fracture frequency was also greater in these women with 32.2% suffering fractures during the study period compared with 18.8% of women with tTG <16.9U/mL.

2.5.4 Improvement of bone mineral density on a gluten-free diet

Many of the studies that have investigated the relationship between bone density and CD have measured BMD at initial diagnosis. However, research contradicts this method (unless followed up with a second DXA) as BMD has been shown to increase in CD patients within the first year of strict adherence to a GFD (Bathrellou et al., 2018). After this initial increase in the first year, no further significant improvements in BMD have been found in either adults (Bathrellou et al., 2018) or children (Kirsaçlioğlu et al., 2016).

A retrospective study in Brazil (Kotze et al., 2016) identified 41 female patients with CD with a mean age 46.1 ± 14.8 years old. Osteopenia and osteoporosis were identified in 56.1% and 29.2% of women respectively in the first DXA scan. Patients received their second DXA scan after a median time of 5 years (1-13 years); osteopenia and osteoporosis were still identified in 58.9% and 28.2% of

patients despite vitamin D and calcium supplements being offered to patients after first densitometry (no information regarding supplementation adherence). Although there was a slight improvement in BMD, particularly in the spine, there was no significant reduction in the incidence of osteoporosis or osteopenia and during this follow up period 25% of the osteoporotic patients developed fractures. It is important to note that due to the age range of participants in this study, 12% of patients started menopause during the follow up period.

In comparison, a prospective study by Pantaleoni and colleagues (2014) investigating bone density in 169 patients (23 males; 146 females), with a mean age of 38.9 (17-75) found improvements in BMD after a year on a GFD. At baseline osteopenia was identified in 62 patients with 36 patients diagnosed with BMD indicative of osteoporosis. After a year on a GFD, osteoporosis was only identified in 22 patients, whereas osteopenia was found in 75. There was significant improvement of BMD in the femoral neck in both pre- and post-menopausal women. However, there was no improvement found in the lumbar spine of either group. The researchers in this study reported that all women and men over 30 should receive a DXA at diagnosis of CD.

Many of the studies assessing bone density in patients with CD have focussed on children or post-menopausal women. Even in studies including younger women, few have noted the prevalence of young women with low BMD.

However, a recent study in England between 2013 and 2016 investigated the bone density of young adults (Pritchard et al., 2018). This study reported BMD of 260 patients who received a DXA no earlier than 12 months after diagnosis. Patients were identified through serology and duodenal biopsy and at the time of diagnosis were provided with calcium and/or vitamin D supplements if deficient. The researchers identified common risks of osteoporosis with BMD reducing with age, female sex, lower BMI and previous history of fracture. However, they also noted that half of participants under 50 years and all patients under 20 years of age

had low BMD despite having a normal BMI. BMD did also not appear to be associated with baseline vitamin D or with the supplementation of calcium and vitamin D.

2.5.5 Theories behind low bone mineral density in CD

Some of the proposed mechanisms linking poor BMD in people with CD include nutrient malabsorption, activity of pro-inflammatory cytokines, secondary hyperparathyroidism and uncoupled bone formation and resorption (Kotze et al., 2016).

Malabsorption

It was initially believed that BMD is impaired in people with CD due to the observed malabsorption resulting from intestinal damage (Lucendo & García-Manzanares, 2013). This malabsorption is believed to affect the absorption of calcium and vitamin D, together with other micronutrients required for bone such as vitamin K, iodine and phosphorus. However, lower BMD is found even in patients with no intestinal symptoms (Lucendo & García-Manzanares, 2013), affecting up to 75% of patients regardless of their symptomatic presentation (Lerner & Matthias, 2016a). Low BMD has been reported in over half of asymptomatic patients, including those with stage 1 and 2 Marsh scores, showing minimal to moderate damage to the intestine (Lerner & Matthias, 2016a).

It is important to note that lactose intolerance, which may result in reduced calcium intake, commonly accompanies CD. Although this condition should resolve in most people once gut damage has healed; inadequate advice given to patients results in many people continuing to exclude dairy products from their diet permanently. Research suggests that patients with CD have inadequate intakes of calcium rich foods, such as dairy products, even after commencing a strict GFD (Blazina et al., 2010).

Inflammation: pro-inflammatory cytokines

Both local and systemic inflammation are also suggested to play important roles in the lower BMD observed in CD (Bathrellou et al., 2018). Although inflammation is reduced in CD patients after commencing a GFD, it is often only abrogated and not completely resolved (Barone et al., 2022). Pro-inflammatory cytokines such as interleukin (IL) -1 and -6, and tumour necrosis factor α (TNF-α) are involved in osteoblast inhibition and the stimulation of osteoclasts (Kamycheva et al., 2017; Larussa et al., 2017). IL-6 has been demonstrated to have roles in bone resorption through recruitment of osteoclast precursors and stimulation of their differentiation (Di Stefano et al., 2013). IL-1 and TNF-α also stimulate osteoclastogenesis and therefore promote bone resorption. This inflammation is thought to be a major contributing factor to low BMD in CD; with ongoing inflammation on a GFD possibly implicated in the lack of complete recovery of BMD.

Secondary hyperparathyroidism

In untreated CD, PTH is often high due to malabsorption of calcium. PTH stimulates osteoclast activity and increases bone resorption to release calcium (Walsh et al., 2018). Raised PTH also results in increased activity of renal enzyme 1α-hydroxylase which activates vitamin D by converting 25-hydroxyvitamin D into 1,25-dihydroxyvitamin D; this enhances the absorption of calcium in the intestine (Di Stefano et al., 2013). However, in patients with active CD this is inefficient due to the damage to the small intestine affecting calcium absorption. In addition to its effect in untreated CD, raised PTH is also observed in patients maintaining a strict GFD, despite assumed mucosal recovery (Bianchi & Bardella, 2008). These findings suggest that PTH may continue to contribute to impaired BMD even in treated CD patients.

Immune system: M-cells

Another theory behind the relationship between low BMD and CD is the role of M-cells. Also known as microfold or membranous cells (Ohno, 2015), M-cells are specialised cells present in the follicle-associated epithelium (FAE) covering Peyer's Patches of the intestine (Corr et al., 2008). It is estimated that M-cells represent about 10% of the FAE cells covering the gut-associated lymphoid tissues (GALT) (Walsh & Choi, 2014). M-cells act as "gatekeepers", controlling the uptake and transport of antigens from the lumen of the intestine into the GALT (Kanaya et al., 2018). M-cells differ structurally from the enterocytes surrounding them; normal microvilli are absent on their apical membrane, but they instead possess short microfolds (hence the name microfold cell). On their basal membrane they also have deep invaginations which form sac-like structures (M-cell pockets) which contain immune cells such as lymphocytes and dendritic cells (DCs) (Ohno, 2015). M-cells are specialised to phagocytose ingested macromolecules, antigens and pathogens, and transcytose these to the underlying dendritic cells, APCs which will 'show' these antigens to the immune system (Mabbott et al., 2013; Mesin et al., 2012).

Differentiation of M-cells from $LGR5^{+}$ stem cells is stimulated by the secretion of RANKL from sub-epithelial stromal/dome cells (Kanaya et al., 2018). The ability of RANKL to stimulate the number and activity of M-cells in intestinal tissue, as well as promote bone resorption, may lead to a vicious circle of intestinal damage concurrent with bone loss (fig. 2.4). This is further supported by research finding a lower OPG:RANKL ratio in patients with CD who displayed lower BMD (Fiore et al., 2006). This would explain the relationship between CD and osteoporosis and the inability of a strict GFD to prevent osteopenia and osteoporosis.

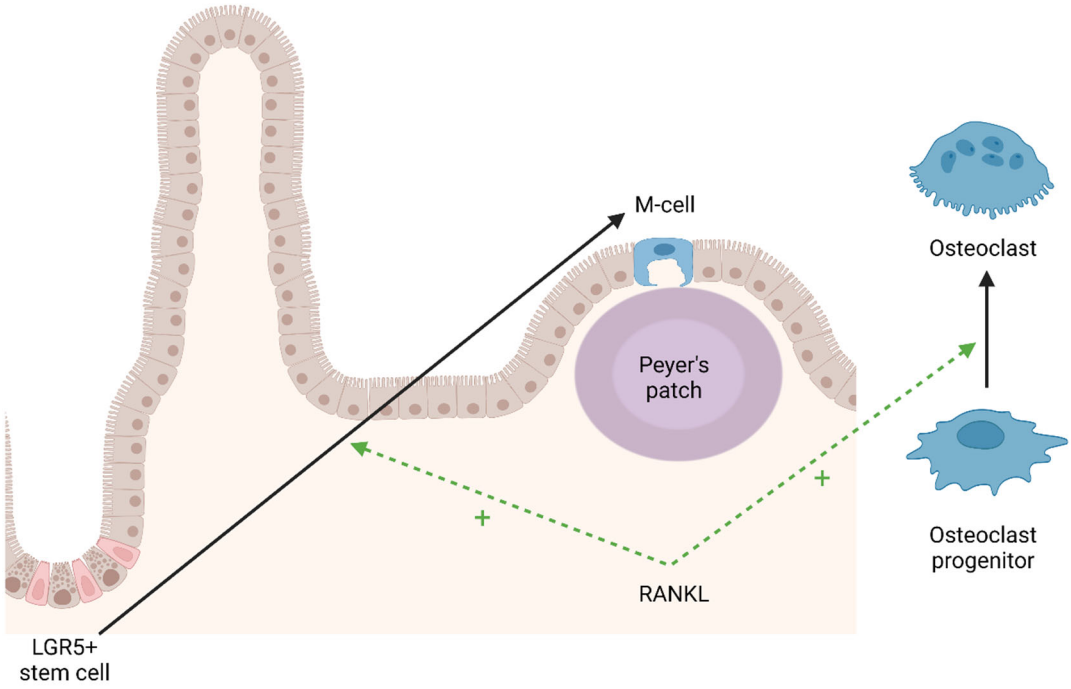

Figure 2.4 RANKL stimulation of osteoclast differentiation from osteoclast progenitor cells and M-cell differentiation from LGR5+ stem cells (Created with BioRender.com)

2.6 Conclusion

The physiology behind low BMD in patients with CD is currently not well understood, with previous suggestions regarding malabsorption and hyperparathyroidism not able to explain the findings in patients following a strict GFD. With an ageing population it is becoming increasingly important to understand the risks of developing osteoporosis to minimise the potential economic burden. The role of RANKL in both the translocation of gluten across the lamina propria and the differentiation of osteoclasts suggest that it may have a significant role in both the pathogenesis of CD and osteoporosis.

2.7 References

Aaron, L., Torsten, M., & Patricia, W. (2019). Autoimmunity in celiac disease: Extra-intestinal manifestations. *Autoimmunity Reviews*, *September 2018*, 0–1. https://doi.org/10.1016/J.AUTREV.2018.09.010

Abadie, V., & Jabri, B. (2014). IL-15: a central regulator of coeliac disease immunopathology. *Immunol*, *260*(1), 221–234. https://doi.org/10.1016/j.asieco.2008.09.006.EAST

Abadie, V., Kim, S. M., Lejeune, T., Palanski, B. A., Ernest, J. D., Tastet, O., Voisine, J., Discepolo, V., Marietta, E. V., Hawash, M. B. F., Ciszewski, C., Bouziat, R., Panigrahi, K., Horwath, I., Zurenski, M. A., Lawrence, I., Dumaine, A., Yotova, V., Grenier, J. C., … Jabri, B. (2020). IL-15, gluten and HLA-DQ8 drive tissue destruction in coeliac disease. *Nature*, *578*(7796), 600–604. https://doi.org/10.1038/s41586-020-2003-8

Aboulaghras, S., Piancatelli, D., Oumhani, K., Balahbib, A., Bouyahya, A., & Taghzouti, K. (2022). Pathophysiology and immunogenetics of celiac disease. *Clinica Chimica Acta*, *528*(February), 74–83. https://doi.org/10.1016/j.cca.2022.01.022

Abu-Janb, N., & Jaana, M. (2020). Facilitators and barriers to adherence to gluten-free diet among adults with celiac disease: a systematic review. *Journal of Human Nutrition and Dietetics*, *33*(6), 786–810. https://doi.org/10.1111/jhn.12754

Adelman, D. C., Murray, J., Wu, T. T., Mäki, M., Green, P. H., & Kelly, C. P. (2018). Measuring change in small intestinal histology in patients with celiac disease. *American Journal of Gastroenterology*, *113*(3), 339–347. https://doi.org/10.1038/ajg.2017.480

Admou, B., Essaadouni, L., Krati, K., Zaher, K., Sbihi, M., Chabaa, L., Belaabidia, B., & Alaoui-Yazidi, A. (2012). Atypical celiac disease: From recognizing to

managing. *Gastroenterology Research and Practice*, *July*. https://doi.org/10.1155/2012/637187

Agardh, D., Björck, S., Agardh, C. D., & Lidfeldt, J. (2009). Coeliac disease-specific tissue transglutaminase autoantibodies are associated with osteoporosis and related fractures in middle-aged women. *Scandinavian Journal of Gastroenterology*, *44*(5), 571–578. https://doi.org/10.1080/00365520902718929

Aguiar, E. V., Santos, F. G., Krupa-Kozak, U., & Capriles, V. D. (2021). Nutritional facts regarding commercially available gluten-free bread worldwide: Recent advances and future challenges. *Critical Reviews in Food Science and Nutrition*. https://doi.org/10.1080/10408398.2021.1952403

Akin, E., & Ersoy, O. (2012). Capsule endoscopy in celiac disease. *Gastroenterology Research and Practice*, *2012*. https://doi.org/10.1155/2012/676073

Aksnes, L., & Fluge, G. (1981). Morphological and morphometric assessment of human duodenal biopsies maintained in organ culture: In vitro influences of gluten in coeliac disease. *Scandinavian Journal of Gastroenterology*, *16*(4), 555–567. https://doi.org/10.3109/00365528109182012

Al-Sunaid, F. F., Al-Homidi, M. M., Al-Qahtani, R. M., Al-Ashwal, R. A., Mudhish, G. A., Hanbazaza, M. A., & Al-Zaben, A. S. (2021). The influence of a gluten-free diet on health-related quality of life in individuals with celiac disease. *BMC Gastroenterology*, *21*(1), 1–9. https://doi.org/10.1186/s12876-021-01908-0

Al-Toma, A., Volta, U., Auricchio, R., Castillejo, G., Sanders, D. S., Cellier, C., Mulder, C. J., & Lundin, K. E. A. (2019). European Society for the Study of Coeliac Disease (ESsCD) guideline for coeliac disease and other gluten-related disorders. *United European Gastroenterology Journal*, *7*(5), 583–613. https://doi.org/10.1177/2050640619844125

Amarasekara, D. S., Yu, J., & Rho, J. (2015a). Bone Loss Triggered by the Cytokine Network in Inflammatory Autoimmune Diseases. *Journal of Immunology Research*, *2015*. https://doi.org/10.1155/2015/832127

Amarasekara, D. S., Yu, J., & Rho, J. (2015b). Bone Loss Triggered by the Cytokine Network in Inflammatory Autoimmune Diseases. *Journal of Immunology Research*, *2015*, 1–12. https://doi.org/10.1155/2015/832127

Aronsson, C. A, Lee, H. S., Hård Af Segerstad, E. M., Uusitalo, U., Yang, J., Koletzko, S., Liu, E., Kurppa, K., Bingley, P. J., Toppari, J., Ziegler, A. G., She, J. X., Hagopian, W. A., Rewers, M., Akolkar, B., Krischer, J. P., Virtanen, S. M., Norris, J. M., & Agardh, D. (2019). Association of gluten intake during the first 5 years of life with incidence of celiac disease autoimmunity and celiac disease among children at increased risk. *JAMA - Journal of the American Medical Association*, *322*(6), 514–523. https://doi.org/10.1001/jama.2019.10329

Bach, J. F. (2018). The hygiene hypothesis in autoimmunity: The role of pathogens and commensals. *Nature Reviews Immunology*, *18*(2), 105–120. https://doi.org/10.1038/nri.2017.111

Baggus, E. M. R., Hadjivassiliou, M., Cross, S., Penny, H., Urwin, H., Watson, S., Woodward, J. M., & Sanders, D. S. (2020). How to manage adult coeliac disease: Perspective from the NHS England Rare Diseases Collaborative Network for Non-Responsive and Refractory Coeliac Disease. *Frontline Gastroenterology*, *11*(3), 235–242. https://doi.org/10.1136/flgastro-2019-101191

Bajinka, O., Tan, Y., Abdelhalim, K. A., Özdemir, G., & Qiu, X. (2020). Extrinsic factors influencing gut microbes, the immediate consequences and restoring eubiosis. *AMB Express*, *10*(1). https://doi.org/10.1186/s13568-020-01066-8

Balk, J., Connorton, J. M., Wan, Y., Lovegrove, A., Moore, K. L., Uauy, C., Sharp, P. A., & Shewry, P. R. (2019). Improving wheat as a source of iron and zinc for global nutrition. *Nutrition Bulletin*, *44*(1), 53–59. https://doi.org/10.1111/nbu.12361

Barone, M. V., Zanzi, D., Maglio, M., Nanayakkara, M., Santagata, S., Lania, G., Miele, E., Ribecco, M. T. S., Maurano, F., Auricchio, R., Gianfrani, C., Ferrini, S., Troncone, R., & Auricchio, S. (2011). Gliadin-mediated proliferation and

innate immune activation in celiac disease are due to alterations in vesicular trafficking. *PLoS ONE*, *6*(2). https://doi.org/10.1371/journal.pone.0017 039

Barone, M. V., Auricchio, R., Nanayakkara, M., Greco, L., Troncone, R., & Auricchio, S. (2022). Pivotal Role of Inflammation in Celiac Disease. *International Journal of Molecular Sciences*, *23*(13), 1–17. https://doi.org/10.339 0/ijms23137177

Bascuñán, K. A., Vespa, M. C., & Araya, M. (2017). Celiac disease: understanding the gluten-free diet. *European Journal of Nutrition*, *56*(2), 449–459. https://doi.org/10.1007/s00394-016-1238-5

Bathrellou, E., Kontogianni, M. D., & Panagiotakos, D. B. (2018). Celiac disease and non-celiac gluten or wheat sensitivity and health in later life: A review. *Maturitas*, *112*(January), 29–33. https://doi.org/10.1016/j.maturitas.2018.03.014

Bianchi, M. L., & Bardella, M. T. (2008). Bone in celiac disease. *Osteoporosis International*, *19*(12), 1705–1716. https://doi.org/10.1007/s00198-008-0624-0

Biesiekierski, J. R. (2017). What is gluten? *Journal of Gastroenterology and Hepatology (Australia)*, *32*, 78–81. https://doi.org/10.1111/jgh.13703

Biesiekierski, J. R., Newnham, E. D., Shepherd, S. J., Muir, J. G., & Gibson, P. R. (2014). Characterization of adults with a self-diagnosis of nonceliac gluten sensitivity. *Nutrition in Clinical Practice*, *29*(4), 504–509. https://doi.org/10.11 77/0884533614529163

Bikle, D., & Christakos, S. (2020). New aspects of vitamin D metabolism and action — addressing the skin as source and target. *Nature Reviews Endocrinology*, *16*(4), 234–252. https://doi.org/10.1038/s41574-019-0312-5

Bittker, S. (2015). Exposure to excessive oral vitamin D in youth: a risk factor for celiac disease in later life? *Journal of Allergy and Asthma*, *2*(1), 2. https://doi.org/10.7243/2054-9873-2-2

Bittker, S. S. (2020). Elevated levels of 1,25-dihydroxyvitamin D in plasma as a missing risk factor for celiac disease. *Clinical and Experimental*

Gastroenterology, *13*, 1–15. https://doi.org/10.2147/CEG.S222353

Bituh, M., Žižić, V., Krbavčić, I. P., Zadro, Z., & Barić, I. C. (2011). Gluten-free products are insufficient source of folate and vitamin B12 for coeliac patients. *Food Technology and Biotechnology*, *49*(4), 511–516.

Blazina, Š., Bratanič, N., Šampa, A. Š., Blagus, R., & Orel, R. (2010). Bone mineral density and importance of strict gluten-free diet in children and adolescents with celiac disease. *Bone*, *47*(3), 598–603. https://doi.org/10.1016/j.bone.2010.06.008

Bolland, M. J., Grey, A., & Rowbotham, D. S. (2016). Outcomes of bone density measurements in coeliac disease. *New Zealand Medical Journal*, *129*(1429), 40–44.

Branchi, F., Ferretti, F., Orlando, S., Tontini, G. E., Penagini, R., Vecchi, M., & Elli, L. (2020). Small-bowel capsule endoscopy in patients with celiac disease, axial versus lateral/panoramic view: Results from a prospective randomized trial. *Digestive Endoscopy*, *32*(5), 778–784. https://doi.org/10.1111/den.13575

Briot, K., Geusens, P., Em Bultink, I., Lems, W. F., & Roux, C. (2017). Inflammatory diseases and bone fragility. *Osteoporosis International*, *28*(12), 3301–3314. https://doi.org/10.1007/s00198-017-4189-7

Brough, L., & Skeaff, S. (2020). Iodine status in New Zealand. In P. A. C. Richards, *Iodophor: Tamed Iodine: The Status of Iodine in Tasmania*. Gatekeeper Press

Brown, P., McNeill, R., Leung, W., Radwan, E., & Willingale, J. (2011). Current and Future Economic Burden of Osteoporosis in New Zealand. *Applied Health Economic Health Policy*, *9*(2), 111–123.

Burkhardt, J. G., Chapa-Rodriguez, A., & Bahna, S. L. (2018). Gluten sensitivities and the allergist: Threshing the grain from the husks. *Allergy: European Journal of Allergy and Clinical Immunology*, *73*(7), 1359–1368. https://doi.org/10.1111/all.13354

Cabrera-Chávez, F., Rouzaud-Sández, O., Sotelo-Cruz, N., & Calderón De La Barca,

A. M. (2008). Transglutaminase treatment of wheat and maize prolamins of bread increases the serum IgA reactivity of celiac disease patients. *Journal of Agricultural and Food Chemistry*, *56*(4), 1387–1391. https://doi.org/10.1021/jf072 4163

Caio, G., & Volta, U. (2012). Coeliac disease: Changing diagnostic criteria? *Gastroenterology and Hepatology from Bed to Bench*, *5*(3), 119–122.

Caminero, A., Galipeau, H. J., McCarville, J. L., Johnston, C. W., Bernier, S. P., Russell, A. K., Jury, J., Herran, A. R., Casqueiro, J., Tye-Din, J. A., Surette, M. G., Magarvey, N. A., Schuppan, D., & Verdu, E. F. (2016). Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity. *Gastroenterology*, *151*(4), 670–683. https://doi.org/10.1053/j.gastro.2016.06.041

Capacci, S., Leucci, A. C., & Mazzocchi, M. (2018). There is no such thing as a (gluten-)free lunch: Higher food prices and the cost for coeliac consumers. *Economics and Human Biology*, *30*(March 2017), 84–91. https://doi.org/10.1016 /j.ehb.2018.06.001

Caputo, I., Secondo, A., Lepretti, M., Paolella, G., Auricchio, S., Barone, M. V., & Esposito, C. (2012). Gliadin Peptides Induce Tissue Transglutaminase Activation and ER-Stress through Ca2+ Mobilization in Caco-2 Cells. *PLoS ONE*, *7*(9). https://doi.org/10.1371/journal.pone.0045209

Cardo, A., Churruca, I., Lasa, A., Navarro, V., Vázquez-Polo, M., Perez-Junkera, G., & Larretxi, I. (2021). Nutritional imbalances in adult celiac patients following a gluten-free diet. *Nutrients*, *13*(8), 1–18. https://doi.org/10.3390/nu13082877

Carey, J. J., & Delaney, M. F. (2010). T-Scores and Z-Scores. *Clinical Reviews in Bone and Mineral Metabolism*, *8*, 113–121. https://doi.org/10.1007/s12018-009-9064-4

Caruso, R., Pallone, F., Stasi, E., Romeo, S., & Monteleone, G. (2013). Appropriate nutrient supplementation in celiac disease. *Annals of Medicine*, *45*(8), 522–531. https://doi.org/10.3109/07853890.2013.849383

Casella, G., Villanacci, V., Di Bella, C., Bassotti, G., Bold, J., & Rostami, K. (2018). Non celiac gluten sensitivity and diagnostic challenges. *Gastroenterology and Hepatology from Bed to Bench*, *11*(3), 197–202.

Casellas, F., Rodrigo, L., Lucendo, A. J., Fernández-Bañares, F., Molina-Infante, J., Vivas, S., Rosinach, M., Dueñas, C., & López-Vivancos, J. (2015). Benefit on health-related quality of life of adherence to gluten-free diet in adult patients with celiac disease. *Revista Espanola de Enfermedades Digestivas*, *107*(4), 196–201.

Castillo, N. E., Theethira, T. G., & Leffler, D. A. (2015). The present and the future in the diagnosis and management of celiac disease. *Gastroenterology Report*, *3*(1), 3–11. https://doi.org/10.1093/gastro/gou065

Catassi, C., Elli, L., Bonaz, B., Bouma, G., Carroccio, A., Castillejo, G., Cellier, C., Cristofori, F., de Magistris, L., Dolinsek, J., Dieterich, W., Francavilla, R., Hadjivassiliou, M., Holtmeier, W., Körner, U., Leffler, D. A., Lundin, K. E. A., Mazzarella, G., Mulder, C. J., … Fasano, A. (2015). Diagnosis of non-celiac gluten sensitivity (NCGS): The salerno experts' criteria. *Nutrients*, *7*(6), 4966–4977. https://doi.org/10.3390/nu7064966

Catassi, C., Kryszak, D., Bhatti, B., Sturgeon, C., Helzlsouer, K., Clipp, S. L., Gelfond, D., Puppa, E., Sferruzza, A., & Fasano, A. (2010). Natural history of celiac disease autoimmunity in a USA cohort followed since 1974. *Annals of Medicine*, *42*(7), 530–538. https://doi.org/10.3109/07853890.2010.514285

Catassi, C., Verdu, E. F., Bai, J. C., & Lionetti, E. (2022). Coeliac disease. *The Lancet*, *399*(10344), 2413–2426. https://doi.org/10.1016/S0140-6736(22)00794-2

Catassi, G. N., Vallorani, M., Cerioni, F., Lionetti, E., & Catassi, C. (2020). A negative fallout of COVID-19 lockdown in Italy: Life-threatening delay in the diagnosis of celiac disease. *Digestive and Liver Disease*, *52*(10). https://doi.org/10.1016/j.dld.2020.05.016

Cernat, E., & Furman, M. (2018). The value of single and combination serological

tests in diagnosing paediatric coeliac disease in the era of the latest ESPGHAN guidelines. *Current Allergy & Clinical Immunology*, *31*(3), 142–145.

Charlesworth, R. P. G. (2020). Diagnosing coeliac disease: Out with the old and in with the new? *World Journal of Gastroenterology*, *26*(1), 1–10. https://doi.org/10.3748/wjg.v26.i1.1

Chen, Y., Liu, X., Ma, W., Zhou, W., Wu, W., Kelly, C. P., Silvester, J., Zheng, D., & Ji, G. (2020). Increased risk of celiac disease in southern china. *Gastroenterology*, *158*(6), S-1120. https://doi.org/10.1016/s0016-5085(20)33470-3

Chew, C. K., & Clarke, B. L. (2018). Causes of low peak bone mass in women. *Maturitas*, *111*(September 2017), 61–68. https://doi.org/10.1016/j.maturitas.2017.12.010

Chin, K. Y., & Ima-Nirwana, S. (2013). Calcaneal quantitative ultrasound as a determinant of bone health status: What properties of bone does it reflect? *International Journal of Medical Sciences*, *10*(12), 1778–1783. https://doi.org/10.7150/ijms.6765

Choung, R. S., Lamba, A., Marietta, E. V., See, J. A., Larson, J. J., King, K. S., Van Dyke, C. T., Rubio-Tapia, A., & Murray, J. A. (2020). Effect of a gluten-free diet on quality of life in patients with nonclassical versus classical presentations of celiac disease. *Journal of Clinical Gastroenterology*, *54*(7), 620–625. https://doi.org/10.1097/MCG.0000000000001277

Choung, R. S., Unalp-Arida, A., Ruhl, C. E., Brantner, T. L., Everhart, J. E., & Murray, J. A. (2017). Less Hidden Celiac Disease But Increased Gluten Avoidance Without a Diagnosis in the United States: Findings From the National Health and Nutrition Examination Surveys From 2009 to 2014. *Mayo Clinic Proceedings*, *92*(1), 30–38. https://doi.org/10.1016/j.mayocp.2016.10.012

Churruca, I., Miranda, J., Lasa, A., Bustamante, M., Larretxi, I., & Simon, E. (2015). Analysis of body composition and food habits of Spanish celiac women. *Nutrients*, *7*(7), 5515–5531. https://doi.org/10.3390/nu7075234

Ciacci, C., & Zingone, F. (2015). The Perceived Social Burden in Celiac Disease. *Diseases*, *3*(2), 102–110. https://doi.org/10.3390/diseases3020102

Ciclitira, P. J., Evans, D. J., & Fagg, N. L. K. (1984). Clinical testing of gliadin fractions in coeliac patients. *Clinical Science*, *66*(3), 357–364. https://doi.org/10.1042/cs0660357

Codex Alimentarius Commission. (2008). *Codex standard for foods for special dietary use for persons intolerant to gluten*. Available from: http://www.codexalimentarius.net /download/standards/291/cxs_118e. pdf.

Coeliac New Zealand. (n.d.-a). *Do you need help with diagnosis and treatment?* Available from: Coeliac New Zealand. https://coeliac.org.nz/get-diagnosed/

Coeliac New Zealand. (n.d.-b). *How Do You Eat Gluten Free?* Available from: https://coeliac.org.nz/how-to-eat-gluten-free/

Coeliac New Zealand. (2017). *What is Coeliac Disease?* Available from: *https://www.coeliac.org.nz/coeliac-disease/what-is-coeliac-disease*.

Cohen, I. S., Day, A. S., & Shaoul, R. (2019). Gluten in celiac disease more or less? *Rambam Maimonides Medical Journal*, *10*(1), 1–6. https://doi.org/10.5041/RMMJ.10360

Collado, M. C., Donat, E., Ribes-Koninckx, C., Calabuig, M., & Sanz, Y. (2009). Specific duodenal and faecal bacterial groups associated with paediatric coeliac disease. *Journal of Clinical Pathology*, *62*(3), 264–269. https://doi.org/10.1136/jcp.2008.061366

Collin, P., Vilppula, A., Luostarinen, L., Holmes, G. K. T., & Kaukinen, K. (2018). Review article: coeliac disease in later life must not be missed. *Alimentary Pharmacology and Therapeutics*, *47*(5), 563–572. https://doi.org/10.1111/apt.14490

Cook, B., Oxner, R., Chapman, B., Whitehead, M., & Burt, M. (2004). A thirty-year (1970–1999) study of coeliac disease in the Canterbury region of New Zealand. *The New Zealand Medical Journal*, *117*(1189), 8.

Corr, S. C., Gahan, C. C. G. M., & Hill, C. (2008). M-cells: Origin, morphology and role in mucosal immunity and microbial pathogenesis. *FEMS Immunology and Medical Microbiology*, *52*(1), 2–12. https://doi.org/10.1111/j.1574-695X.2007.00359.x

Correa-Rodríguez, M., Rio-Valle, J. S., González-Jiménez, E., & Rueda-Medina, B. (2016). The Effects of Body Composition, Dietary Intake, and Physical Activity on Calcaneus Quantitative Ultrasound in Spanish Young Adults. *Biological Research for Nursing*, *18*(4), 439–444. https://doi.org/10.1177/1099800416634884

Cronin, C. C., & Shanahan, F. (2001). Why is celiac disease so common in Ireland? *Perspectives in Biology and Medicine*, *44*(3), 342–352. https://doi.org/10.1353/pbm.2001.0045

Czaja-Bulsa, G. (2015). Non coeliac gluten sensitivity - A new disease with gluten intolerance. *Clinical Nutrition*, *34*(2), 189–194. https://doi.org/10.1016/j.clnu.2014.08.012

de Bruin, I. J. A., Vranken, L., Wyers, C. E., van der Velde, R. Y., Trienekens, T. A. M., Kaarsemaker, S., Janzing, H. M. J., Wolters, F. L., Wouda, S., Geusens, P. P. M. M., & van den Bergh, J. P. W. (2020). The Prevalence of Celiac Disease in a Fracture Liaison Service Population. *Calcified Tissue International*, *107*(4), 327–334. https://doi.org/10.1007/s00223-020-00725-z

de Sousa, T., Ribeiro, M., Sabença, C., & Igrejas, G. (2021). The 10,000-Year Success Story of Wheat! *Foods*, *10*(9), 2124. https://doi.org/10.3390/foods10092124

De Souza, M. C. P., Deschênes, M. E., Laurencelle, S., Godet, P., Roy, C. C., & Djilali-Saiah, I. (2016). Pure oats as part of the canadian gluten-free diet in celiac disease: The need to revisit the issue. *Canadian Journal of Gastroenterology and Hepatology*, *2016*. https://doi.org/10.1155/2016/1576360

Delvecchio, M., Bizzoco, F., Lapolla, R., Gentile, A., Carrozza, C., Barone, M., Simonetti, S., Giordano, P., Dargenio, V. N., Cristofori, F., & Francavilla, R.

(2021). Iodine absorption in celiac children: A longitudinal pilot study. *Nutrients*, *13*(3), 1–10. https://doi.org/10.3390/nu13030808

Dewar, D. H., Amato, M., Ellis, H. J., Pollock, E. L., Gonzalez-Cinca, N., Wieser, H., & Ciclitira, P. J. (2006). The toxicity of high molecular weight glutenin subunits of wheat to patients with coeliac disease. *European Journal of Gastroenterology and Hepatology*, *18*(5), 483–491. https://doi.org/10.1097/00042737-200605000-00005

Di Cagno, R., De Angelis, M., De Pasquale, I., Ndagijimana, M., Vernocchi, P., Ricciuti, P., Gagliardi, F., Laghi, L., Crecchio, C., Guerzoni, M., Gobbetti, M., & Francavilla, R. (2011). Duodenal and faecal microbiota of celiac children: Molecular, phenotype and metabolome characterization. *BMC Microbiology*, *11*, 1–21. https://doi.org/10.1186/1471-2180-11-219

Di Stefano, M., Mengoli, C., Bergonzi, M., & Corazza, G. R. (2013). Bone mass and mineral metabolism alterations in adult celiac disease: Pathophysiology and clinical approach. *Nutrients*, *5*(11), 4786–4799. https://doi.org/10.3390/nu5114786

Dimidi, E., Kabir, B., Singh, J., Ageridou, A., Foster, C., Ciclitira, P., Dubois, P., & Whelan, K. (2021). Predictors of adherence to a gluten-free diet in celiac disease: Do knowledge, attitudes, experiences, symptoms, and quality of life play a role? *Nutrition*, *90*, 111249. https://doi.org/10.1016/j.nut.2021.111249

Dos Santos, S., & Lioté, F. (2017). Osteoarticular manifestations of celiac disease and non-celiac gluten hypersensitivity. *Joint Bone Spine*, *84*(3), 263–266. https://doi.org/10.1016/j.jbspin.2016.09.007

Dunne, M. R., Byrne, G., Chirdo, F. G., & Feighery, C. (2020). Coeliac Disease Pathogenesis: The Uncertainties of a Well-Known Immune Mediated Disorder. *Frontiers in Immunology*, *11*(July), 1–14. https://doi.org/10.3389/fimmu.2020.01374

Edmonds, S. (2016). *Coeliac suffers hard hit by high gluten-free prices*. Stuff.

Available from: https://www.stuff.co.nz/business/money/83102687/coeliac-suffers-hard-hit-by-high-glutenfree-prices

El Miedany, Y. (2022). Osteoporosis Update for Primary Care Physicians. In *New Horizons in Osteoporosis Management* (pp. 433–453). Springer. https://doi.org/10.1007/978-3-030-87950-1_16

Emilsson, L., Wijmenga, C., Murray, J. A., & Ludvigsson, J. F. (2015). Autoimmune disease in first-degree relatives and spouses of individuals with celiac disease. *Clinical Gastroenterology and Hepatology*, *13*(7), 1271-1277.e2. https://doi.org/10.1016/j.cgh.2015.01.026

Enaud, R., Tetard, C., Dupuis, R., Laharie, D., Lamireau, T., Zerbib, F., Rivi, P., Shili-mismoudi, S., & Poullenot, F. (2022). Compliance with Gluten Free Diet Is Associated with Better Quality of Life in Celiac Disease. *Nutrients, 14*(6), 1210. https://doi.org/10.3390/nu14061210

England, C. Y., & Nicholls, A. M. (2004). Advice available on the Internet for people with coeliac disease: An evaluation of the quality of websites. *Journal of Human Nutrition and Dietetics*, *17*(6), 547–559. https://doi.org/10.1111/j.1365277X.2004.00561.x

Ensrud, K. E., Vo, T. N., Burghardt, A. J., Schousboe, J. T., Cauley, J. A., Taylor, B. C., Hoffman, A. R., Orwoll, E. S., Lane, N. E., & Langsetmo, L. (2018). Weight loss in men in late life and bone strength and microarchitecture: a prospective study. *Osteoporosis International*, *26*, 1549–1557. https://doi.org/10.1007/s00198-018-4489-6

Fan, Y., & Pedersen, O. (2021). Gut microbiota in human metabolic health and disease. *Nature Reviews Microbiology*, *19*(1), 55–71. https://doi.org/10.1038/s41579-020-0433-9

Fasano, A., Sapone, A., Zevallos, V., & Schuppan, D. (2015). Nonceliac Gluten and Wheat Sensitivity. *Gastroenterology*, *148*, 1195–1204. https://doi.org/10.1016/j.mayocp.2015.07.009

Fiore, C. E., Pennisi, P., Ferro, G., Ximenes, B., Privitelli, L., Mangiafico, R. A., Santoro, F., Parisi, N., & Lombardo, T. (2006). Altered Osteoprotegerin/RANKL Ratio and Low Bone Mineral Density in Celiac Patients on Long-term Treatment with Gluten-free Diet. *Horm Metab Res*, *38*(6), 417–422. https://doi.org/10.1055/s-2006-944548

Food Standards Australia New Zealand. (2022). *Australia New Zealand Food Standards Code: Standard 2.9.5. Food for special medical purposes*. Canberra, Australia: Australian Government. Available from: https://www.foodstandards.gov.au/code/Documents/2.9.5%20FSMPs%20v157.pdf.

Freedman, A. R., Macartney, J. C., Nelufer, J. M., & Ciclitira, P. J. (1987). Timing of infiltration of T lymphocytes induced by gluten into the small intestine in coeliac disease. *Journal of Clinical Pathology*, *40*(7), 741–745. https://doi.org/10.1136/jcp.40.7.741

Freire, R., Ingano, L., Serena, G., Cetinbas, M., Anselmo, A., Sapone, A., Sadreyev, R. I., Fasano, A., & Senger, S. (2019). Human gut derived-organoids provide model to study gluten response and effects of microbiota-derived molecules in celiac disease. *Scientific Reports*, *9*(1), 1–15. https://doi.org/10.1038/s41598-019-43426-w

Fuchs, V., Kurppa, K., Huhtala, H., Laurila, K., Mäki, M., Collin, P., Salmi, T., Luostarinen, L., Saavalainen, P., & Kaukinen, K. (2019). Serology-based criteria for adult coeliac disease have excellent accuracy across the range of pre-test probabilities. *Alimentary Pharmacology and Therapeutics*, *49*(3), 277–284. https://doi.org/10.1111/apt.15109

Galli, G., Lahner, E., Conti, L., Esposito, G., Sacchi, M. C., & Annibale, B. (2018). Risk factors associated with osteoporosis in a cohort of prospectively diagnosed adult coeliac patients. *United European Gastroenterology Journal*, *6*(8), 1161–1168. https://doi.org/10.1177/2050640618784340

García-Nieto, V. M. (2014). A History of Celiac Disease. In L Rodrigo & LS Peña

(Eds.), *Celiac Disease and Non-Celiac Gluten Sensitivity* (pp. 45–59). OmniaScience Monographs. https://doi.org/10.3926/oms.226

Gibson, P. R., Skodje, G. I., & Lundin, K. E. A. (2017). Non-coeliac gluten sensitivity. *Journal of Gastroenterology and Hepatology*, *32*, 86–89. https://doi.org/10.1111/jgh.13705

Gidrewicz, D., Trevenen, C. L., Lyon, M., & Decker Butzner, J. (2017). Normalization time of celiac serology in children on a gluten-free diet. *Journal of Pediatric Gastroenterology and Nutrition*, *64*(3), 362–367. https://doi.org/10.1097/MPG.0000000000001270

Girbovan, A., Sur, G., Samasca, G., & Lupan, I. (2017). Dysbiosis a risk factor for celiac disease. *Medical Microbiology and Immunology*, *206*(2), 83–91. https://doi.org/10.1007/s00430-017-0496-z

Giuberti, G., & Gallo, A. (2018). Reducing the glycaemic index and increasing the slowly digestible starch content in gluten-free cereal-based foods: a review. *International Journal of Food Science and Technology*, *53*(1), 50–60. https://doi.org/10.1111/ijfs.13552

Gładyś, K., Dardzińska, J., Guzek, M., Adrych, K., Kochan, Z., & Małgorzewicz, S. (2021). Expanded role of a dietitian in monitoring a gluten-free diet in patients with celiac disease: Implications for clinical practice. *Nutrients*, *13*(6). https://doi.org/10.3390/nu13061859

Gładyś, K., Dardzińska, J., Guzek, M., Adrych, K., & Małgorzewicz, S. (2020). Celiac dietary adherence test and standardized dietician evaluation in assessment of adherence to a gluten-free diet in patients with celiac disease. *Nutrients*, *12*(8), 1–10. https://doi.org/10.3390/nu12082300

Golley, S., Corsini, N., Topping, D., Morell, M., & Mohr, P. (2015). Motivations for avoiding wheat consumption in Australia: Results from a population survey. *Public Health Nutrition*, *18*(3), 490–499. https://doi.org/10.1017/S1368980014000652

Graziano, M., & Rossi, M. (2018). An update on the cutaneous manifestations of coeliac disease and non-coeliac gluten sensitivity. *International Reviews of Immunology*, *37*(6), 1–10. https://doi.org/10.1080/08830185.2018.1533008

Grober, U., Reichrath, J., Holick, M. F., & Kisters, K. (2014). Vitamin K : an old vitamin in a new perspective. *Dermato-Endocrinoogyl, 6*(1). https://doi.org/10.4161/19381972.2014.968490

Gu, Q., Yang, H., & Shi, Q. (2017). Macrophages and bone inflammation. *Journal of Orthopaedic Translation*, *10*, 86–93. https://doi.org/10.1016/j.jot.2017.05.002

Guennouni, M., Admou, B., El Khoudri, N., Bourrhouat, A., Zogaam, L. G., Elmoumou, L., & Hilali, A. (2022). Gluten contamination in labelled gluten-free, naturally gluten-free and meals in food services in low-, middle- and high-income countries: a systematic review and meta-analysis. *British Journal of Nutrition*, *127*(10), 1528–1542. https://doi.org/10.1017/S0007114521002488

Gutowski, E. D., Weiten, D., Green, K. H., Rigaux, L. N., Bernstein, C. N., Graff, L. A., Walker, J. R., Duerksen, D. R., & Silvester, J. A. (2020). Can individuals with celiac disease identify gluten-free foods correctly? *Clinical Nutrition ESPEN*, *36*, 82–90. https://doi.org/10.1016/j.clnesp.2020.01.012

Hære, P., Høie, O., Schulz, T., Schönhardt, I., Raki, M., & Lundin, K. E. A. (2016). Long-term mucosal recovery and healing in celiac disease is the rule – not the exception. *Scandinavian Journal of Gastroenterology*, *51*(12), 1439–1446. https://doi.org/10.1080/00365521.2016.1218540

Hall, N. J., Rubin, G., & Charnock, A. (2009). Systematic review: Adherence to a gluten-free diet in adult patients with coeliac disease. *Alimentary Pharmacology and Therapeutics*, *30*(4), 315–330. https://doi.org/10.1111/j.1365-2036.2009.04053.x

Halmos, E. P., Deng, M., Knowles, S. R., Sainsbury, K., Mullan, B., & Tye-Din, J. A. (2018). Food knowledge and psychological state predict adherence to a gluten-free diet in a survey of 5310 Australians and New Zealanders with coeliac

disease. *Alimentary Pharmacology and Therapeutics*, *February*, 78–86. https://doi.org/10.1111/apt.14791

Hammad, L. F. (2013). Quantitative Ultrasound Measurements Of Stiffness Index In Young Adult Females. *Archives of Medicine*, *5*(1), 1–5. https://doi.org/10.3823/107

Hammad, L. F. (2016). Measurements of bone mineral density and stiffness index in young Saudi females. *Pakistan Journal of Medical Sciences*, *32*(2), 399–402. https://doi.org/10.12669/pjms.322.9757

Hardy, M. Y., Tye-Din, J. A., Stewart, J. A., Schmitz, F., Dudek, N. L., Hanchapola, I., Purcell, A. W., & Anderson, R. P. (2015). Ingestion of oats and barley in patients with celiac disease mobilizes cross-reactive T cells activated by avenin peptides and immuno-dominant hordein peptides. *Journal of Autoimmunity*, *56*, 56–65. https://doi.org/10.1016/j.jaut.2014.10.003

Hasan, B., Thompson, W. G., Almasri, J., Wang, Z., Lakis, S., Prokop, L. J., Hensrud, D. D., Frie, K. S., Wirtz, M. J., Murad, A. L., Ewoldt, J. S., & Murad, M. H. (2019). The effect of culinary interventions (cooking classes) on dietary intake and behavioral change: A systematic review and evidence map. *BMC Nutrition*, *5*(1), 1–9. https://doi.org/10.1186/s40795-019-0293-8

Heaney, R. P., Abrams, S., Dawson-Hughes, B., Looker, A., Marcus, R., Matkovic, V., & Weaver, C. (2000). Peak bone mass. *Osteoporosis International*, *11*(12), 985–1009. https://doi.org/10.1007/s001980070020

Høiberg, M. P., Rubin, K. H., Hermann, A. P., Brixen, K., & Abrahamsen, B. (2016). Diagnostic devices for osteoporosis in the general population: A systematic review. *Bone*, *92*, 58–69. https://doi.org/10.1016/j.bone.2016.08.011

Holtmeier, W., & Caspary, W. F. (2006). Celiac disease. *Orphanet Journal of Rare Diseases*, *1*(1). https://doi.org/10.1186/1750-1172-1-3

Hopper, A. D., Hurlstone, D. P., Leeds, J. S., McAlindon, M. E., Dube, A. K., Stephenson, T. J., & Sanders, D. S. (2006). The occurrence of terminal ileal

histological abnormalities in patients with coeliac disease. *Digestive and Liver Disease*, *38*(11), 815–819. https://doi.org/10.1016/j.dld.2006.04.003

Horan, M. P., Chai, S. Y., Munusamy, N., Tay, K. H., Wienholt, L., Tye-Din, J. A., Varney, M., & Badrick, T. (2018). High rates of variation in HLA-DQ2/DQ8 testing for coeliac disease: results from an RCPAQAP pilot program. *Journal of Clinical Pathology*, *71*(10), 900–905. https://doi.org/10.1136/jclinpath-2018-205209

Hou, K., Wu, Z. X., Chen, X. Y., Wang, J. Q., Zhang, D., Xiao, C., Zhu, D., Koya, J. B., Wei, L., Li, J., & Chen, Z. S. (2022). Microbiota in health and diseases. *Signal Transduction and Targeted Therapy*, *7*(1), 135. https://doi.org/10.1038/s41392-022-00974-4

Husby, S., Koletzko, S., Korponay-Szabó, I. R., Mearin, M. L., Phillips, A., Shamir, R., Troncone, R., Giersiepen, K., Branski, D., Catassi, C., Lelgeman, M., Mäki, M., Ribes-Koninckx, C., Ventura, A., & Zimmer, K. P. (2012). European society for pediatric gastroenterology, hepatology, and nutrition guidelines for the diagnosis of coeliac disease. *Journal of Pediatric Gastroenterology and Nutrition*, *54*(1), 136–160. https://doi.org/10.1097/MPG.0b013e31821a23d0

Husby, Steffen, Koletzko, S., Korponay-Szabó, I. R., Kurppa, K., Mearin, M. L., Ribes-Koninckx, C., Shamir, R., Troncone, R., Auricchio, R., Castillejo, G., Christensen, R., Dolinsek, J., Gillett, P., Hróbjartsson, A., Koltai, T., Maki, M., Nielsen, S. M., & Popp, A. (2020). European Society Paediatric Gastroenterology, Hepatology and Nutrition guidelines for diagnosing coeliac disease 2020. *Journal of Pediatric Gastroenterology and Nutrition*, *70*(1), 141–156. https://doi.org/10.1097/MPG.0000000000002497

Iida, T., Chikamura, C., Aoi, S., Ikeda, H., Matsuda, Y., Oguri, Y., Ono, Y., Katada, K., & Ishizaki, F. (2010). A study on the validity of quantitative ultrasonic measurement used the bone mineral density values on dual-energy X-ray absorptiometry in young and in middle-aged or older women. *Radiological*

Physics and Technology, *3*(2), 113–119. https://doi.org/10.1007/s12194-010-0086-x

International Osteoporosis Foundation. (2017). *Facts and Statistics*. Available from: https://www.iofbonehealth.org/facts-statistics#category-26

Iseme, R. A., Mcevoy, M., Kelly, B., Agnew, L., Walker, F. R., & Attia, J. (2017). Is osteoporosis an autoimmune mediated disorder? *Bone Reports*, *7*(August), 121–131. https://doi.org/10.1016/j.bonr.2017.10.003

Jansson-Knodell, C. L., Hujoel, I. A., West, C. P., Taneja, V., Prokop, L. J., Rubio-Tapia, A., & Murray, J. A. (2019). Sex Difference in Celiac Disease in Undiagnosed Populations: A Systematic Review and Meta-analysis. *Clinical Gastroenterology and Hepatology*, *17*(10), 1954-1968.e13. https://doi.org/10.1016/j.cgh.2018.11.013

Jonkers, I. H., & Wijmenga, C. (2017). Context-specific effects of genetic variants associated with autoimmune disease. *Human Molecular Genetics*, *26*(R2), R185–R192. https://doi.org/10.1093/hmg/ddx254

Kamycheva, E., Goto, T., & Camargo, C. A. (2017). Celiac disease is associated with reduced bone mineral density and increased FRAX scores in the US National Health and Nutrition Examination Survey. *Osteoporosis International*, *28*(3), 781–790. https://doi.org/10.1007/s00198-016-3791-4

Kanaya, T., Sakakibara, S., Jinnohara, T., Hachisuka, M., Tachibana, N., Hidano, S., Kobayashi, T., Kimura, S., Iwanaga, T., Nakagawa, T., Katsuno, T., Kato, N., Akiyama, T., Sato, T., Williams, I. R., & Ohno, H. (2018). Development of intestinal M cells and follicle-associated epithelium is regulated by TRAF6-mediated NF-κB signaling. *The Journal of Experimental Medicine*, *215*(2), 501–519. https://doi.org/10.1084/jem.20160659

Khaleghi, S., Ju, J. M., Lamba, A., & Murray, J. A. (2016). The potential utility of tight junction regulation in celiac disease: focus on larazotide acetate. *Therapeutic Advances in Gastroenterology*, *9*(1), 37–49. https://doi.org/10.1177/1756283X15616576

Kim, J., Koo, B. K., & Knoblich, J. A. (2020). Human organoids: model systems for human biology and medicine. *Nature Reviews Molecular Cell Biology*, *21*(10), 571–584. https://doi.org/10.1038/s41580-020-0259-3

King, J. A., Jeong, J., Underwood, F. E., Quan, J., Panaccione, N., Windsor, J. W., Coward, S., Debruyn, J., Ronksley, P. E., Shaheen, A. A., Quan, H., Godley, J., Veldhuyzen Van Zanten, S., Lebwohl, B., Ng, S. C., Ludvigsson, J. F., & Kaplan, G. G. (2020). Incidence of Celiac Disease Is Increasing over Time: A Systematic Review and Meta-analysis. *American Journal of Gastroenterology*, *115*(4), 507–525. https://doi.org/10.14309/ajg.0000000000000523

Kirsaçlioğlu, C. T., Kuloğlu, Z., Tanca, A., Küçük, N. Ö., Aycan, Z., Öcal, G., Ensari, A., Kalayci, A. G., & Girgin, N. (2016). Bone mineral density and growth in children with coeliac disease on a gluten free-diet. *Turkish Journal of Medical Sciences*, *46*(6), 1816–1821. https://doi.org/10.3906/sag-1508-52

Kotze, L. M. S., Skare, T., Vinholi, A., Jurkonis, L., & Nisihara, R. (2016). Impact of a gluten-free diet on bone mineral density in celiac patients. *Revista Espanola de Enfermedades Digestivas*, *108*(2), 84–88. https://doi.org/10.17235/reed.2015.3953/2015

Kreutz, J. M., Adriaanse, M. P. M., van der Ploeg, E. M. C., & Vreugdenhil, A. C. E. (2020). Narrative review: Nutrient deficiencies in adults and children with treated and untreated celiac disease. *Nutrients*, *12*(2). https://doi.org/10.3390/nu12020500

Kuja-Halkola, R., Lebwohl, B., Halfvarson, J., Wijmenga, C., Magnusson, P. K. E., & Ludvigsson, J. F. (2016). Heritability of non-HLA genetics in coeliac disease: A population-based study in 107 000 twins. *Gut*, *65*(11), 1793–1798. https://doi.org/10.1136/gutjnl-2016-311713

Kumar, J., Kumar, M., Pandey, R., & Chauhan, N. S. (2017). Physiopathology and Management of Gluten-Induced Celiac Disease. *Journal of Food Science*, *82*(2), 270–277. https://doi.org/10.1111/1750-3841.13612

Kvamme, J.-M., Sørbye, S., Florholmen, J., & Halstensen, T. S. (2022). Population-based screening for celiac disease reveals that the majority of patients are undiagnosed and improve on a gluten-free diet. *Scientific Reports*, *12*(1), 12647. https://doi.org/10.1038/s41598-022-16705-2

Larussa, T., Suraci, E., Imeneo, M., Marasco, R., & Luzza, F. (2017). Normal bone mineral density associates with duodenal mucosa healing in adult patients with celiac disease on a gluten-free diet. *Nutrients*, *9*(2). https://doi.org/10.3390/nu9020098

Laszkowska, M., Mahadev, S., Sundström, J., Lebwohl, B., Green, P. H. R., Michaelsson, K., & Ludvigsson, J. F. (2018). Systematic review with meta-analysis: the prevalence of coeliac disease in patients with osteoporosis. *Alimentary Pharmacology & Therapeutics*, *March*, 1–8. https://doi.org/10.1111/apt.14911

Lebwohl, B., & Rubio-Tapia, A. (2021). Epidemiology, Presentation, and Diagnosis of Celiac Disease. *Gastroenterology*, *160*(1), 63–75. https://doi.org/10.1053/j.gastro.2020.06.098

Lebwohl, B., Sanders, D. S., & Green, P. H. R. (2018). Coeliac disease. *The Lancet*, *391*, 70–81. https://doi.org/10.1016/S0140-6736(17)31796-8

Lebwohl, B., Tennyson, C. A., Holub, J. L., Lieberman, D. A., Neugut, A. I., & Green, P. H. R. (2012). Sex and racial disparities in duodenal biopsy to evaluate for celiac disease. *Gastrointestinal Endoscopy*, *76*(4), 779–785. https://doi.org/10.1016/j.gie.2012.05.011

Lee, A. R., Wolf, R. L., Lebwohl, B., Ciaccio, E. J., & Green, P. H. R. (2019). Persistent economic burden of the Gluten free diet. *Nutrients*, *11*(2), 1–8. https://doi.org/10.3390/nu11020399

Lerner, A., & Matthias, T. (2016a). Gut-bone Cross Talks and Implications in Celiac Disease. *International Journal of Celiac Disease*, *4*(1), 19–23. https://doi.org/10.12691/ijcd-4-1-4

Lerner, A., & Matthias, T. (2016b). GUT-the Trojan Horse in Remote Organs' Autoimmunity. *Journal of Clinical & Cellular Immunology*, *07*(02), 1–10. https://doi.org/10.4172/2155-9899.1000401

Lerner, B. A., Vo, L. P., Yates, S., Rundle, A. G., Green, P. H. R., & Lebwohl, B. (2019). Detection of Gluten in Gluten-Free Labeled Restaurant Food: Analysis of Crowd-Sourced Data. *American Journal of Gastroenterology*, *114*(5), 792–797. https://doi.org/10.14309/ajg.0000000000000202

Lewis, S. K., & Semrad, C. E. (2019). Capsule Endoscopy and Enteroscopy in Celiac Disease. *Gastroenterology Clinics of North America*, *48*(1), 73–84. https://doi.org/10.1016/j.gtc.2018.09.005

Li, C., Sun, J., & Yu, L. (2022). Diagnostic value of calcaneal quantitative ultrasound in the evaluation of osteoporosis in middle-aged and elderly patients. *Medicine*, *101*(2). https://doi.org/10.1097/MD.0000000000028325

Lindfors, K., Ciacci, C., Kurppa, K., Lundin, K. E. A., Makharia, G. K., Mearin, M. L., Murray, J. A., Verdu, E. F., & Kaukinen, K. (2019). Coeliac disease. *Nature Reviews Disease Primers*, *5*(1), 3. https://doi.org/10.1038/s41572-018-0054-z

Liu, E., Dong, F., Barón, A. E., Taki, I., Norris, J. M., Frohnert, B. I., Hoffenberg, E. J., & Rewers, M. (2017). High incidence of celiac disease in a long-term study of adolescents with susceptibility genotypes. *Gastroenterology*, *152*(6), 1329-1336.e1. https://doi.org/10.1053/j.gastro.2017.02.002

Liu, W., & Zhang, X. (2015). Receptor activator of nuclear factor-κB ligand (RANKL)/RANK/osteoprotegerin system in bone and other tissues (Review). *Molecular Medicine Reports*, *11*(5), 3212–3218. https://doi.org/10.3892/mmr.2015.3152

Lockyer, S., & Spiro, A. (2020). The role of bread in the UK diet: An update. *Nutrition Bulletin*, *45*(2), 133–164. https://doi.org/10.1111/nbu.12435

Lohi, S., Mustalahti, K., Kaukinen, K., Laurila, K., Collin, P., Rissanen, H., Lohi, O., Bravi, E., Gasparin, M., Reunanen, A., & Mäki, M. (2007). Increasing

prevalence of coeliac disease over time. *Alimentary Pharmacology and Therapeutics*, *26*(9), 1217–1225. https://doi.org/10.1111/j.1365-2036.2007.03502.x

Loi, F., Córdova, L. A., Pajarinen, J., Lin, T. hua, Yao, Z., & Goodman, S. B. (2016). Inflammation, fracture and bone repair. *Bone*, *86*, 119–130. https://doi.org/10.1016/j.bone.2016.02.020

Lucendo, A. J., & García-Manzanares, A. (2013). Bone mineral density in adult coeliac disease: an updated review. *Revista Española de Enfermedades Digestivas : Organo Oficial de La Sociedad Española de Patología Digestiva*, *105*(3), 154–162. https://doi.org/10.4321/S1130-01082013000300006

Mabbott, N. A., Donaldson, D. S., Ohno, H., Williams, I. R., & Mahajan, A. (2013). Microfold (M) cells: Important immunosurveillance posts in the intestinal epithelium. *Mucosal Immunology*, *6*(4), 666–677. https://doi.org/10.1038/mi.2013.30

Makharia, G. K., Chauhan, A., Singh, P., & Ahuja, V. (2022). Review article : Epidemiology of coeliac disease. *Alimentary Pharmacology and Therapeutics*, *56 Suppl 1*(3–17), 3–17. https://doi.org/10.1111/apt.16787

Makharia, G. K., Verma, A. K., Amarchand, R., Bhatnagar, S., Das, P., Goswami, A., Bhatia, V., Ahuja, V., Datta Gupta, S., & Anand, K. (2011). Prevalence of celiac disease in the northern part of India: A community based study. *Journal of Gastroenterology and Hepatology (Australia)*, *26*(5), 894–900. https://doi.org/10.1111/j.1440-1746.2010.06606.x

Martín-Masot, R., Nestares, M. T., Diaz-Castro, J., López-Aliaga, I., Alférez, M. J. M., Moreno-Fernandez, J., & Maldonado, J. (2019). Multifactorial etiology of anemia in celiac disease and effect of gluten-free diet: A comprehensive review. *Nutrients*, *11*(11), 1–15. https://doi.org/10.3390/nu11112557

Martin, T. J., & Sims, N. A. (2015). RANKL/OPG; Critical role in bone physiology. *Reviews in Endocrine and Metabolic Disorders*, *16*(2), 131–139. https://doi.org/10.1007/s11154-014-9308-6

Matthias, T., Neidhöfer, S., Pfeiffer, S., Prager, K., Reuter, S., & Gershwin, M. E. (2011). Novel trends in celiac disease. *Cellular & Molecular Immunology*, *8*(2), 121–125. https://doi.org/10.1038/cmi.2010.68

Mazzarella, G., Stefanile, R., Camarca, A., Giliberti, P., Cosentini, E., Marano, C., Iaquinto, G., Giardullo, N., Auricchio, S., Sette, A., Troncone, R., & Gianfrani, C. (2008). Gliadin Activates HLA Class I-Restricted CD8+ T Cells in Celiac Disease Intestinal Mucosa and Induces the Enterocyte Apoptosis. *Gastroenterology*, *134*(4), 1017–1027. https://doi.org/10.1053/j.gastro.2008.01.008

Mesin, L., Sollid, L. M., & Di Niro, R. (2012). The intestinal B-cell response in celiac disease. *Frontiers in Immunology*, *3*(OCT), 1–12. https://doi.org/10.3389/fimmu.2012.00313

Mijatov, M. A. K., & Mičetić-Turk, D. (2016). Dietary intake in adult female coeliac disease patients in Slovenia. *Zdravstveno Varstvo*, *55*(2), 86–93. https://doi.org/10.1515/sjph-2016-0013

Ministry for Primary Industries. (2021). *Folic acid fortification: Summary and analysis of submissions on New Zealand Food Safety Discussion Paper No: 2019/08* (Issue ISSN No: 2253-394X). https://doi.org/10.1016/S0140-6736(05)77074-4

Miranda, J., Lasa, A., Bustamante, M. A., Churruca, I., & Simon, E. (2014). Nutritional Differences Between a Gluten-free Diet and a Diet Containing Equivalent Products with Gluten. *Plant Foods for Human Nutrition*, *69*(2), 182–187. https://doi.org/10.1007/s11130-014-0410-4

Montoro-Huguet, M. A., Santolaria-Piedrafita, S., Cañamares-Orbis, P., & García-Erce, J. A. (2021). Iron Deficiency in Celiac Disease: Prevalence, Health Impact, and Clinical Management. *Nutrients*, *13*(10), 3437. https://doi.org/10.3390/nu13103437

Moore, S. G., Donnelly, J. K., Jones, S., & Cade, J. E. (2018). Effect of educational

interventions on understanding and use of nutrition labels: A systematic review. *Nutrients*, *10*(10), 1–14. https://doi.org/10.3390/nu10101432

Myhrstad, M. C. W., Slydahl, M., Hellmann, M., Garnweidner-Holme, L., Lundin, K. E. A., Henriksen, C., & Telle-Hansen, V. H. (2021). Nutritional quality and costs of gluten-free products: A case-control study of food products on the Norwegian market. *Food and Nutrition Research*, *65*(March). https://doi.org/10.29219/fnr.v65.6121

New Zealand Institute of Plant and Food Research. (2013). *The Concise New Zealand Food Composition Tables. 10th edition.* Palmerston North: New Zealand Institute of Plant and Food Research and The New Zealand Ministry of Health. Available from: www.foodcomposition.co.nz

Ohno, H. (2015). Intestinal M cells. *Journal of Biochemistry*, *159*(2), 151–160. https://doi.org/10.1093/jb/mvv121

Owen, D. R., & Owen, D. A. (2018). Celiac disease and other causes of duodenitis. *Archives of Pathology and Laboratory Medicine*, *142*(1), 35–43. https://doi.org/10.5858/arpa.2016-0608-RA

Oxentenko, A. S., & Murray, J. a. (2015). Celiac Disease: ten things that every gastroenterologist should know. *Clinical Gastroenterology and Hepatology : The Official Clinical Practice Journal of the American Gastroenterological Association*, *13*(8), 1396–1404; quiz e127-9. https://doi.org/10.1016/j.cgh.2014.07.024

Pagani, A., Nai, A., Silvestri, L., & Camaschella, C. (2019). Hepcidin and anemia: a tight relationship. *Frontiers in Physiology*, *10*(October), 1–7. https://doi.org/10.3389/fphys.2019.01294

Paganizza, S., Zanotti, R., D'Odorico, A., Scapolo, P., & Canova, C. (2019). Is adherence to a gluten-free diet by adult patients with celiac disease influenced by their knowledge of the gluten content of foods? *Gastroenterology Nursing*, *42*(1), 55–64. https://doi.org/10.1097/SGA.0000000000000368

Park, K. A., Park, Y. H., Suh, M. H., & Choi-Kwon, S. (2015). Lifestyle and genetic predictors of stiffness index in community-dwelling elderly Korean men and women. *Asian Nursing Research*, *9*(3), 251–258. https://doi.org/10.1016/j.anr.2015.05.006

Parzanese, I., Qehajaj, D., Patrinicola, F., Aralica, M., Chiriva-Internati, M., Stifter, S., Elli, L., & Grizzi, F. (2017). Celiac disease: From pathophysiology to treatment. *World Journal of Gastrointestinal Pathophysiology*, *8*(2), 27–38. https://doi.org/10.4291/wjgp.v8.i2.27

Pelizzaro, F., Marsilio, I., Fassan, M., Piazza, F., Barberio, B., D'odorico, A., Savarino, E. V., Farinati, F., & Zingone, F. (2021). The risk of malignancies in celiac disease - A literature review. *Cancers*, *13*(21), 1–34. https://doi.org/10.3390/cancers13215288

Peña, A. S. (2015). What is the best histopathological classification for celiac disease? Does it matter? *Gastroenterology and Hepatology from Bed to Bench*, *8*(4), 239–243.

Penny, H. A., Baggus, E. M. R., Rej, A., Snowden, J. A., & Sanders, D. S. (2020). Non-responsive coeliac disease: A comprehensive review from the NHS england national centre for refractory coeliac disease. *Nutrients*, *12*(1). https://doi.org/10.3390/nu12010216

Pinto-Sánchez, M. I., Causada-Calo, N., Bercik, P., Ford, A. C., Murray, J. A., Armstrong, D., Semrad, C., Kupfer, S. S., Alaedini, A., Moayyedi, P., Leffler, D. A., Verdú, E. F., & Green, P. (2017). Safety of adding oats to a gluten-free diet for patients with celiac disease: systematic review and meta-analysis of clinical and observational studies. *Gastroenterology*, *153*(2), 395-409.e3. https://doi.org/10.1053/j.gastro.2017.04.009

Pisani, P., Greco, A., Conversano, F., Renna, M. D., Casciaro, E., Quarta, L., Costanza, D., Muratore, M., & Casciaro, S. (2017). A quantitative ultrasound approach to estimate bone fragility: A first comparison with dual X-ray

absorptiometry. *Measurement: Journal of the International Measurement Confederation*, *101*, 243–249. https://doi.org/10.1016/j.measurement.2016.07.033

Plugis, N. M., & Khosla, C. (2015). Therapeutic approaches for celiac disease. *Best Practice & Research. Clinical Gastroenterology*, *29*(3), 503–521. https://doi.org/10.1016/j.bpg.2015.04.005

Pritchard, L., Wilson, S., Griffin, J., Pearce, G., & Lewis, S. (2018). Prevalence of reduced bone mineral density in adults with coeliac disease–are we missing opportunities for detection in patients below 50 years of age? *Scandinavian Journal of Gastroenterology*, *22*(2017), 143–144. https://doi.org/10.1080/00365521.2018.1543447

Radawiec, A., Szulc, W., & Rutkowska, B. (2021). Selenium biofortification of wheat as a strategy to improve human nutrition. *Agriculture*, *11*(2), 1–12. https://doi.org/10.3390/agriculture11020144

Raiteri, A., Granito, A., Giamperoli, A., Catenaro, T., Negrini, G., & Tovoli, F. (2022). Current guidelines for the management of celiac disease: A systematic review with comparative analysis. *World Journal of Gastroenterology*, *28*(1), 154–175. https://doi.org/10.3748/wjg.v28.i1.154

Reilly, N. R., Husby, S., Sanders, D. S., & Green, P. H. R. (2018). Coeliac disease: To biopsy or not? *Nature Reviews Gastroenterology and Hepatology*, *15*(1), 60–66. https://doi.org/10.1038/nrgastro.2017.121

Rewers, M., Hagopian, W., She, J., Schatz, D., Ziegler, A., Toppari, J., Akolkar, B., Krischer, J., & TEDDY Study Group. (2018). The Environmental Determinants of Diabetes in the Young (TEDDY) Study: 2018 Update. *Current Diabetes Reports*, *18*(12), 136. https://doi.org/10.1007/s11892-018-1113-2

Robertson, A., Godavitarne, C., & Peters, J. (2017). Bone quantification. *Orthopaedics and Trauma*, *31*(5), 326–329. https://doi.org/10.1016/j.mporth.2017.07.009

Rodrigo, L., Pérez-Martinez, I., Lauret-Braña, E., & Suárez-González, A. (2018). Descriptive study of the different tools used to evaluate the adherence to a gluten-free diet in celiac disease patients. *Nutrients*, *10*(11), 1777.

Rozenberg, S., Bruyère, O., Bergmann, P., Cavalier, E., Gielen, E., Goemaere, S., Kaufman, J. M., Lapauw, B., Laurent, M. R., De Schepper, J., & Body, J. J. (2020). How to manage osteoporosis before the age of 50. *Maturitas*, *138*(February), 14–25. https://doi.org/10.1016/j.maturitas.2020.05.004

Rubio-Tapia, A., Kyle, R. A., Kaplan, E. L., Johnson, D. R., Page, W., Erdtmann, F., Brantner, T. L., Kim, W. R., Phelps, T. K., Lahr, B. D., Zinsmeister, A. R., Melton, L. J., & Murray, J. A. (2009). Increased Prevalence and Mortality in Undiagnosed Celiac Disease. *Gastroenterology*, *137*(1), 88–93. https://doi.org/10.1053/j.gastro.2009.03.059

Rustagi, S., Choudhary, S., Khan, S., & Jain, T. (2020). Consequential effect of gluten-free diet on health-related quality of life in celiac populace-a meta-analysis. *Current Research in Nutrition and Food Science*, *8*(3), 1025–1036. https://doi.org/10.12944/CRNFSJ.8.3.29

Sabença, C., Ribeiro, M., de Sousa, T., Poeta, P., Bagulho, A. S., & Igrejas, G. (2021). Wheat/gluten-related disorders and gluten-free diet misconceptions: a review. *Foods*, *10*(8), 1765. https://doi.org/10.3390/foods10081765.

Sainsbury, K., Halmos, E. P., Knowles, S., Mullan, B., & Tye-Din, J. A. (2018). Maintenance of a gluten free diet in coeliac disease: The roles of self-regulation, habit, psychological resources, motivation, support, and goal priority. *Appetite*, *125*, 356–366. https://doi.org/10.1016/j.appet.2018.02.023

Satherley, R. M., Higgs, S., & Howard, R. (2017). Disordered eating patterns in coeliac disease: a framework analysis. *Journal of Human Nutrition and Dietetics*, *30*(6), 724–736. https://doi.org/10.1111/jhn.12475

Scherf, K. A., Koehler, P., & Wieser, H. (2016). Gluten and wheat sensitivities - An overview. *Journal of Cereal Science*, *67*, 2–11. https://doi.org/10.1016/j.jcs.2015

.07.008

Schraders, K., Zatta, G., Kruger, M., Coad, J., Weber, J., Brough, L., & Thomson, J. (2019). Quantitative ultrasound and dual x-ray absorptiometry as indicators of bone mineral density in young women and nutritional factors affecting it. *Nutrients, 11*(10), 2336. https://doi.org/10.3390/nu12010030

Schuppan, D., Pickert, G., Ashfaq-Khan, M., & Zevallos, V. (2015). Non-celiac wheat sensitivity: Differential diagnosis, triggers and implications. *Best Practice and Research: Clinical Gastroenterology*, *29*(3), 469–476. https://doi.org/10.1016/j.bpg.2015.04.002

Sellitto, M., Bai, G., Serena, G., Fricke, W. F., Sturgeon, C., Gajer, P., White, J. R., Koenig, S. S. K., Sakamoto, J., Boothe, D., Gicquelais, R., Kryszak, D., Puppa, E., Catassi, C., Ravel, J., & Fasano, A. (2012). Proof of concept of microbiome-metabolome analysis and delayed gluten exposure on celiac disease autoimmunity in genetically at-risk infants. *PLoS ONE*, *7*(3). https://doi.org/10.1371/journal.pone.0033387

Sharma, N., Bhatia, S., Chunduri, V., Kaur, S., Sharma, S., Kapoor, P., Kumari, A., & Garg, M. (2020). Pathogenesis of Celiac Disease and Other Gluten Related Disorders in Wheat and Strategies for Mitigating Them. *Frontiers in Nutrition*, *7*(February), 1–26. https://doi.org/10.3389/fnut.2020.00006

Shenoy, S., Chawla, J. K., & Sandhu, J. S. (2014). Multisite quantitative ultrasound: It's comparison with dual energy X-ray absorptiometry in the diagnosis of osteoporosis. *Journal of Orthapedics and Allied Sciences*, *2*(2), 40–44. https://doi.org/10.4103/2319-2585.145598

Singh, P., Arora, A., Strand, T. A., Leffler, D. A., Catassi, C., Green, P. H., Kelly, C. P., Ahuja, V., & Makharia, G. K. (2018). Global Prevalence of Celiac Disease: Systematic Review and Meta-analysis. *Clinical Gastroenterology and Hepatology*, *16*(6), 823-836.e2. https://doi.org/10.1016/j.cgh.2017.06.037

Skodje, G. I., Minelle, I. H., Rolfsen, K. L., Iacovou, M., Lundin, K. E. A., Veierød,

M. B., & Henriksen, C. (2019). Dietary and symptom assessment in adults with self-reported non-coeliac gluten sensitivity. *Clinical Nutrition ESPEN*, *31*, 88–94. https://doi.org/10.1016/j.clnesp.2019.02.012

Skodje, G. I., Sarna, V. K., Minelle, I. H., Rolfsen, K. L., Muir, J. G., Gibson, P. R., Veierød, M. B., Henriksen, C., & Lundin, K. E. A. (2018). Fructan, Rather Than Gluten, Induces Symptoms in Patients With Self-Reported Non-Celiac Gluten Sensitivity. *Gastroenterology*, *154*(3), 529-539.e2. https://doi.org/10.1053/j.gastro.2017.10.040

Sobczyńska-Malefora, A., Delvin, E., McCaddon, A., Ahmadi, K. R., & Harrington, D. J. (2021). Vitamin B12 status in health and disease: a critical review. Diagnosis of deficiency and insufficiency–clinical and laboratory pitfalls. *Critical Reviews in Clinical Laboratory Sciences*, *58*(6), 399–429. https://doi.org/10.1080/10408363.2021.1885339

Stefanelli, G., Viscido, A., Longo, S., Magistroni, M., & Latella, G. (2020). Persistent iron deficiency anemia in patients with celiac disease despite a gluten-free diet. *Nutrients*, *12*(8), 1–19. https://doi.org/10.3390/nu12082176

Stoven, S., Murray, J. A., & Marietta, E. V. (2013). Latest in vitro and in vivo models of celiac disease. *Expert Opinion on Drug Discovery*, *8*(4), 445–457. https://doi.org/10.1517/17460441.2013.761203

Stricker, S., De Laffolie, J., Rudloff, S., Komorowski, L., & Zimmer, K. P. (2019). Intracellular localization of microbial transglutaminase and its influence on the transport of gliadin in enterocytes. *Journal of Pediatric Gastroenterology and Nutrition*, *68*(3), E43–E50. https://doi.org/10.1097/MPG.0000000000002171

Sun, H., Chow, E. C. Y., Liu, S., Du, Y., & Pang, K. S. (2008). The Caco-2 cell monolayer: Usefulness and limitations. *Expert Opinion on Drug Metabolism and Toxicology*, *4*(4), 395–411. https://doi.org/10.1517/17425255.4.4.395

Talarico, V., Giancotti, L., Mazza, G. A., Miniero, R., & Bertini, M. (2021). Iron deficiency anemia in celiac disease. *Nutrients*, *13*(5), 1–11. https://doi.org/10

.3390/nu13051695

Tanpowpong, P., & Camargo, C. A. (2014). Early-life vitamin D deficiency and childhood-onset coeliac disease. *Public Health Nutrition*, *17*(4), 823–826. https://doi.org/10.1017/S1368980013003510

Tanpowpong, P., Ingham, T., Lampshire, P., Kirchberg, F. F., Epton, M. J., Crane, J., Camargo Jr, C. A., & the New Zealand Asthma and Allergy Cohort Study Group. (2012). Coeliac disease and gluten avoidance in New Zealand children. *Archives of Disease in Childhood*, *97*(1), 12–16. https://doi.org/10.1136/archdischild-2011-300248

International Society for Clinical Densitometry (ISCD). (2019). *Official Position Statement 2019, Adult*. Available from: https://iscd.org/learn/official-positions/adult-positions/

Trimpou, P., Bosaeus, I., Bengtsson, B. Å., & Landin-Wilhelmsen, K. (2010). High correlation between quantitative ultrasound and DXA during 7 years of follow-up. *European Journal of Radiology*, *73*(2), 360–364. https://doi.org/10.1016/j.ejrad.2008.11.024

Tye-Din, J. (2018). Interpreting tests for coeliac disease. *Aust J Gen Pract*, *47*(1–2), 28–33. https://doi.org/10.31128/AFP-10-17-4357

Tye-Din, J. A., Galipeau, H. J., & Agardh, D. (2018). Celiac disease: A review of current concepts in pathogenesis, prevention, and novel therapies. *Frontiers in Pediatrics*, *6*(November), 1–19. https://doi.org/10.3389/fped.2018.00350

Valitutti, F., & Fasano, A. (2019). Breaking Down Barriers: How Understanding Celiac Disease Pathogenesis Informed the Development of Novel Treatments. *Digestive Diseases and Sciences*, *64*(7), 1748–1758. https://doi.org/10.1007/s10620-019-05646-y

van Beurden, Y. H., van Gils, T., van Gils, N. A., Kassam, Z., Mulder, C. J. J., & Aparicio-Pag??s, N. (2016). Serendipity in refractory celiac disease: Full recovery of duodenal villi and clinical symptoms after fecal microbiota

transfer. *Journal of Gastrointestinal and Liver Diseases*, *25*(3), 385–388. https://doi.org/10.15403/jgld.2014.1121.253.cel

Verdu, E. F., Galipeau, H. J., & Jabri, B. (2015). Novel players in coeliac disease pathogenesis: role of the gut microbiota. *Nat Rev Gastroenterol Hepatol*, *12*(9), 497–506. https://doi.org/10.1038/nrgastro.2015.90

Vici, G., Belli, L., Biondi, M., & Polzonetti, V. (2016). Gluten free diet and nutrient deficiencies: A review. *Clinical Nutrition*, *35*(6), 1236–1241. https://doi.org/10.1016/j.clnu.2016.05.002

Vici, G., Camilletti, D., & Polzonetti, V. (2020). Possible role of vitamin D in celiac disease onset. *Nutrients*, *12*(4), 1–17. https://doi.org/10.3390/nu12041051

Wacklin, P., Kaukinen, K., Tuovinen, E., Collin, P., Lindfors, K., Partanen, J., Mäki, M., & Mättuö, J. (2013). The duodenal microbiota composition of adult celiac disease patients is associated with the clinical manifestation of the disease. *Inflammatory Bowel Diseases*, *19*(5), 934–941. https://doi.org/10.1097/MIB.0b013e3182802 9a9

Walsh, M. C., & Choi, Y. (2014). Biology of the RANKL-RANK-OPG system in immunity, bone, and beyond. *Frontiers in Immunology*, *5*(511), 1–11. https://doi.org/10.3389/fimmu.2014.00511

Walsh, M. C., Takegahara, N., Kim, H., & Choi, Y. (2018). Updating osteoimmunology: Regulation of bone cells by innate and adaptive immunity. *Nature Reviews Rheumatology*, *14*(3), 146–156. https://doi.org/10.1038/nrrheum.2017.213

Wawrzyniak, N., & Suliburska, J. (2021). Nutritional and health factors affecting the bioavailability of calcium: A narrative review. *Nutrition Reviews*, *79*(12), 1307–1320. https://doi.org/10.1093/nutrit/nuaa138

Weaver, C. M., Gordon, C. M., Janz, K. F., Kalkwarf, H. J., Lappe, J. M., Lewis, R., O'Karma, M., Wallace, T. C., & Zemel, B. S. (2016). The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle

factors: a systematic review and implementation recommendations. *Osteoporosis International*, *27*(4), 1281–1386. https://doi.org/10.1007/s00198-015-3440-3

Whittle, C. R., Woodside, J. V., Cardwell, C. R., McCourt, H. J., Young, I. S., Murray, L. J., Boreham, C. A., Gallagher, A. M., Neville, C. E., & McKinley, M. C. (2012). Dietary patterns and bone mineral status in young adults: The Northern Ireland Young Hearts Project. *British Journal of Nutrition*, *108*(8), 1494–1504. https://doi.org/10.1017/S0007114511006787

Wieser, H., Ruiz-Carnicer, Á., Segura, V., Comino, I., & Sousa, C. (2021b). Challenges of monitoring the gluten-free diet adherence in the management and follow-up of patients with celiac disease. *Nutrients*, *13*(7), 9–13. https://doi.org/10.33 90/nu13072274

Wieser, H., Segura, V., Ruiz-Carnicer, Á., Sousa, C., & Comino. (2021a). Food safety and cross-contamination of gluten-free products: A narrative review. *Nutrients*, *13*(7), 2244. https://doi.org/10.3390/nu13072244

Wolf, R. L., Lebwohl, B., Lee, A. R., Zybert, P., Reilly, N. R., Cadenhead, J., Amengual, C., & Green, P. H. R. (2018). Hypervigilance to a gluten-free diet and decreased quality of life in teenagers and adults with celiac disease. *Digestive Diseases and Sciences*, *63*(6), 1438–1448. https://doi.org/10.1007/s10620-018-4936-4

Wünsche, J., Lambert, C., Gola, U., & Biesalski, H. K. (2018). Consumption of gluten free products increases heavy metal intake. *NFS Journal*, *12*, 11–15. https://doi.org/10.1016/j.nfs.2018.06.001

Zammit, C. S., Kurien, M., Sanders, D. S., & Sidhu, R. (2020). What is the role of small bowel capsule endoscopy in established coeliac disease? *Clinics and Research in Hepatology and Gastroenterology*, *44*(5), 753–761. https://doi.org/10.1016/j.clinre.2019.11.011

Zammit, C. S., Sanders, D. S., & Sidhu, R. (2018). Capsule endoscopy for patients

with coeliac disease. *Expert Review of Gastroenterology and Hepatology*, *12*(8), 779–790. https://doi.org/10.1080/17474124.2018.1487289

Zarkadas, M., Cranney, A., Case, S., Molloy, M., Switzer, C., Graham, I. D., Butzner, J. D., Rashid, M., Warren, R. E., & Burrows, V. (2006). The impact of a gluten-free diet on adults with coeliac disease: Results of a national survey. *Journal of Human Nutrition and Dietetics*, *19*(1), 41–49. https://doi.org/10.1111/j.1365-277X.2006.00659.x

Zhou, C., Gao, F., Gao, J., Yuan, J., Lu, J., Sun, Z., Xu, M., Engel, J., Hui, W., Gilissen, L., & Chen, H. (2020). Prevalence of coeliac disease in Northwest China: heterogeneity across Northern Silk road ethnic populations. *Alimentary Pharmacology and Therapeutics*, *51*(11), 1116–1129. https://doi.org/10.1111/apt.15737

Zingone, F., & Ciacci, C. (2018). The value and significance of 25(OH) and 1,25(OH) vitamin D serum levels in adult coeliac patients: A review of the literature. *Digestive and Liver Disease*, *50*(8), 757–760. https://doi.org/10.1016/j.dld.2018.04.005

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Katie Schraders
Name and title of main supervisor:	Professor Marlena C. Kruger
In which chapter is the manuscript/published work?	Chapter 3
What percentage of the manuscript/published work was contributed by the student?	90%

Describe the contribution that the student has made to the manuscript/published work:

Involved in the study design, execution of the study, data collection and interpretation and wrote first manuscript draft and ethics protocol

Please select one of the following three options:

- ○ **The manuscript/published work is published or in press**
  Please provide the full reference of the research output:
- ○ **The manuscript is currently under review for publication**
  Please provide the name of the journal:
- ◉ **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:	Katie Schraders Digitally signed by Katie Schraders Date: 2022.08.28 18:35:26 +12'00'	Main supervisor's signature:	Marlena Kruger Digitally signed by Marlena Kruger DN: cn=Marlena Kruger, c=NZ, o=Massey University, ou=School of Health Sciences, email=m.c.kruger@massey.ac.nz Date: 2022.08.27 09:07:57 +12'00'

This form should be placed at the beginning of each relevant thesis chapter.

CHAPTER 3

Use of an Organoid Model to Investigate the Gut-Bone Axis in Coeliac Disease

3.1 Abstract:

Bone health is often not fully resolved even when individuals with coeliac disease (CD) adopt a gluten-free diet (GFD) intended to resolve gut inflammation and promote optimal nutrient absorption. This may be the result of a persistent dysfunctional gut-bone axis in which the bone tissue continues to produce cellular signals which affect gut function and recovery. The elucidation of the cell signalling pathways in the gut-bone axis is challenged by both the inaccessibility of the cells, such as M-cells which are implicated in the gut-bone axis and Paneth cells which create a niche for the stem cells involved in the regeneration and recovery of the small intestine, and the lack of readily available human tissue. Small intestinal organoids ("mini-guts") offer the opportunity to study otherwise inaccessible gut cells and to maintain and expand gut tissue grown in culture.

The use of murine tissue allowed the development of the methodology for culturing, passaging and re-seeding small intestinal organoids, and for organoids to be triggered to express M-cells after exposure of the organoid tissue to RANK-ligand (RANKL). The establishment of immunocytochemical techniques permitted M-cells to be easily identified from their expression of the M-cell-specific glycoprotein 2. Confocal microscopy techniques meant that the integrity of tight junctions (TJ) could be assessed using antibodies and florescent second markers for the TJ proteins, occludin and zonula occludens-1 (ZO-1). The images from the confocal microscopy were used to create Z-series confocal stacks and 3-

dimensional (3D) stereo-images of the mouse organoids so the organoid structure could be reconstructed and optically resolved to investigate live cells.

Had it been possible to acquire human tissue biopsies, the intention was to expand the methodology for use in organoids derived from tiny amounts of human tissue from individuals with and without CD. It was hoped that the expanded cell mass would allow investigation of the role of M-cells in transepithelial transport of gluten and products of its digestion and the effects of gliadin peptides on tight junction integrity and gut barrier function.

3.2 Introduction:

Coeliac disease (CD) is an incurable immune-mediated disease that is triggered by exposure to gluten in genetically sensitive individuals. CD is associated with a specific genotype (*HLA-DQ2* and/or *HLA-DQ8* genes), the presence of autoantibodies (anti-tissue transglutaminase and anti-endomysial antibodies) and the presence of definitive signs of intestinal damage and inflammation seen in histological samples from endoscopic biopsies of the duodenum (Walker et al., 2017). Individuals with CD commonly suffer from nutrient malabsorption as a result of villous atrophy and the reduced surface area in the intestine (Lerner & Matthias, 2016), as well as defects in epithelial integrity (Rauhavirta et al., 2014). Compromised bone density is a common finding at the time of diagnosis; it has been reported that more than 75% of people with untreated CD are defined as having either osteopenia or osteoporosis (Corazza et al., 2005). In addition to impaired absorption of nutrients needed for bone such as vitamin D, calcium (Krupa-Kozak & Drabińska, 2016) and B vitamins (Clarke et al., 2015), patients with CD often develop hyperparathyroidism caused by low serum calcium (Dos Santos & Lioté, 2017).

Although there is some improvement in bone health when CD patients adopt and strictly adhere to a gluten-free diet (GFD), poor bone health is often

never fully resolved (Szymczak et al., 2012), suggesting that the underlying mechanism for this impairment is ongoing. One cause of this could be that gut inflammation is not fully resolved on a GFD (Bathrellou et al., 2018), resulting in inhibition of bone formation by osteoblasts and stimulation of bone resorption by osteoclasts (Kamycheva et al., 2017; Larussa et al., 2017).

The hypothesis presented here is that persistent poor bone health is due to bone tissue continuing to produce cellular signals that affect the function of both the gut and bone, causing a dysfunctional gut-bone axis. Essentially, that these signals promote bone resorption and gut inflammation leading to compromised nutrient absorption and a net state of bone loss.

3.2.1 Mechanisms of gluten action

Gluten in wheat consists mainly of alcohol-insoluble glutenins and alcohol-soluble gliadins. Gluten is fairly resistant to proteolytic digestion by gastrointestinal enzymes because it contains a high number of glutamine and proline residues (Cabanillas, 2020), 30% and 15% of the amino acid content, respectively (Schumann et al., 2017). This results in the presence of long gluten-derived peptides in the lumen of the small intestine which can access the underlying lamina propria through either the transepithelial route or passively, due to a compromised ("leaky") epithelial barrier function (Lebwohl et al., 2017). The mechanisms of transepithelial movement are not completely understood, but it is thought that the gliadin peptides have increased access to the lamina propria in people with CD (Caio et al., 2019; Valitutti & Fasano, 2019).

In the lamina propria, the immunogenic gluten peptides are deamidated by tissue transglutaminase (tTG) to form glutamic acid residues (figure 3.1). This deamidation process enhances binding of gluten peptides, increasing their affinity for the human leukocyte antigens HLA-DQ2 and HLA-DQ8 present on antigen

presenting cells (APCs) (Kahaly et al., 2018). Almost 100% of CD cases carry variants of the HLA class II genes, HLA-DQA1 and HLA-DQB1.

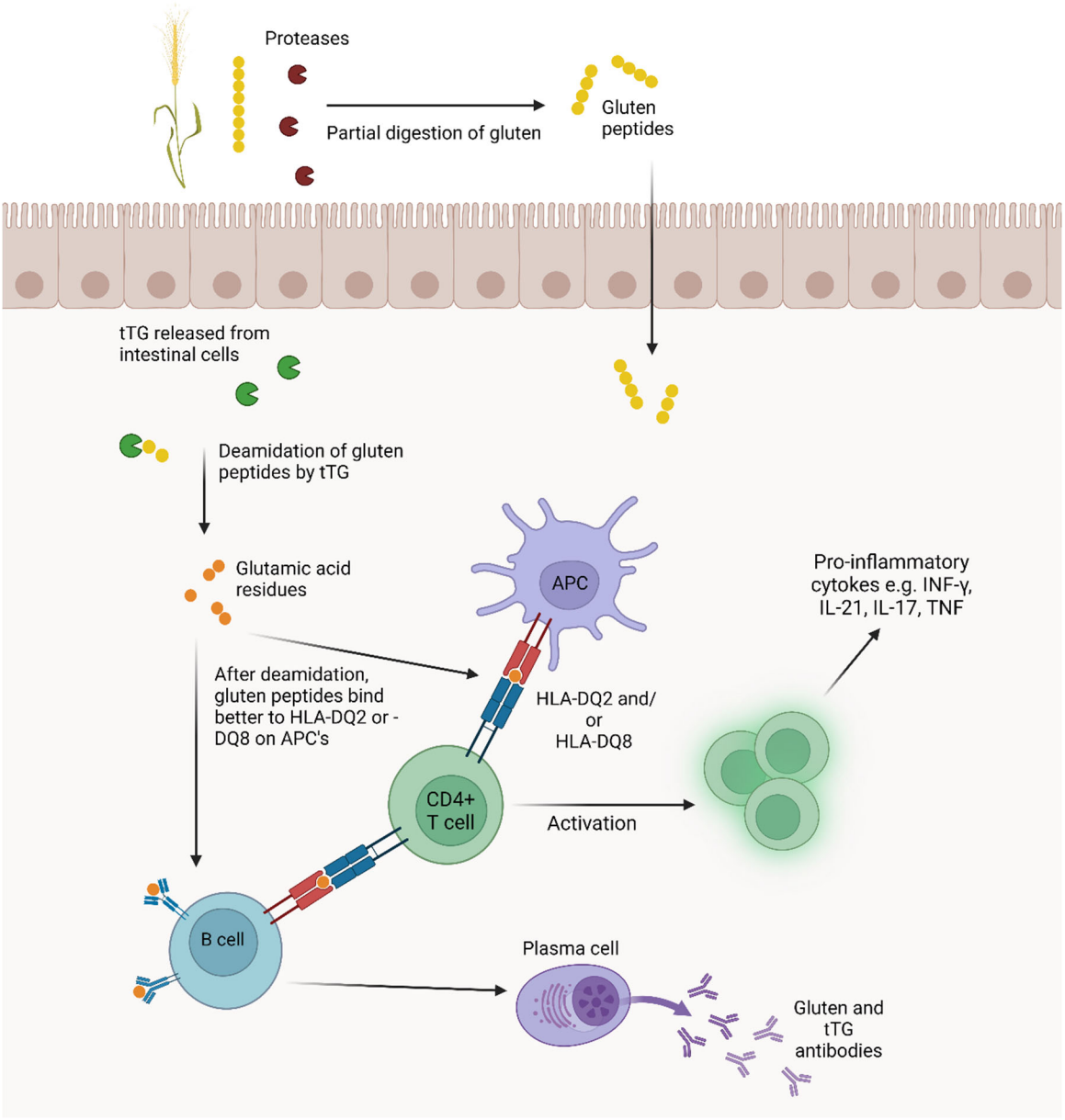

Figure 3.1 Activation of the immune system after ingestion of gluten. Long gluten-derived gliadin peptides are translocated across the epithelial barrier to the lamina propria where they are deamidated by tissue transglutaminase (tTG). Deamidation allows for better binding with HLA-DQ2 or DQ8 on antigen presenting cells (APCs). Gliadin peptides are presented to CD4+ T-cells by APCs triggering the immune response and subsequent inflammatory milieu (Created with BioRender.com)

These genes encode the α and β chains of the HLA-DQ2 and HLA-DQ8 proteins that are expressed on the surface of APCs. More than 90% of people with CD are DQ2 positive with most of the remaining cases being DQ8 positive (Lebwohl et al., 2018). Although the DQ2/DQ8 genotype is necessary for CD to develop, it should be noted that presence of this genotype does not indicate a person has CD since 40% of people in Europe and the Americas carry these alleles, whereas CD affects only ~1% of those populations. APCs expressing these HLA variants present the gluten peptides to CD4+ T-lymphocytes (Kahaly et al., 2018; Lebwohl et al., 2018). B-lymphocytes are also suggested to act as APCs in CD; both gluten-specific and tTG-specific B-lymphocytes recognise their antigens via B-cell receptors (BCRs), internalise them and present them to gluten-specific CD4+ cells. B-lymphocytes also differentiate into plasma cells secreting anti-gluten and anti-tTG antibodies (Björck et al., 2015). Once activated, gluten-specific CD4+ T-cells start secreting inflammatory cytokines, including IFNγ and IL-21, thereby creating an inflammatory milieu in the small intestinal lamina propria.

In addition to these effects of gluten peptides on the immune system, gut permeability is also directly increased by the prolamin component of gluten, gliadin. Gliadin upregulates the zonulin pathway which triggers the disassembly of the protein ZO-1 from the tight junction complex to increase paracellular movement of luminal contents into the lamina propria (Fasano, 2011). This effect of gliadin is therefore implicated in the pathogenesis of CD.

3.2.2 The dysfunctional gut-bone axis: a potential mechanism of low BMD in Coeliac Disease.

M-cells are specialised cells which cover Peyer's patches in the small intestine and act as antigen-sampling cells transporting antigens from the lumen of the small intestine into the gut associated lymphoid tissues (GALT) (Williams & Owen, 2015). Large invaginations in the basolateral membrane of M-cells often

contain immune cells, bringing them relatively close to the apical membrane (Dillon & Lo, 2019). M-cells endocytose antigens, microbiota and pathogens present in the lumen of the small intestine at their apical membrane and encase them in vesicles which are transported to the basolateral membrane where they are delivered to underlying lymphocytes and APCs, such as dendritic cells. These APCs then present the sampled antigens to immune cells (Sakhony et al., 2015). Some intestinal pathogens use M cells in the gut and lungs as their route of entry to invade the host and cause infections (Miller et al., 2007). A possible role for M-cells in the transcytosis of gliadin peptides has not previously been proposed nor studied, but we hypothesise that such a link might explain the relationship between CD and persistent poor bone health.

3.2.3 The intestinal organoid model

Prior to the recent development of techniques to prepare and maintain self-renewing epithelial organoids in tissue culture (Sato et al., 2009), the majority of studies of cell function in the small intestinal epithelium have used either intact tissue, predominantly from mice or rats, or tumor cell lines. Caco-2 cells, for example, have been extensively studied as a model for small intestine function despite the fact that they are a single cell type, not comparable to any of the cell types present in the small intestine and are actually derived from a colon cancer (Sun et al., 2008).

M-cells are particularly difficult to study in the small intestine where they comprise less than 1 in 7,000 of the cells present in the epithelium (Haber et al., 2017). Intestinal organoids, cultured *in vitro* from either intestinal crypts or from stem cells, provide a powerful model to directly observe cell function in living tissue which has a similar cellular composition, organisation, and function of the intestinal epithelium *in vivo* (Sato et al., 2009). LGR5+ stem cells at the base of the small intestinal crypts are responsible for the production of all cell types present in

the epithelium which lines the small intestine (Barker et al., 2007). M-cell differentiation from LGR5+ stem cells in the intestinal crypts is stimulated by Receptor Activator of Nuclear Factor-κβ ligand (RANKL) in a Spi-B dependent manner (Li et al., 2012).

The primary function of RANKL signalling is in bone marrow mesenchymal stem cells where it negatively regulates bone formation by osteoblasts. Bone is continuously remodelled by osteoblasts and osteoclasts, the latter being responsible for bone resorption (Yao et al., 2020); cell differentiation to mature osteoclasts is controlled by RANKL and macrophage colony stimulating factor (M-CSF) (Ono et al., 2020). However, RANKL is also selectively expressed by the subepithelial stromal cells beneath the follicle-associated epithelia (FAE) in the small intestine where it triggers the differentiation of M-cells from LGR5+ stem cells (Knoop et al., 2009). It is this link with gut and bone health that this PhD research study was designed to investigate further. The hypothesis was that the ability of RANKL to stimulate the number and activity of M-cells in intestinal tissue and therefore, potentially, the transepithelial transport of gliadin, as well as promoting bone resorption, may lead to a vicious cycle of intestinal damage and concurrent bone loss. This would explain the relationship between CD and osteoporosis and the inability of a strict GFD to completely restore bone mineral density (BMD).

3.2.4 How can M-cells be identified?

Glycoprotein 2 (GP2) is a cell surface marker expressed in M-cells which is not present in other gut epithelial cell types, so it can be used to identify M-cells using immunocytochemistry to characterize their expression of glycoprotein 2 (Hase et al., 2009). Without RANKL in the medium, organoids express virtually no M-cells. Addition of RANKL has been shown to increase M-cell numbers in a concentration and time-dependent manner, first seen at day 4 and peaking at

day 7. RANKL was reported to increase M-cell numbers in murine organoids from 0.2 ± 0.4% (negligible) in controls, to 36.0 ± 12.9% in the RANKL-treated organoids (Rouch et al., 2016).

The main advantage of the organoid model is the ability to grow, expand and maintain intestinal (or other) tissue for weeks in tissue culture, without loss of function. In the case of human tissue, this is a vast improvement to previous studies on intact human biopsy material, which is rarely available and has a functional lifespan of approximately 2 hours and cannot be maintained in culture. In addition, access to the basolateral membrane of the epithelium is impossible in intact tissue so that transepithelial transport cannot be investigated.

This chapter describes the establishment of the methodology for *in vitro* production of organoids from mouse small intestine, their growth and expansion in culture, immunocytochemical labelling to specifically identify M-cells using confocal imaging and 3-dimensional image reconstruction. These methodologies were established in murine tissue, with the original plan to apply them to human organoids derived from biopsy fragments, both from patients with and without CD. The chapter concludes with a description of the proposed experimental plan to use this model to study the transepithelial movement of gliadin peptides, monitored by multiwavelength fluorescence confocal imaging and gliadin peptide quantitation by ELISA. These studies were not possible to undertake because of the SARS-CoV-2 lockdown in March 2020; following the lockdown it was still not possible to do practical laboratory work within the university safety guidelines even if human biopsy material had been available, which was not the case. The proposed work on human tissue was intended to test the hypothesis that M-cell expression would differ between tissue derived from CD and non-CD patients both in the number of M-cells and their function which could be monitored by immunohistochemistry of fixed sections with the remainder of the biopsy material being used for organoid preparation.

3.3 Materials and methods

3.3.1 Organoid preparation

Animal care

Female C57B6 mice (6-10 weeks old) were collected from the animal house on the morning of each experiment. Mice had access to food and water ad libitum and were euthanised via cervical dislocation within an hour of collection. All study protocols were approved by Massey University Animals Ethics Committee, Protocol 18/56.

Crypt isolation

The murine crypt isolation was adapted from the protocol of Sato et al. (2009). The small intestine from the duodenum to the caecum was quickly removed from the euthanised mouse and placed in fresh ice-cold phosphate-buffered solution (PBS). Pieces were cut longitudinally, and the luminal side was gently scraped with the edge of double glass coverslips until no more debris came off. The tissue was then cut into small 0.5cm pieces and placed in 5 mL fresh ice-cold PBS and washed. This washing step was repeated a further 3 times, with the supernatant discarded after each wash.

After the final wash, the tissue was moved into 15 mL of ice-cold 2 mM EDTA Crypt Isolation Medium (described in appendix A) in a falcon tube and placed horizontally in ice on a shaker for 30 minutes to 'rock like a baby'.

After 30 minutes on the shaker, the tube was shaken more vigorously for 10 seconds by hand, then transferred to a sterile culture dish (labelled 1). Intestinal pieces were quickly removed using tweezers and placed into a 50 mL Falcon tube containing 15 mL of fresh ice-cold sterile PBS. This tube was again shaken for 10 seconds before the contents were transferred to a clean culture dish (labelled 2). This process was repeated 3-5 times and the tissue in each culture dish was

inspected under a dissecting microscope. If no fraction (3, 4 or 5) contained a significant number of individual crypts, a further fraction 6 was produced. The crypt fraction with the highest number of healthy-looking crypts and the smallest amount of single cell debris and villi was chosen, with the contents filtered through a 70 µm sterile filter into a sterile 50 mL Falcon tube. The dish was rinsed with fresh PBS to ensure all crypts were collected. The fraction was split into two balanced sterile 15 mL tubes and centrifuged at 300g for 5 minutes at 4°C.

The supernatant was removed, and the pellet resuspended in 5-7 mL of chilled medium. This was centrifuged at 150-200g for 2 minutes at 4°C. This step was repeated one more time.

Culture of organoids

Matrigel (200 µL) was thawed on ice and gently aspirated multiple times using a pipette tip which had been precooled in a falcon tube placed on ice. Being careful not to lose any of the pellet, the supernatant was removed from the tube containing the tissue. The pellet was allowed to dry slightly and then resuspended in the 200µL of Matrigel. Domes of Matrigel were then placed on each of 4-5 sterile 13 mm round coverslips in a sterile 24-well tissue culture plate, with 3-5 small domes (~40 µL) per well, and this was placed in a tissue culture incubator for 5-10 minutes at 37°C. After 10 minutes, 500 µl of complete culture media containing growth factors (described in appendix A) and pre-warmed in the incubator, was added to each of the wells containing coverslips and the plate was incubated at 37°C/5% Co_2 under sterile conditions.

Complete medium was changed every 4 days with growth factors being renewed half-way between these media changes, so that, the entire medium was gently aspirated and replaced at days 4, 8, 12 etc. and 50 µl/well of growth factor mix (REN) (described in appendix A) was added to the existing 500 µl/well of medium at day 2, 6, 10 etc.

Passage of organoids.

Organoids were passaged 7–14 days after seeding by removal of the culture medium then gentle breaking up of the Matrigel by repeatedly aspirating with a 1 mL pipette. The organoids were then resuspended in 1-2 mL of basal culture medium and transferred to a 15 mL Falcon tube. Again, the organoids were gently disrupted using a fire-polished Pasteur pipette. Dead cells and single cells were removed by washing the organoids with 10 mL basal culture medium. Organoids were then mixed with 200 µL Matrigel (as above) and 3-5 ~12 µL domes were placed on each of 4-5 sterile 13 mm coverslips and cultured in a new 24 well culture plate.

Treatment with RANKL

A stock solution of RANKL was prepared by dissolving 10 µg of recombinant mouse RANKL (R&D Systems) in 1 mL of sterile PBS containing 0.1% BSA. 10 µL of this was added to the 500 µL of medium present in each well of organoids (50x dilution of the 10 µg/mL) at day 7 of their culture to get a final concentration of 200 ng/mL. The remaining stock RANKL was frozen in 45 µL aliquots, each sufficient for 4 wells. Culture medium was replaced with fresh medium containing growth factors and RANKL every 2 days until organoids were fixed at day 5 of RANKL exposure for immunocytochemical staining of glycoprotein-2, the M-cell marker.

3.3.2 Preparation for immunocytochemical staining

Fixation

Organoids were fixed and permeabilised prior to preparation for immunocytochemical staining. Organoids were washed 3 times with PBS leaving it in the well for 5 minutes each time. Organoids were then fixed with 2% paraformaldehyde at pH 7.4 (4% paraformaldehyde, mixed 1:1 with PBS) and left

overnight at 4°C. The following day, the fixation medium was removed, and the organoids were washed 3 times with PBS and left with 1 mL of PBS in each well. Parafilm was wrapped tightly around the sides of the 24-well plate to seal it and avoid evaporation and the plate was returned to the fridge until ready for staining.

Permeabilisation

Blocking buffer (BB) was prepared by adding 1 mL of frozen goat serum to 19 mL PBS. This was gently mixed using a vortex mixer to avoid shaking which would cause the solution to foam. The fixed organoids were washed with this BB after removing the storage medium. 10 µl Triton X-100 was added to 2 mL of BB to yield a 0.5% Triton permeabilisation medium. The BB was removed from the organoids and replaced with 250 µL/well of this permeabilisation medium, then left for 60 minutes at room temperature (RT).

Immunocytochemical staining for GP2

Control organoids, and organoids cultured in the presence of RANKL, were fixed and permeabilised then incubated with 250 µL/well of anti-mouse Glycoprotein 2 mouse monoclonal antibody derived from rat (5 µL of stock anti-GP2 (1 mg/mL) in 1 mL BB). Plates were sealed carefully and placed in the fridge overnight.

The fluorescently tagged second antibody solution was prepared by adding 20 µL of Alexa Fluor 488 labelled goat anti-Rat IgG (A-11006) to 2 mL BB (100x dilution) with gentle vortex mixing. After overnight incubation, the first antibody solution was removed from each well and replaced with 250 µL/well of the second antibody solution and incubated at RT for 2 hours. The coverslips were then washed by replacing the media 3 times with BB allowing 5 minutes each time in the medium.

Immunocytochemical staining for Occludin and ZO-1

The staining procedure for the assessment of tight junction integrity was first established using monolayers of Caco-2 cells. To enable us to correlate tight junction integrity, visualized by immunocytochemical staining of tight junction proteins, with transepithelial leakage, Caco-2 cells were used as a model system to establish epithelial growth on Transwell filters, as discussed later in this Chapter. Caco-2 cells were grown in culture for approximately 21 days to achieve post-confluent, 'epithelium-like' monolayers on Transwell filters. The apical fluid was removed, and monolayers were washed 3 times by addition of 200 µL of PBS and then fixed in 2% paraformaldehyde in PBS for 30 minutes at RT. The fixation medium was removed, and the monolayers were again washed 3 times with PBS and stored at 4°C until staining.

For staining, the Transwells were removed from the tissue culture plate and the filters bearing the monolayers were carefully removed from the plastic supports using a fine scalpel blade, making sure the filters did not dehydrate. The cut filters were incubated in 95% ethanol for 30 minutes at 4°C, followed by 3 minutes at RT in acetone that had been stored at -20°C.

3.3.3 Immunostaining protocol

Cells were brought to RT and permeabilised for 15 minutes with 2 mL of 0.2% triton in PBS, then washed 3 times with PBS. Media were aspirated and replaced with 2 mL of BB (comprised of 2% normal goat serum in PBS, filtered through a 0.22 µm filter) and left at RT for 60 minutes. All subsequent media were prepared in BB.

The cell monolayers on Transwell filters were stained by placing the filter, monolayer-side down, on a 200 µL droplet of the following media at RT: mouse anti-occludin antibody (2.5 µg/mL) for 60 minutes, Biotin-XX goat anti-mouse IgG antibody (2.5 µg/mL) for 60

minutes, Alexa Fluor 488 goat anti-rabbit IgG antibody 10μg/mL) for 60 minutes and Streptavidin Alexa Fluor 546 conjugate (10 μg/mL) for 60 minutes. After each incubation, the filters were washed 3 times with BB. Filters were then mounted onto a microscope slide, immersed in Prolong Gold and covered using gentle pressure with a No.1 glass coverslip, to avoid trapping air bubbles. The coverslip was sealed around its edges to the microscope slide using clear nail varnish.

Nuclear staining

Nuclei were stained with 1 μM Hoechst 33258 (2,000x dilution of 2 mM stock stored at -20°C) for 20 minutes at RT.

3.3.4 Microscopy

Fluorescent and transmitted light images were captured using an inverted fluorescence microscope (Nikon TE2000) with a confocal scanhead (Nikon C1) equipped with 405 nm, 488 nm and 561 nm lasers (Coherent Scientific) and a range of focal length objectives including a 60x CFI Plan Apochromat VC water immersion objective (Nikon). Single fluorescence or transmitted light (brightfield) images, or Z-series confocal stacks, were collected using EZ-C1 software (Nikon) and these were processed using NIH ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018) or Volocity software (ThermoFisher) and presented using Adobe Photoshop.

3.4 Results

Intestinal crypts were isolated and placed in tissue culture. Single freshly isolated crypts (as shown in Fig. 3.2A) rapidly sealed into spherical structures (Fig. 2B) and after 3 days in culture, the organoids had grown and new buds could be seen extending from the central mass (Fig. 3.2C). By 5 days in culture, multiple crypts were seen to bud from the central mass (Fig. 3.2D)

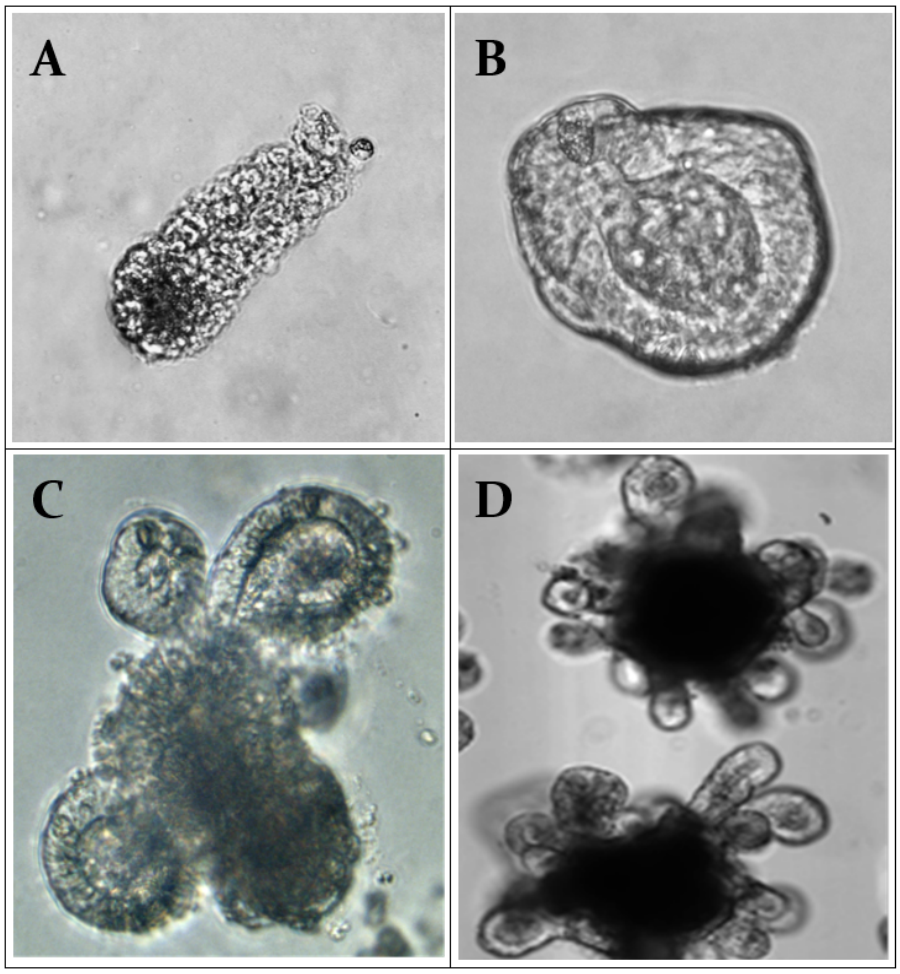

Figure 3.2 Brightfield image of freshly isolated intestinal crypt (A), and developing organoids after 1 day (B), 7 days (C) and 10 days (D) in tissue culture.

Examination of the organoids at high magnification showed the very dense central lumen being full of apoptotic cells that had been sloughed off during the continuous turnover of epithelial cells, which became trapped in the lumen of the organoid and their light-absorbing nuclei (Fig. 3.3). At this stage, the organoids were broken up and re-seeded from the passaged cells.

The size and density of mature organoids is shown in Figure 3.3. This image illustrates how difficult it would be to obtain detailed images of mature organoids with conventional light or fluorescence microscopy.

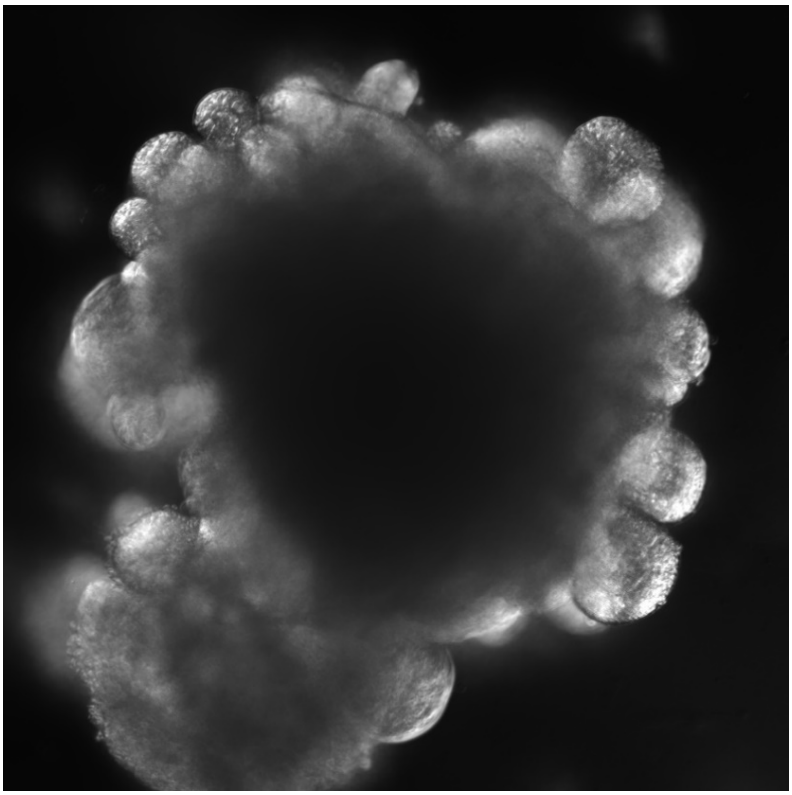

Figure 3.3 Darkfield image of a single intestinal organoid showing the central light-absorbing lumen filled with apoptotic cells that would normally be shed after a 3–5-day lifespan into the lumen of the small intestine.

Reasonably well-defined images can be acquired from intact organoids when viewing intestinal crypts which protrude significantly from the body of the organoid, unlike those seen in Figure 3.3. Figure 3.4 shows a brightfield image of a single organoid crypt showing two Paneth cells at the base of the crypt where they lie adjacent to, and communicate with, the LGR5+ stem cells.

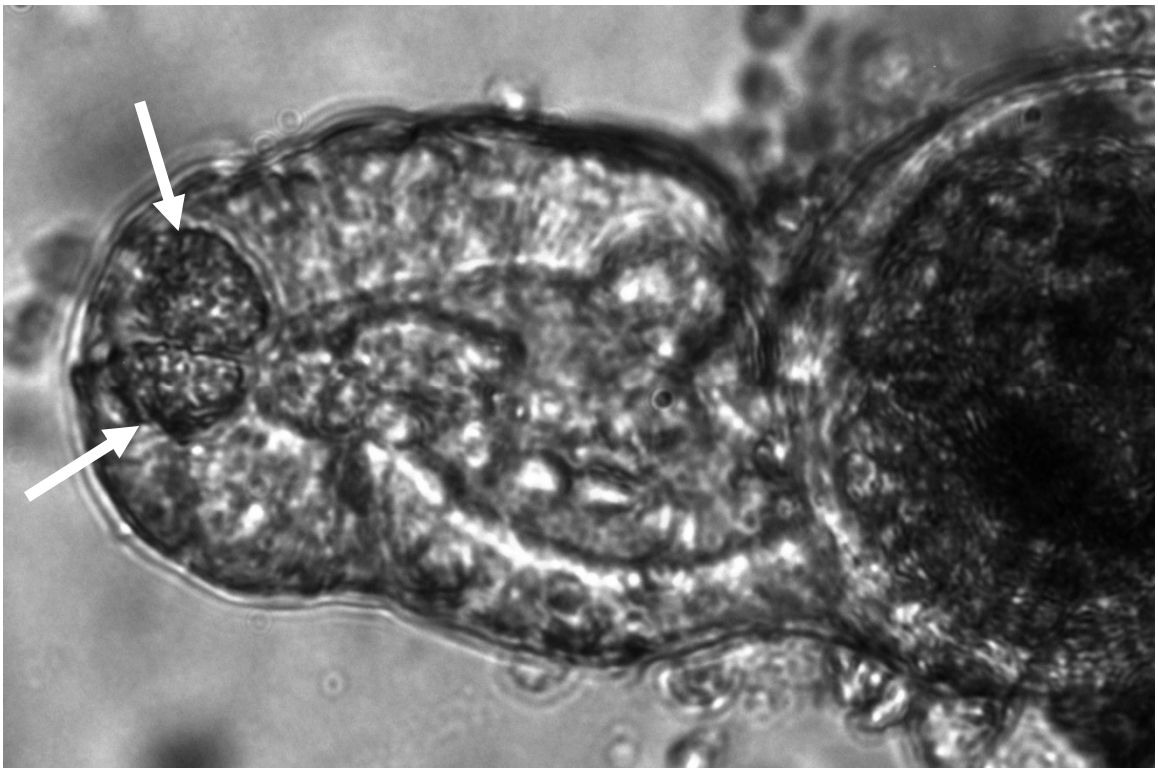

Figure 3.4 A brightfield image of a single crypt. The arrows indicate two Paneth cells.

The use of confocal microscopy allows the imaging of optical sections. These are equivalent to manually cutting sections through the tissue using a microtome, except that the section is selected by the excitation and emitted light passing through confocal apertures so that all emitted light from above and below the plane of focus is not detected. The principle of the confocal microscope is shown in Figure 3.5. Excitation light from the laser, matched to the excitation optimum of each fluorescent probe, passes through a pinhole and is reflected by a dichroic mirror to illuminate the specimen. Light of a longer wavelength (due to energy lost) is emitted by the probe and this passes through the dichroic mirror so that the intensity of that point in the image is detected with high sensitivity by the photomultiplier tube (PMT). The laser is scanned across X and Y dimensions and the intensity of emitted light at each point (pixel) is assembled to make the

complete image. The maximum resolution of the confocal microscope used here was 2048 x 2048 pixels.

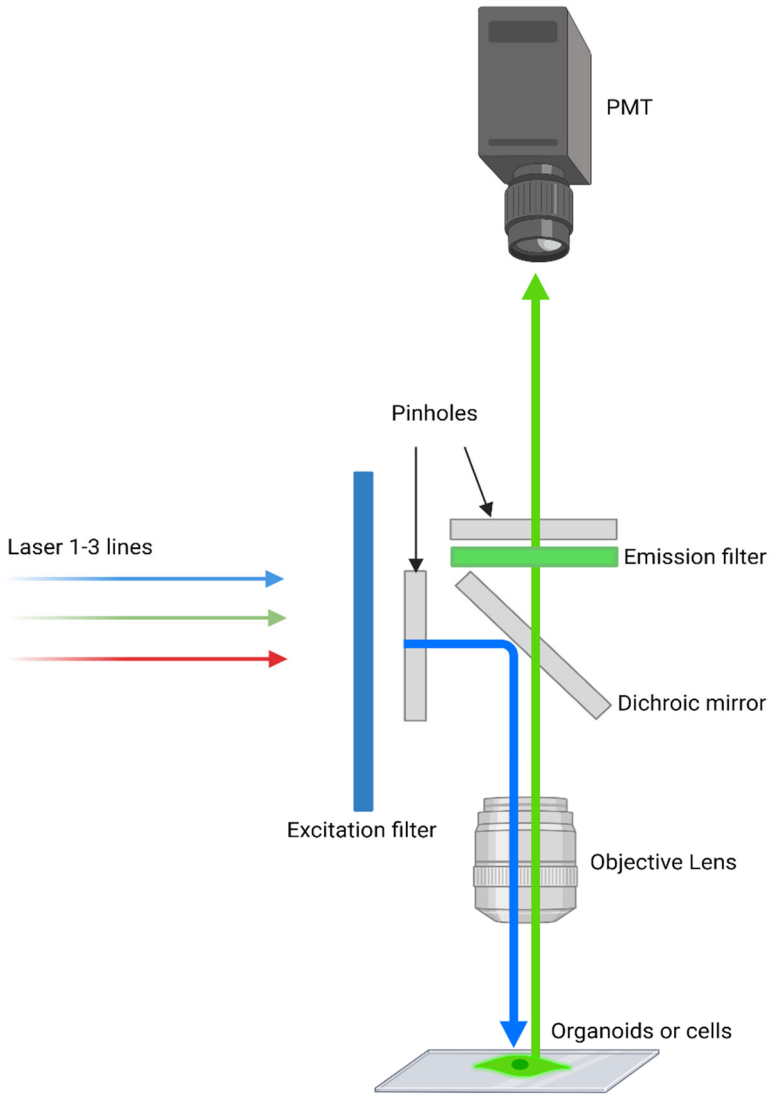

Figure 3.5 Diagrammatic description of a confocal microscope. Excitation light from the selected laser passes via a pinhole and is reflected by a dichroic mirror to illuminate the specimen. The light of longer wavelength emitted by the probe, passes through the dichroic mirror and emission pinhole so that the intensity of that point in the image is detected by the photomultiplier tube (PMT) (Created with BioRender.com)

The ability to reject almost all of the emitted light from above and below the plane of focus allows an optical section to be visualized through a complex structure like an organoid, to produce a clear image of that section which cannot be seen by eye. Figure 3.6 shows a confocal image through a single organoid crypt lying within an intact organoid. The transmitted light image (A) enables the outer epithelial layer of cells to be distinguished from the lumen they surround but at low resolution because of the thickness of the tissue. The cell nuclei are seen in the confocal fluorescence image where the nuclei are labelled with Hoechst 33258 (B). This image is superimposed on the brightfield image in (C). Both the single layer of epithelium with the nuclei close to the outer, basolateral membranes, and the nuclei shed into the crypt lumen, are visible.

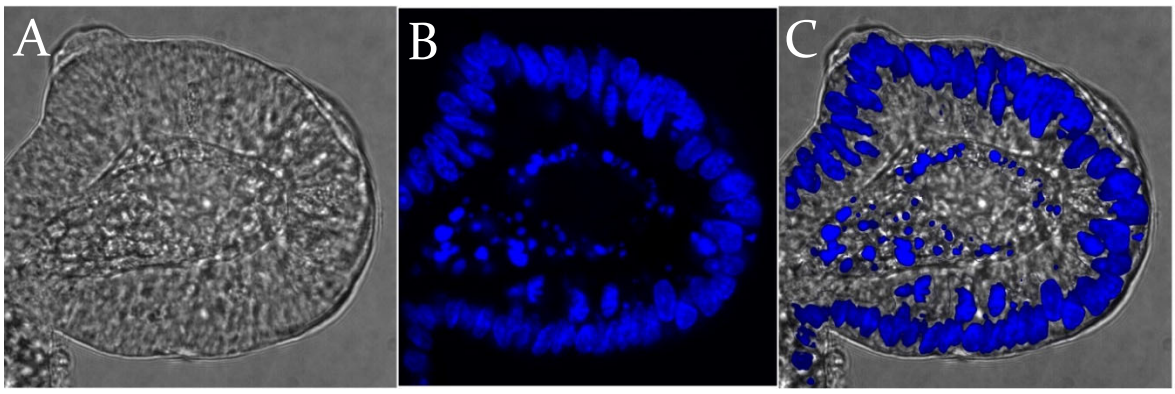

Figure 3.6 Confocal image through a single organoid crypt showing the transmitted light image (A), the fluorescence image of cell nuclei labelled with Hoechst 33258 (B) and the superimposed images (C).

As well as acquiring clear, high resolution optical sections from large, intact organoids using confocal microscopy a Z-series of the organoids was produced. Using the confocal microscope with the fine focus control driven by a calibrated stepping motor, it was possible to obtain a series of images (Z-series) from the surface of the organoid nearest to the objective to the opposite surface of the

organoid furthest from the objective by repeated accurate movement of the focus between sequential images in identical steps. The series of optically sectioned, high-resolution images can be viewed individually or re-constructed into a 3-dimensional (3D) image.

This approach is shown in Figure 3.7 which depicts 4 of a series of 39 confocal images captured with the microscope focus moved in 5µm steps between each image to produce the Z-series. The organoid nuclei were stained to identify individual cells using the fluorescent DNA dye, Hoechst 33258. The nuclei of dead epithelial cells which have been lost by apoptosis into the organoid lumen are visible in Figure 3.7b and 3.7c which show the organoid lumen at 75 µm and 115 µm, respectively, beneath the top surface of the organoid. This is made possible by the ability of the confocal microscope to 'optically section' thick tissue.

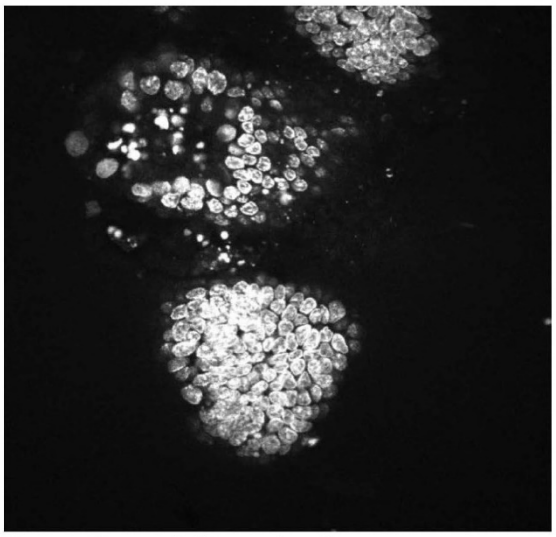

Fig. 3.7a (Image 3): Organoid surface nearest to objective

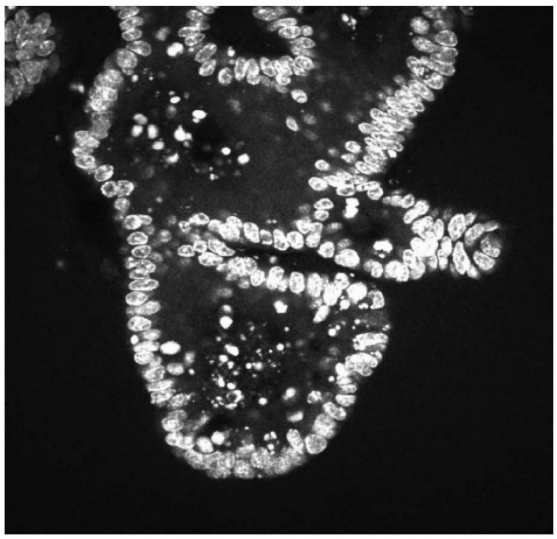

Fig. 3.7b (Image 15): 75µm depth

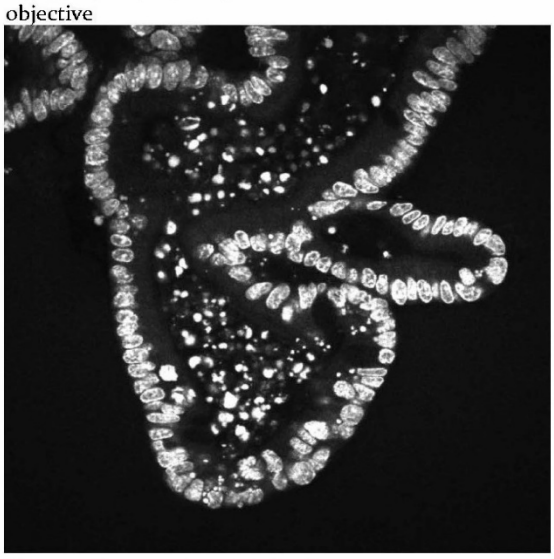

Fig. 3.7c (Image 23): 115µm depth

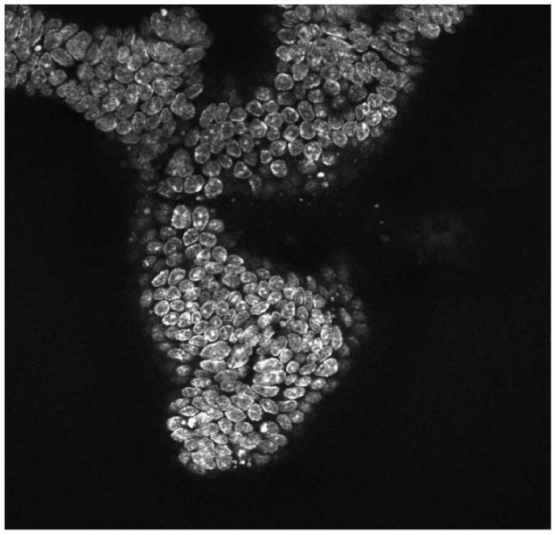

Fig. 3.7d (Image 36): Furthest surface

Figure 3.7 A Z-series of 39 confocal images were captured with the microscope focus moved in 5 µm steps between each image.

The Z-series of confocal images can also be reconstructed in 3D to form pictures of the structure in which all organoid cells can be visualised. Figure 3.8 shows an example of a 3D stereo image which needs to be viewed wearing 3D glasses (attached).

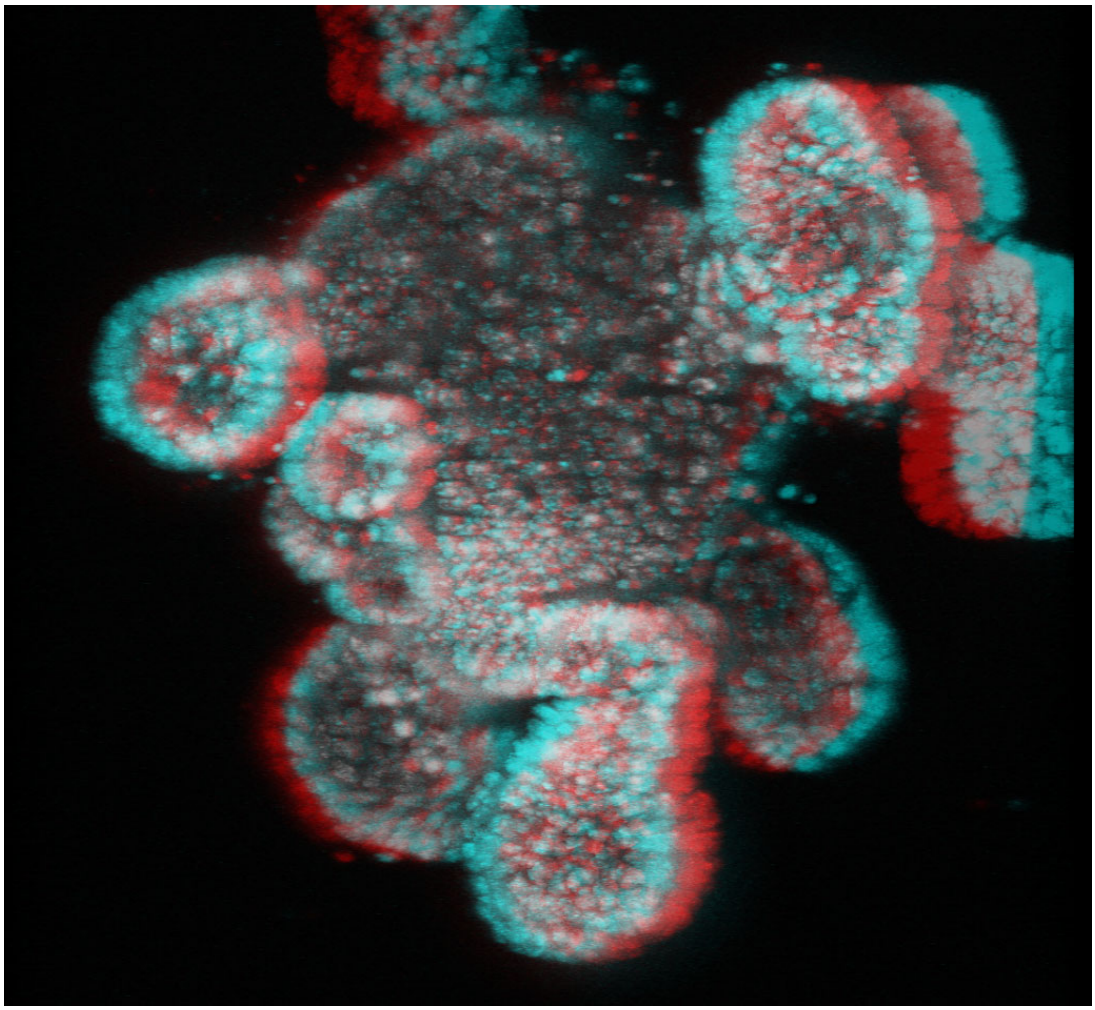

Figure 3.8 3D reconstructed image of an organoid. To be viewed with 3D glasses.

A major part of this study was to investigate the possible role of M-cells in the transepithelial movement of gliadin peptides and the possible link between RANKL signalling in osteopenia/osteoporosis and increased RANKL-induced expression of M-cells in the intestine of CD patients; the 'Gut/Bone Axis'.

Supplementation of organoid culture medium with RANKL was successful, as previously reported (de Lau et al., 2012) in triggering the expression of intestinal M-cells, which were essentially absent from control organoids. These were clearly detected using the immunocytochemical labelling of the M-cell-specific GP2

protein which was visualized in 3-dimensional images of fluorescently tagged organoids using confocal microscopy (Fig. 3.9).

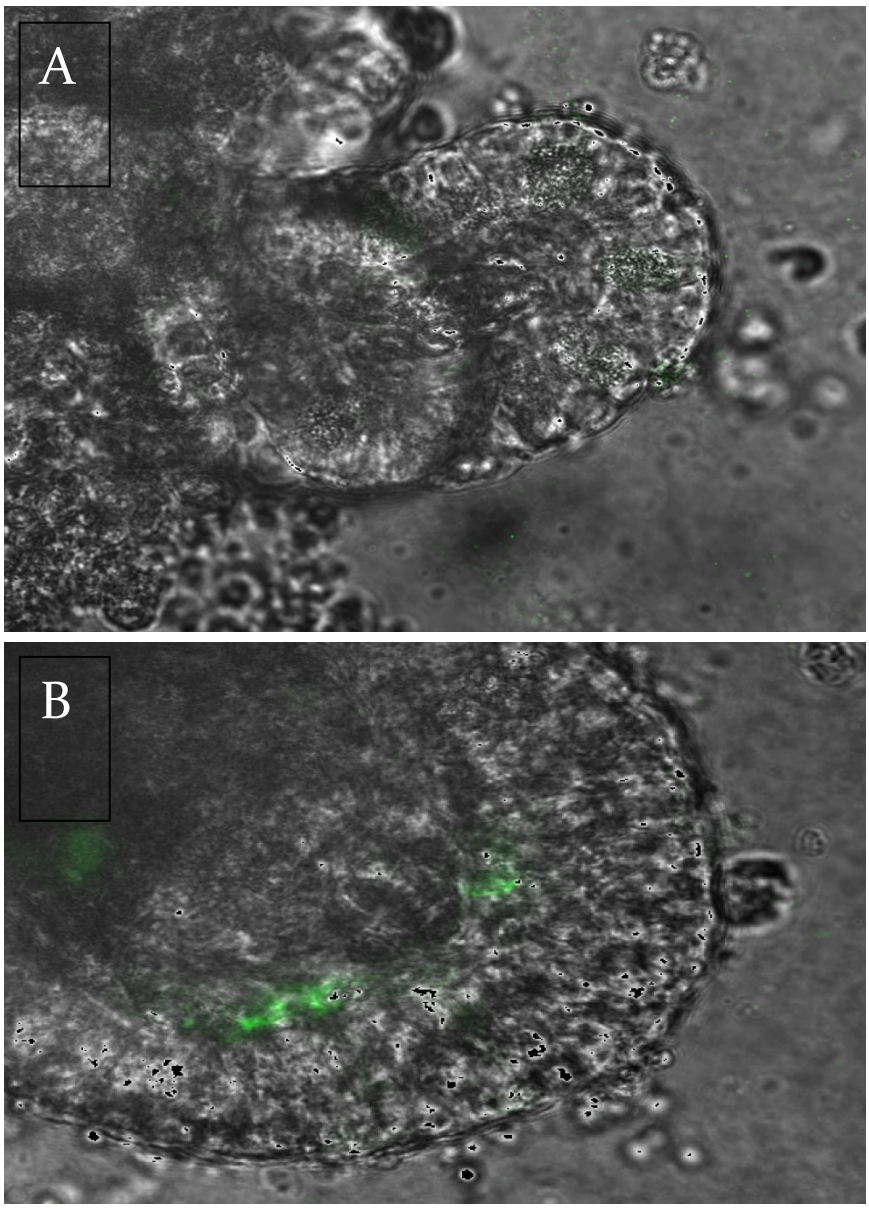

Figure 3.9 RANKL-induced M-cell expression in mouse organoids. Addition of RANK ligand (RANKL) to the medium induced the stem cell-derived production of M-cells (Image B). M-cells were identified by fluorescence immunocytochemistry using a rat monoclonal antibody against the M-cell specific protein, glycoprotein 2, labelled with Alexa fluor 488 goat anti-rat IgG (green). Image A shows a control organoid, not exposed to RANKL.

3.5 Conclusion

The intention of this chapter was to describe the development of the methodology necessary for the studies, described above, in mouse organoids so that they could be utilised in organoids prepared from the much more difficult to obtain human biopsy tissue. The studies using mouse organoids proved successful with RANKL inducing the expression of M-cells. These findings provided confidence that these techniques could be applied to human tissue. The isolation of intestinal crypts and their development into functional organoids in tissue culture would have been easily applied to tiny amounts of human tissue from donated biopsies. Furthermore, the successful disruption and passaging of developed organoids to grow and maintain organoids over weeks in culture would have allowed this approach using human tissue to provide sufficient tissue for long-term studies which would be impossible to achieve using unmodified human biopsy material.

3.6 Application of the techniques in human tissue

When organoids are grown in the artificial extracellular matrix, Matrigel, the apical side of the epithelium faces the lumen of the organoid presenting multiple problems for access to, and sampling of, the apical medium. The use of intact organoids to study the effects of gliadin peptides applied to the apical membranes of organoid epithelium would have necessitated the use of microinjection via a micropipette into the organoid lumen. Exposure of the apical membranes to supplements can only be achieved by microinjection, with a subsequently unknown concentration of injected additives due to dilution by an unknown volume of luminal contents. Sampling of apical media is also tricky because the rapid turnover of the intestinal epithelial cells, with their lifetime of 3-5 days, deposits the debris from the dead cells, as well as cellular secretions and mucus, into the lumen of the organoid.

To establish a more easily used model, we had intended to covert mature organoids into epithelial monolayers grown on Transwell filters in culture. The application of this approach to human tissue would have greatly simplified our ability to apply known concentrations of agonists to the apical surface, specifically sample transepithelial movement to the basolateral surface, measure epithelial integrity as transepithelial electrical resistance (TEER) and visualize the redistribution of tight junction-related proteins using specific antibodies tagged with fluorescent dyes and high-resolution confocal imaging. Application of this approach would have allowed us to address some important questions which it has not been possible to address in human tissue or patients. These included:

- the possible role of M-cells in transepithelial transport of gluten peptides
- the time-course of the effects of gliadin peptides on epithelial leakage
- the involvement of gliadin-induced zonulin signalling and tight junction leakage

In 2018, at the commencement of this PhD no studies had been published on the use of human organoids to study the mechanisms of gluten-induced intestinal cell pathology. In February 2020, a paper was published on changes in gene expression in intestinal stem cells in organoids derived from CD patients (Dieterich et al., 2020). Then, in February 2022, the first publication on the study of inflammatory mechanisms in CD in patient-derived organoids was published (Porpora et al., 2022). The latter used both intact organoids and 'opened up 2D organoids' to bypass the need for microinjection to reach the apical cell membranes, a requirement we planned to address here with the use of organoid-derived monolayers. Neither of these publications addressed the possible mechanisms of transepithelial movement of gluten peptides nor the presumed

roles and mechanisms of gluten-derived peptides in the downregulation of tight junction integrity.

3.6.1 Phase 2 Experimental plan (this was not able to proceed due to the pandemic lockdowns)

The following experiments were planned using small intestinal human biopsy tissue. A collaboration was established with Dr James Irwin, Department of Gastroenterology, Palmerston North Hospital, to acquire this tissue. It was planned that four to six samples would be collected from each consenting patient (healthy and those with CD) having a biopsy to provide tissue to establish the method.

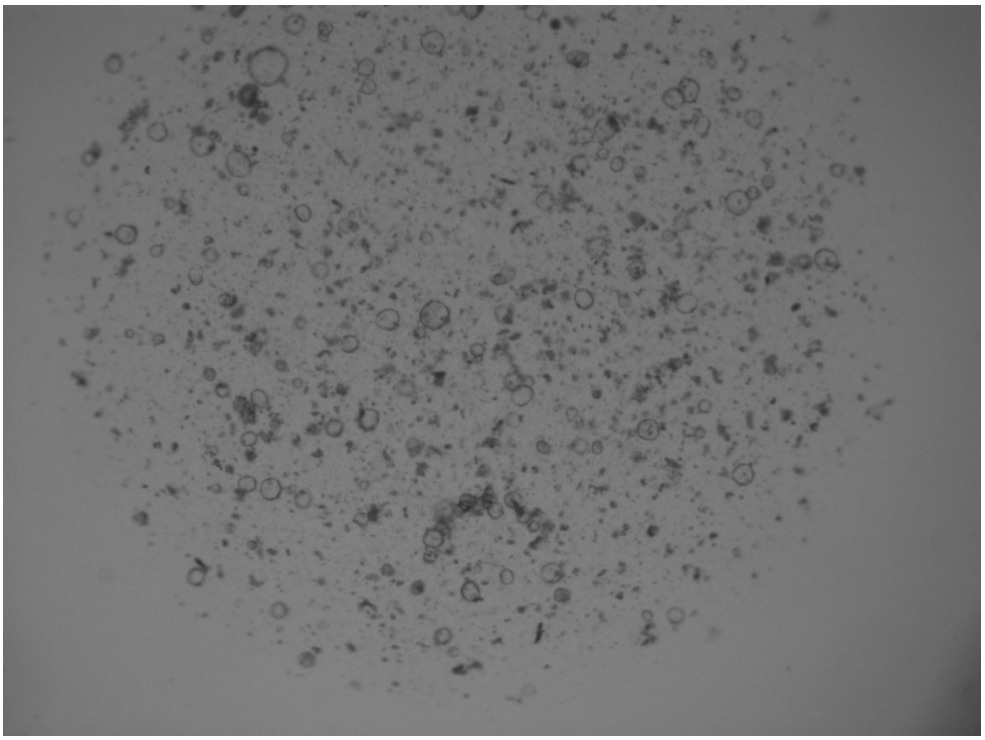

Figure 3.10 Human colon organoids at day 6, after recovery from cryopreserved crypts

In addition, prior to the pandemic, I was invited to visit the Hubrecht Institute in the Netherlands, one of the leading institutes in the world for stem cell

research and pioneers in the field of organoid research, where I was able to both observe and assist with organoid work using human tissue. Figure 3.10 depicts the colon organoids which I helped to establish from crypts which were cryopreserved.

Unfortunately, despite gaining this experience with human organoids, our human experiments were unable to continue due to the constraints on research and ethics applications as a result of the pandemic and subsequent lockdowns. The following explain the planned projects:

1. ***Microinjection of organoids with gliadin peptides***

Hypothesis: Organoids derived from small intestinal tissue from individuals with CD will respond differently to exposure of gliadin peptides.

Plan: The structure of intact intestinal organoids is inverted, with the apical membranes of the epithelium facing the central lumen of the organoid (see Fig. 3.3). This would require microinjection of gliadin peptides into the organoid lumen in order for them to reach the apical membranes of M-cells.

The intention was to carry this out using glass micropipettes of the type used for intracellular microinjection with picolitre/nanolitre volumes of medium being delivered by a PicoPump (World Precision Instruments).

2. ***Increasing the organoid cell mass to prepare monolayers of cells to investigate translocation of gliadin peptides across the gut barrier***

Hypothesis: M-cells are involved in the movement of gluten across the gut barrier in individuals with CD with an increased number of M-cells being present due to increased levels of RANKL.

Plan: A second experimental model would be established from an expanded mass of human derived cells. The intestinal organoids would be used to grow and markedly expand human biopsy tissue samples in culture; the markedly increased cell yield would then have been used to prepare monolayers of cells (Bar-Ephraim

et al., 2020; Dutta & Clevers, 2017; Kasendra et al., 2018). The resulting single-cell preparation would then have been grown on Transwells (Kim et al., 2017) to form a confluent, 'epithelium-like' monolayer of small intestine cells which would allow selective access to, and sampling of, the apical and basolateral medium from the confluent cell monolayer to study the cellular uptake and transcytosis of gliadin peptides directly. This approach would negate the need for microinjection to reach the apical membranes of M-cells. Epithelial integrity could also be monitored in cell monolayers exposed to gliadin peptides by measuring transepithelial electrical resistance (TEER) using electrodes placed on opposite sides of the epithelium. Furthermore, tight junction integrity would have been visualised using immunocytochemical labelling of tight junction proteins, confocal microscopy, and 3D image reconstruction of Z-series.

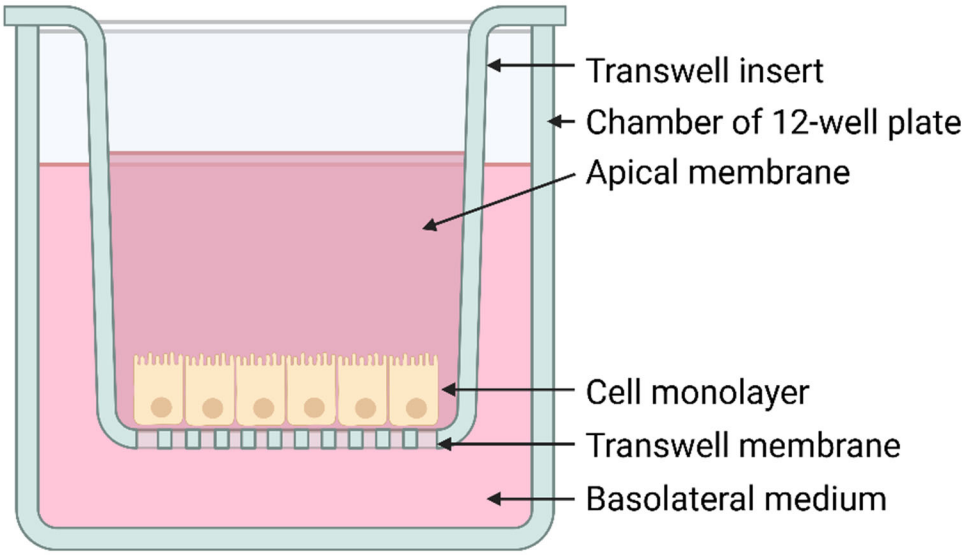

Figure 3.11 Monolayer of organoid-derived epithelial cells adhering to Transwell filter (Created with BioRender.com)

The monolayers (Fig. 3.11) would have been monitored for when confluence was achieved by measuring TEER using an EVOM Epithelial Volt/ohm meter

(World Precision Instruments) (Fig. 3.12). In this model, monolayers would have been considered to be confluent when TEER values exceeded 350 Ω.cm².

In addition, the transepithelial movement of apically added gliadin peptides could be detectable in the basolateral medium using an ELISA assay. This model would have allowed comparison of the movement of gliadin peptides across the epithelial monolayers into the basolateral media of control epithelium with RANKL-treated M-cell expressing epithelium. Electrodes would have been placed in the apical and basolateral medium as shown in Figure 3.12.

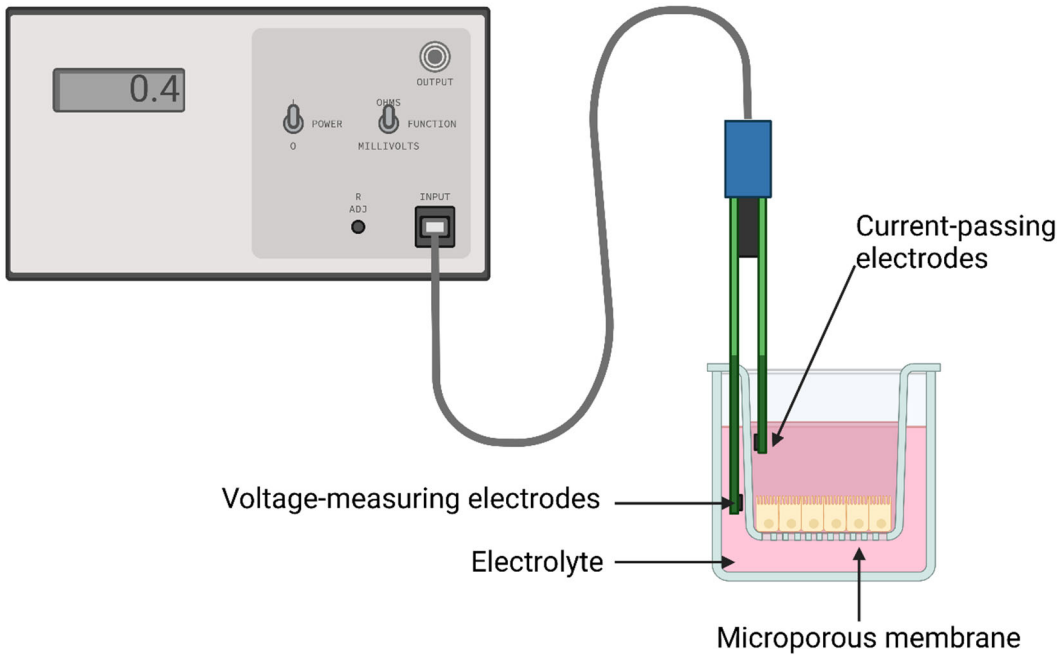

Figure 3.12 Measurement of TEER values of organoid-derived monolayers on Transwell filters (Created with BioRender.com)

Exposure to gliadin peptides

Organoid-derived monolayers would have been differentiated to contain M-cells, by addition to the basolateral medium of RANKL (100 ng/mL). The medium would have been changed every two days to contain fresh RANKL. At 5 days,

gliadin peptides would have been added to the apical medium and the effects on epithelial barrier function tested by measurement of TEER values as described above.

The organoids would have been:

1. Converted into monolayers using the protocol reported by Ranganathan et al. (2019).
2. Repeatedly passaged, as described above
3. Fragmented by transferring to a sterile tube and shaking on ice for 30 minutes, followed by repeated trituration.
4. Combined from several wells, washed with basal medium then centrifuged at 1,000 rpm for 10 min at 4°C.
5. The triturated organoids would then be resuspended in complete medium supplemented with growth factors, Y-27632 (10 μM) and CHIR99021 (5 μM).
6. Prior to the addition of the cell suspension, Transwell filters (Corning) (Fig. 3.11) would have been coated with human collagen Type IV by addition of 100 mL of a collagen (Sigma) solution (35 μm/mL) to the top surface of the filter and incubation at 37°C for 3 hours.
7. Transwells would have been washed and the triturated organoid fragments would have been added to the apical surface of the Transwells.
8. 400 μL of complete medium supplemented with growth factors would have been added as the apical medium and 2.5 mL as the basolateral medium.
9. Transwells would then be incubated in a tissue culture incubator at 37°C with 5% CO_2 and monitored after one week in culture until TEER values exceeded 350 $\Omega.cm^2$. After confluence was achieved and barrier function established, this *in vitro* epithelium expanded via organoid growth from human intestinal biopsies would have provided important information which could not be accessed using other experimental models, nor *in vivo*.

3.7 References

Bar-Ephraim, Y. E., Kretzschmar, K., & Clevers, H. (2020). Organoids in immunological research. *Nature Reviews Immunology*, *20*(5), 279–293. https://doi.org/10.1038/s41577-019-0248-y

Barker, N., Van Es, J. H., Kuipers, J., Kujala, P., Van Den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P. J., & Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature*, *449*(7165), 1003–1007. https://doi.org/10.1038/nature06196

Bathrellou, E., Kontogianni, M. D., & Panagiotakos, D. B. (2018). Celiac disease and non-celiac gluten or wheat sensitivity and health in later life: A review. *Maturitas*, *112*(January), 29–33. https://doi.org/10.1016/j.maturitas.2018.03.014

Björck, S., Lindehammer, S. R., Fex, M., & Agardh, D. (2015). Serum cytokine pattern in young children with screening detected coeliac disease. *Clinical and Experimental Immunology*, *179*(2), 230–235. https://doi.org/10.1111/cei.12454

Cabanillas, B. (2020). Gluten-related disorders: Celiac disease, wheat allergy, and nonceliac gluten sensitivity. *Critical Reviews in Food Science and Nutrition*, *60*(15), 2606–2621. https://doi.org/10.1080/10408398.2019.1651689

Caio, G., Volta, U., Sapone, A., Leffler, D. A., De Giorgio, R., Catassi, C., & Fasano, A. (2019). Celiac disease: A comprehensive current review. *BMC Medicine*, *17*(1), 1–20. https://doi.org/10.1186/s12916-019-1380-z

Clarke, M., Ward, M., Dickey, W., Hoey, L., Molloy, A. M., Waldron, L., Varghese, A., McCann, A., Blayney, J. K., & McNulty, H. (2015). B-vitamin status in relation to bone mineral density in treated celiac disease patients. *Scandinavian Journal of Gastroenterology*, *50*(8), 975–984. https://doi.org/10.3109/00365521.2015.1015603

Corazza, G. R., Di Stefano, M., Mauriño, E., & Bai, J. C. (2005). Bones in coeliac disease: Diagnosis and treatment. *Best Practice and Research Clinical Gastroenterology*, *19*(3), 453–465. https://doi.org/10.1016/j.bpg.2005.01.002

de Lau, W., Kujala, P., Schneeberger, K., Middendorp, S., Li, V. S. W., Barker, N., Martens, A., Hofhuis, F., DeKoter, R. P., Peters, P. J., Nieuwenhuis, E., & Clevers, H. (2012). Peyer's Patch M Cells Derived from Lgr5 + Stem Cells Require SpiB and Are Induced by RankL in Cultured "Miniguts" . *Molecular and Cellular Biology*, *32*(18), 3639–3647. https://doi.org/10.1128/mcb.00434-12

Dieterich, W., Neurath, M. F., & Zopf, Y. (2020). Intestinal ex vivo organoid culture reveals altered programmed crypt stem cells in patients with celiac disease. *Scientific Reports*, *10*(1), 1–10. https://doi.org/10.1038/s41598-020-60521-5

Dillon, A., & Lo, D. D. (2019). M cells: Intelligent engineering of mucosal immune surveillance. *Frontiers in Immunology*, *10*(JUL), 1–13. https://doi.org/10.3389/fimmu.2019.01499

Dos Santos, S., & Lioté, F. (2017). Osteoarticular manifestations of celiac disease and non-celiac gluten hypersensitivity. *Joint Bone Spine*, *84*(3), 263–266. https://doi.org/10.1016/j.jbspin.2016.09.007

Dutta, D., & Clevers, H. (2017). Organoid culture systems to study host–pathogen interactions. *Current Opinion in Immunology*, *48*, 15–22. https://doi.org/10.1016/j.coi.2017.07.012

Fasano, A. (2011). Zonulin and its regulation of intestinal barrier function: The biological door to inflammation, autoimmunity, and cancer. *Physiological Reviews*, *91*(1), 151–175. https://doi.org/10.1152/physrev.00003.2008

Haber, A. L., Biton, M., Rogel, N., Herbst, R. H., Shekhar, K., Smillie, C., Burgin, G., Delorey, T. M., Howitt, M. R., Katz, Y., Tirosh, I., Beyaz, S., Dionne, D., Zhang, M., Raychowdhury, R., Garrett, W. S., Rozenblatt-Rosen, O., Shi, H. N., Yilmaz, O., … Regev, A. (2017). A single-cell survey of the small intestinal epithelium. *Nature*, *551*(7680), 333–339. https://doi.org/10.1038/nature24489

Hase, K., Kawano, K., Nochi, T., Pontes, G. S., Fukuda, S., Ebisawa, M., Kadokura, K., Tobe, T., Fujimura, Y., Kawano, S., Yabashi, A., Waguri, S., Nakato, G., Kimura, S., Murakami, T., Iimura, M., Hamura, K., Fukuoka, S. I., Lowe, A. W.,

… Ohno, H. (2009). Uptake through glycoprotein 2 of FimH + bacteria by M cells initiates mucosal immune response. *Nature*, *462*(7270), 226–230. https://doi.org/10.1038/nature08529

Kahaly, G. J., Frommer, L., & Schuppan, D. (2018). Celiac disease and endocrine autoimmunity – the genetic link. *Autoimmunity Reviews*, *17*(12), 1169–1175. https://doi.org/10.1016/j.autrev.2018.05.013

Kamycheva, E., Goto, T., & Camargo, C. A. (2017). Celiac disease is associated with reduced bone mineral density and increased FRAX scores in the US National Health and Nutrition Examination Survey. *Osteoporosis International*, *28*(3), 781–790. https://doi.org/10.1007/s00198-016-3791-4

Kasendra, M., Tovaglieri, A., Sontheimer-Phelps, A., Jalili-Firoozinezhad, S., Bein, A., Chalkiadaki, A., Scholl, W., Zhang, C., Rickner, H., Richmond, C. A., Li, H., Breault, D. T., & Ingber, D. E. (2018). Development of a primary human Small Intestine-on-a-Chip using biopsy-derived organoids. *Scientific Reports*, *8*(1), 1–14. https://doi.org/10.1038/s41598-018-21201-7

Kim, G.-A., Spence, J. R., & Takayama, S. (2017). Bioengineering for intestinal organoid cultures. *Physiology & Behavior*, *47*, 51–58. https://doi.org/10.1016/j.copbio.2017.05.006.

Knoop, K. A., Kumar, N., Butler, B. R., Sakthivel, S. K., Taylor, R. T., Nochi, T., Akiba, H., Yagita, H., Kiyono, H., & Williams, I. R. (2009). RANKL Is Necessary and Sufficient to Initiate Development of Antigen-Sampling M Cells in the Intestinal Epithelium. *The Journal of Immunology*, *183*(9), 5738–5747. https://doi.org/10.4049/jimmunol.0901563

Krupa-Kozak, U., & Drabińska, N. (2016). Calcium in Gluten-Free Life: Health-Related and Nutritional Implications. *Foods*, *5*(3), 51. https://doi.org/10.3390/foods5030051

Larussa, T., Suraci, E., Imeneo, M., Marasco, R., & Luzza, F. (2017). Normal bone mineral density associates with duodenal mucosa healing in adult patients

with celiac disease on a gluten-free diet. *Nutrients*, *9*(2). https://doi.org/10.3390/nu9020098

Lebwohl, B., Sanders, D. S., & Green, P. H. R. (2017). Coeliac disease. *The Lancet*, *6736*(17), 1–12. https://doi.org/10.1016/S0140-6736(17)31796-8

Lebwohl, B., Sanders, D. S., & Green, P. H. R. (2018). Coeliac disease. *The Lancet*, *391*, 70–81. https://doi.org/10.1016/S0140-6736(17)31796-8

Lerner, A., & Matthias, T. (2016). Gut-bone Cross Talks and Implications in Celiac Disease. *International Journal of Celiac Disease*, *4*(1), 19–23. https://doi.org/10.12691/ijcd-4-1-4

Li, V. S. W., Schneeberger, K., Hofhuis, F., Clevers, H., Martens, A., Peters, P. J., Nieuwenhuis, E., Kujala, P., DeKoter, R. P., de Lau, W., Middendorp, S., & Barker, N. (2012). Peyer's Patch M Cells Derived from Lgr5+ Stem Cells Require SpiB and Are Induced by RankL in Cultured "Miniguts." *Molecular and Cellular Biology*, *32*(18), 3639–3647. https://doi.org/10.1128/mcb.00434-12

Lindfors, K., Ciacci, C., Kurppa, K., Lundin, K. E. A., Makharia, G. K., Mearin, M. L., Murray, J. A., Verdu, E. F., & Kaukinen, K. (2019). Coeliac disease. *Nature Reviews Disease Primers*, *5*(1), 3. https://doi.org/10.1038/s41572-018-0054-z

Miller, H., Zhang, J., KuoLee, R., Patel, G. B., & Chen, W. (2007). Intestinal M cells: The fallible sentinels? *World Journal of Gastroenterology*, *13*(10), 1477–1486. https://doi.org/10.3748/wjg.v13.i10.1477

Ono, T., Hayashi, M., Sasaki, F., & Nakashima, T. (2020). RANKL biology: Bone metabolism, the immune system, and beyond. *Inflammation and Regeneration*, *40*(1), 1–16. https://doi.org/10.1186/s41232-019-0111-3

Porpora, M., Conte, M., Lania, G., Bellomo, C., Rapacciuolo, L., Chirdo, F. G., Auricchio, R., Troncone, R., Auricchio, S., Barone, M. V., & Nanayakkara, M. (2022). Inflammation Is Present, Persistent and More Sensitive to Proinflammatory Triggers in Celiac Disease Enterocytes. *International Journal of Molecular Sciences*, *23*(4). https://doi.org/10.3390/ijms23041973

Ranganathan, S., Doucet, M., Grassel, C. L., Delaine-Elias, B. O., Zachos, N. C., & Barry, E. M. (2019). Evaluating shigella flexneri pathogenesis in the human enteroid model. *Infection and Immunity*, *87*(4), 1–14. https://doi.org/10.1128/IAI.00740-18

Rauhavirta, T., Lindfors, K., Koskinen, O., Laurila, K., Kurppa, K., Saavalainen, P., Mäki, M., Collin, P., & Kaukinen, K. (2014). Impaired epithelial integrity in the duodenal mucosa in early stages of celiac disease. *Translational Research*, *164*(3), 223–231. https://doi.org/10.1016/j.trsl.2014.02.006

Rouch, J. D., Scott, A., Lei, N. Y., Solorzano-Vargas, R. S., Wang, J., Hanson, E. M., Kobayashi, M., Lewis, M., Stelzner, M. G., Dunn, J. C. Y., Eckmann, L., & Martín, M. G. (2016). Development of functional microfold (M) cells from intestinal stem cells in primary human enteroids. *PLoS ONE*, *11*(1). https://doi.org/10.1371/journal.pone.0148216

Sakhony, O. S., Rossy, B., Gusti, V., Pham, A. J., Vu, K., & Lo, D. D. (2015). M cell-derived vesicles suggest a unique pathway for trans-epithelial antigen delivery. *Tissue Barriers*, *3*(1), 1–2. https://doi.org/10.1080/21688370.2015.1004975

Sato, T., Vries, R. G., Snippert, H. J., Van De Wetering, M., Barker, N., Stange, D. E., Van Es, J. H., Abo, A., Kujala, P., Peters, P. J., & Clevers, H. (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*, *459*(7244), 262–265. https://doi.org/10.1038/nature07935

Schumann, M., Siegmund, B., Schulzke, J. D., & Fromm, M. (2017). Celiac Disease: Role of the Epithelial Barrier. *Cell Mol Gastroenterol Hepatol*, *3*(2), 150–162. https://doi.org/10.1016/j.j cmgh.2016.12.006

Sun, H., Chow, E. C. Y., Liu, S., Du, Y., & Pang, K. S. (2008). The Caco-2 cell monolayer: Usefulness and limitations. *Expert Opinion on Drug Metabolism and Toxicology*, *4*(4), 395–411. https://doi.org/10.1517/17425255.4.4.395

Szymczak, J., Bohdanowicz-Pawlak, A., Waszczuk, E., & Jakubowska, J. (2012). Low bone mineral density in adult patients with coeliac disease. *Endokrynologia*

Polska, *63*(4), 270–276.

Valitutti, F., & Fasano, A. (2019). Breaking Down Barriers: How Understanding Celiac Disease Pathogenesis Informed the Development of Novel Treatments. *Digestive Diseases and Sciences*, *64*(7), 1748–1758. https://doi.org/10.1007/s10620-019-05646-y

Walker, M. M., Ludvigsson, J. F., & Sanders, D. S. (2017). Coeliac disease: Review of diagnosis and management. *Medical Journal of Australia*, *207*(4), 173–178. https://doi.org/10.5694m/ja16.00788

Williams, I. R., & Owen, R. L. (2015). M Cells: Specialized Antigen Sampling Cells in the Follicle-Associated Epithelium. *Mucosal Immunology: Fourth Edition*, *1–2*(Figure 1), 211–229. https://doi.org/10.1016/B978-0-12-415847-4.00013-6

Yao, D., Huang, L., Ke, J., Zhang, M., Xiao, Q., & Zhu, X. (2020). Bone metabolism regulation: Implications for the treatment of bone diseases. *Biomedicine and Pharmacotherapy*, *129*(May), 110494. https://doi.org/10.1016/j.biopha.2020.110494

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Katie Schraders
Name and title of main supervisor:	Professor Marlena C. Kruger
In which chapter is the manuscript/published work?	Chapter 4
What percentage of the manuscript/published work was contributed by the student?	85%

Describe the contribution that the student has made to the manuscript/published work:
Led the study design, statistical analyses and wrote first manuscript draft and ethics protocol

Please select one of the following three options:

- ○ **The manuscript/published work is published or in press**
  Please provide the full reference of the research output:
- ○ **The manuscript is currently under review for publication**
  Please provide the name of the journal:
- ◉ **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:	Katie Schraders Digitally signed by Katie Schraders Date: 2022.08.28 18:36:48 +12'00'	Main supervisor's signature:	Marlena Kruger Digitally signed by Marlena Kruger DN: cn=Marlena Kruger, c=NZ, o=Massey University, ou=School of Health Sciences, email=m.c.kruger@massey.ac.nz Date: 2022.08.27 09:08:25 +12'00'

This form should be placed at the beginning of each relevant thesis chapter.

The organoid work in chapter 3 aimed to investigate our underlying hypothesis behind the observation of low bone mineral density (BMD) in individuals with coeliac disease (CD) demonstrated in international findings. Chapter 4 presents the planned protocol of the *Close to the Bone* study which aimed to investigate BMD in individuals with CD in New Zealand, comparing them to healthy controls and confirm if observations of low BMD in this group, seen internationally, are also seen in New Zealand. This study was also designed to include a third participant group of non-coeliac gluten sensitive women (NCGS) who strictly adhere to a gluten-free diet (GFD), for whom a diagnosis of CD has been excluded. This novel approach ensures that the GFD itself is not responsible for any observed differences in BMD.

This chapter was submitted to BMC Public Health and although initially accepted was declined for publication due to not meeting the scope of the journal as a result of being a small study population and not having sufficient fundings.

CHAPTER 4

Close to the Bone: Study Protocol

Study Protocol for a cross-sectional study on the Gut-Bone Axis in Coeliac Disease and Non-Coeliac Gluten Sensitivity: The Close to the Bone Study

4.1 Abstract

Introduction: Reduced bone mineral density (BMD) has been identified in individuals diagnosed with Coeliac Disease (CD) across a range of ages. The reasons are not clear and even when a gluten-free diet (GFD) is strictly adhered to and abdominal symptoms have resolved, bone health remains compromised. Possible explanations include nutritional inadequacy of a GFD, protracted inflammation and compromised nutrient absorption prior to diagnosis or irreversible effects in the gut-bone signalling axis.

Methods and analysis: This cross-sectional study will be the first to assess BMD in premenopausal women with CD and Non-Coeliac Gluten Sensitivity (NCGS) compared with healthy controls in New Zealand. One hundred and forty-one women, aged 18-40, will be recruited from the lower North Island, New Zealand, as 3 groups of 47 age-matched participants; those with CD, NCGS (both adhering to a GFD) and healthy controls (consuming gluten). Participants will have BMD, bone turnover, inflammatory markers and nutrient status assessed to assess the risk of osteoporosis and possible mechanisms linking it with CD; data about lifestyle factors associated with BMD will also be collected.

Ethics and dissemination: Potential participants will complete a screening questionnaire to determine eligibility and, if eligible, will provide written consent before participating. Ethics approval has been granted by Massey University Human Ethics Committee (reference: Southern A, Application SOA 18/73). The

results of this study will be disseminated through conferences and peer-reviewed journals.

Trial registration: Australian New Zealand Clinical Trials Registry; registration number ACTRN1261900 1542189. Date registered 08.11.2019.

4.1.1 Strengths and limitations of this study

- This is the first observational study assessing the relationship between bone density and coeliac disease (CD) in premenopausal women in New Zealand; prior to menopause it is possible for women to make lifestyle changes that could potentially delay the rate of age-related bone loss.
- The inclusion of the non-coeliac gluten sensitive (NCGS) group allows evaluation of the effects of a gluten-free diet (GFD) on bone density in the absence of CD-related inflammatory response
- Inclusion of DXA assessment of forearm bone density allows a comparison of an alternative less-invasive method of assessing BMD
- The specificity of the inclusion criteria and exclusion of other conditions which may affect bone density may hinder enrolment as many individuals with CD also have other autoimmune conditions.

4.2 Introduction

Coeliac disease (CD) is a lifelong immune-mediated disease that is triggered by exposure to gluten in genetically predisposed individuals, predominantly affecting females (2-3:1) (Singh et al., 2018). CD is associated with a specific genotype (HLA-DQ2 and/or HLA-DQ8 genes), the presence of autoantibodies (anti-tissue transglutaminase and anti-endomysial antibodies) together with definitive signs of intestinal damage and inflammation observed in histological samples from endoscopic biopsies of the duodenum (Krupa-Kozak, 2014). The prevalence of CD in New Zealand is one of the highest in the world at 1.2% of the

total population (Burkhardt et al., 2018) and is increasing, for reasons which are not understood.

CD characteristically affects the gut, causing small intestinal inflammation and villous atrophy which compromises absorption of nutrients including calcium and vitamin D (Lerner & Matthias, 2016), affected individuals frequently have low bone mineral density (BMD) (Laszkowska et al., 2018) and thus a high risk of developing osteoporosis. Inflammation of the gut understandably affects nutrient absorption, but despite some initial improvement of bone health in the first 12 months following adoption of a gluten-free diet (GFD), bone health continues to be compromised even in those individuals who totally exclude gluten from their diet (Bathrellou et al., 2018; Pantaleoni et al., 2014). The prevalence of low bone density in individuals with CD in New Zealand has previously been assessed in a single study (Bolland et al., 2016) which investigated patients aged 47±15 referred for a DXA scan after diagnosis of CD. However, as it is not routine for patients with CD to be referred for a DXA scan in New Zealand, these results may underrepresent the true prevalence of low BMD in this group and explain why the findings of this study are different to many studies carried out elsewhere in both postmenopausal (Kotze et al., 2016) and premenopausal women (Pritchard et al., 2017). Further research is therefore warranted to investigate this relationship in the New Zealand population.

This study aims to investigate BMD in premenopausal women (aged 18-40) with CD, who have been consuming a GFD for a minimum of 12 months, compared to an age matched healthy control group and a group of women with NCGS, who have been adhering to a GFD diet for a minimum of 12 months. NCGS is a recently recognised clinical condition in which CD and wheat allergy have been excluded, but the affected individual experiences intestinal and extra-intestinal symptoms in response to gluten (Biesiekierski & Iven, 2015; Leonard et al., 2017). A condition such as NCGS offers the opportunity to investigate the effect of a rigorous GFD on

nutrient intake and bone health in the absence of the gut-related inflammatory pathology distinctive of CD.

In recent years, in many countries, there has been a dramatic increase in the number of people excluding gluten from their diet. However, there are concerns about the nutritional adequacy of the GF diet of individuals with diagnosed CD, particularly the intake of fibre and micronutrients, such as vitamin D, vitamin B12, folate, iron and calcium (Vici et al., 2016), which are implicated in bone health. In addition to concerns about nutrients which may be naturally lacking in GF foods, there are additional concerns regarding some GF products being exempt from fortification.

Although there is no official estimate about the number of people consuming a GFD in New Zealand, this is now one of the most popular dietary trends in modern history (Newberry et al., 2017). A 2015 Nielsen survey of the dietary habits of 30,000 adults living in 60 countries worldwide identified that 23% avoided foods containing gluten (Nielsen, 2015). With this diet becoming increasingly popular it has become more imperative than ever to assess the nutritional implications of typical GF diets in order to provide appropriate dietary guidelines.

4.2.1 Hypotheses

- Bone health, as indicated by markers of bone turnover and assessment of bone density, will be compromised in individuals with a positive diagnosis of CD compared to individuals with NCGS, and BMD of both groups will be less than healthy controls.
- Bone health will be associated with markers of inflammation.
- Intake of micronutrients associated with optimal bone health will be inadequate (in individuals with CD and those with NCGS, and less than in healthy controls)

4.2.2 Aims

- To assess bone health and bone metabolism in individuals with CD and with NCGS, compared to healthy controls.
- To determine dietary intake and nutritional status related to requirements for optimal bone health in individuals with CD and with NCGS.

4.3 Methods and analysis

4.3.1 Study design

The study began in December 2019 and will continue until 141 participants are enrolled (47 in each group).

Screening for this study consists of an online questionnaire that can be completed from the participant's home. Once selected for the study, eligible participants are asked to complete a four-day diet record and attend a single visit to the Human Nutrition Research Unit at Massey University in Palmerston North.

The visit to the research unit begins with fasted venepuncture, followed by the participants being provided with breakfast. Participant's diet records are submitted and reviewed prior to their visit so queries can be addressed on the day of the visit. During the visit, participants also complete online questionnaires, have (non-fasted) capillary blood and urine samples collected, undergo a quantitative ultrasound of their heel, have height and weight recorded and DXA scans.

4.3.2 Sample size calculations

G* Power 3 software (Faul et al., 2007) was used to calculate a sample size of 39 women for each group of the study. Power calculations were used to determine a difference in bone density between people with CD and healthy controls with an effect size 0.41 at power 0.95 and a probability of type 1 error 0.05 (Szymczak et al., 2012). Absence of prior research provided limitations in establishing a sample size required for the comparison of women with NCGS with the other groups. Previous

research investigating risk of osteoporosis in this group has investigated both genders and did not compare findings with age- and sex-matched healthy controls or consider the potential influence of ethnicity. Based on the previous research, we hypothesise that BMD will be higher in the NCGS group than CD (Carroccio et al., 2014), but lower than healthy controls. Although sample size is calculated at 117 (39 in each group), factoring in incomplete data sets/misdiagnosis and the possibility of undetected CD in the healthy control group, the target enrolment is 141 (47 in each group). This will allow us to detect whether any difference in BMD between these groups is significant.

4.3.3 Inclusion/exclusion criteria

Participants for this study will consist of women aged 18-40 years, from three distinct groups: individuals positively diagnosed (by biopsy) with CD who have been consuming a strict GFD for at least 12 months; individuals with NCGS who have had a negative diagnosis for CD (by serology and biopsy and meet the Salerno Experts' Criteria for diagnosis (Catassi et al., 2015) and consuming a strict GFD for at least 12 months; and healthy controls who eat a gluten-containing diet. Women are selected for this study as both CD and NCGS predominantly affect women. A strict GFD means all gluten is intentionally excluded from the diet so gluten intake is below trace levels.

Women are excluded if they are currently or had previously used medication which affects bone metabolism (e.g. corticosteroids, bisphosphonates, selective oestrogen receptor modulators); have any medical conditions known to affect bone health; are currently pregnant or lactating; or have any known history of inflammatory conditions other than CD and NCGS. In addition, women with a body mass index (BMI) >30 (derived from screening questionnaire) are also excluded to counteract the possible impact of body weight on bone density.

4.3.4 Data collection

Biomarker analysis

Fasted venepuncture blood samples (3 samples, total of 26mL) are collected between 8:30 and 9:30am by a qualified and experienced phlebotomist, using sterile vacutainer flashback needles; this timing allows for minimised variability in bone marker results as C-terminal telopeptide of type I collagen (CTX) has a circadian rhythm (Szulc et al., 2017). Participants are requested to fast overnight, consuming nothing but water for a minimum of 8 hours before their visit.

Serum is collected to analyse vitamin D and thyroglobulin (Tg) and markers of CD, tissue transglutaminase (tTG) and endomysial antibodies (EMA). Blood samples are allowed to coagulate and are centrifuged at 3000g for 10 minutes at 4°C within 2 hours. tTG and EMA will allow adherence to the GFD to be measured (as this is otherwise self-reported), in addition to identifying any participants in the healthy control group who may have undiagnosed CD.

Plasma is collected in vacutainers containing EDTA anticoagulant to measure CTX and parathyroid hormone (PTH) and placed on ice to await processing. Additional plasma is collected in vacutainers containing lithium-heparin for the analysis of calcium, high sensitivity C-reactive protein (hsCRP), folate, B12, thyroid stimulating hormone (TSH), thyroxine (T_4), triiodothyronine (free T_3) and anti-thyroid peroxidase antibodies (anti-TPO). Vacutainers for plasma samples are centrifuged at 3000g for 10 minutes at 4°C within 2 hours.

Both serum and plasma samples are frozen in aliquots within 2 hours and stored at -80°C to await processing at the end of the study. Once all samples are collected these will be shipped on dry ice to Canterbury Health Laboratories, Christchurch, New Zealand for analysis.

Trained staff also collect a capillary blood sample from the finger using a lancet and use a point-of-care Hemocue Hb 201+ system to measure whole blood

haemoglobin concentration as a proxy for iron status. Participants are requested to wash their hands with warm water to ensure adequate blood flow in capillaries before the sample is collected.

Iodine and creatinine excretion are also measured through urine spot samples collected from participants during their visit. These are frozen at -20°C for later analysis.

Anthropometry

Participants height is measured to the nearest 0.1cm using a wall-mounted rolled stadiometer (Seca Medical Measuring Systems, Chino, CA, USA) and weight is measured using standard electronic floor scales (Life Measurements Inc., Concord, CA, USA). In addition, body composition is assessed using DXA Horizon A (Hologic Inc., Bedford, MA, USA).

Bone densitometry

Dual X-Ray Absorptiometry (DXA)

DXA is the gold standard for the diagnosis of low BMD, as recognised by the World Health Organization (Høiberg et al., 2016). Although ionising radiation doses during DXA are relatively low, all research participants of childbearing age are asked if they could be pregnant. This question is included in the Participant Screening Questionnaire and asked again immediately prior to the DXA scan. The Participant Information Sheet (appendix B) also informs potential research participants that ionising radiation presents a risk to the unborn child. DXA is performed by a qualified operator who is trained to perform DXA scans, and scan results are reviewed and approved by a consultant radiologist to ensure accurate results and interpretation.

BMD will be measured using DXA for whole body, lumbar spine (L1-L4), left proximal femur (femoral neck, wards triangle, and trochanter), left forearm (distal 1/3), as well as total body composition using a Hologic Horizon series, fan beam X-ray Bone Densitometer, model A. (Hologic Inc., Bedford, MA, USA). Bone mineral content (BMC, grams), bone mineral density (BMD, grams/centimetre^2) and z-scores will be determined for each participant. Calibration of the DXA is completed according to manufacturer's instructions, daily, with a spine phantom, with precision determined using the coefficient of variation of 0.45-0.54% for all measures.

Quantitative Ultrasound (QUS)

QUS uses sound waves providing a non-invasive, immediate and easily understood result for the participants. QUS has been shown to correlate well with DXA (Høiberg et al., 2016), however, recent studies in a similar population of healthy young women suggest that women identified as having low BMD by DXA were not always identified as at risk using QUS (Schraders et al., 2019); as such participants are advised that these results are only an indication of the quality of their bone at one site (the heel).

An Achilles QUS ultrasonometer (Lunar Achilles Insight, GE Lunar Corporation Inc., Madison, WI, USA) is used to establish bone quality in the calcaneus (heel) of the non-dominant leg (to minimise variability). Calibration is completed daily according to the manufacturer's instructions.

Questionnaires

The initial screening questionnaire is provided to participants prior to their visit to the research unit, online via Qualtrics software (Qualtrics, Provo, Utah). Once completed the research team is notified via an automated email that the questionnaire has been completed. If any further clarification is required, the

research team follow-up participants by email to confirm eligibility. Then the research team contact the participant to arrange their visit to the research unit.

Two questionnaires are provided at the visit to the research unit, one to assess the participant's perceived risk of developing osteoporosis and their understanding of the condition. A further questionnaire consists of questions about the participant's own health, date of diagnostic testing and symptoms (for participants with CD and NCGS), history of fracture and gut conditions, sunlight exposure (which affects vitamin D synthesis), physical activity and other lifestyle behaviours which influence bone health. In addition, the health questionnaire contains a short food frequency questionnaire and questions about alcohol and soft drink consumption and use of nutritional supplements. Questionnaires are provided in digital format through Qualtrics software; however, hard copies are available to participants who prefer this format. Although the participant completes the questionnaires in privacy, a researcher is available to answer any questions the participant might have.

Dietary intake

A 4-day diet record template is provided to the participants by email prior to their visit to the research unit. This includes instructions and an example to illustrate the level of detail required. To maintain consistency between participants, all participants are provided with the same template and instructions which advise participants to include 2-3 weekdays and 1 weekend day. Participants are requested to provide as much detail as possible including brands, preparation, cooking methods, recipes used, and amount consumed.

Due to the burden of completing a diet record (Shim et al., 2014), participants are asked to complete this for ideally 4 days, with a minimum of 3 days. Participants are asked to estimate intakes using household measures but are advised that weighing intakes would provide a more accurate picture of their

intake. Participants are told they will be provided with the results of the nutrient analysis/daily average intake from their dietary record to incentivise accuracy.

In addition to the template for dietary intake, an additional page of the diet record asks participants about their current supplement intake. Diet records are reviewed at the participant's visit to the research unit so that any clarifications can be made. Diet records are evaluated for nutrient intake using Foodworks 10 Professional (Xyris Software Pty, Brisbane, Australia), by nutritionists experienced in dietary assessment. Due to limitations in the database for GF products, new foods and recipes will be added/adapted using additional food composition databases.

4.3.5 Data analysis plan

Data will be analysed using Statistical Analysis Software (SAS) (Version 9.4) (SAS Institute Inc., Cary, NC, USA). Normality of data distribution will be assessed using the Shapiro-Wilk test and normality plots. Participant characteristics of the three groups including BMD and bone markers will be described and compared using the mean (95% confidence intervals) for normally distributed data and median (and interquartile range) for non-normally distributed data. Multivariate multiple regression analysis will be used to determine associations between variables, including nutrient intake, and parameters of bone health; possible confounding factors will be considered.

4.3.6 Ethics and dissemination

Patient and public involvement

A gut health advisory/stakeholder group of individuals with CD and NCGS was consulted during the design process of this study. In addition, members of the gastroenterology department at MidCentral Health and a research dietitian with clinical speciality in gastroenterology were consulted.

Dissemination of results

Results will be published in peer-reviewed journals and presented at relevant conferences such as the NZ Coeliac Society conference. All participants will be provided with their individual results with an explanation. Participants with results outside normal parameters for their age group will also receive a letter advising them to discuss their results with a doctor to determine if further investigation is necessary. A final summary report of the research study will be sent to all participants.

The research team has good links with several national and local organisations (including social media groups) providing support for individuals with CD. As well as aiding the recruitment of research participants, these links will be used for dissemination of the study results and their implications. The team will ensure that results are made available to the Ministry of Health and Food Safety Authority in order to guide decision making and policies around food fortification and dietary recommendations.

Data storage and security

Paper documents such as consent forms will be stored securely in a locked filing cabinet in a locked office and only accessed by members of the research team as required for research purposes. Personally identified information will be kept physically separate from de-identified research data, which will be identified by a unique study ID (participant number). Electronic data will be regarded as confidential, stored in secure files, be password protected and backed up in separate locations to university-managed secure computing facilities. Data will be ultimately destroyed according to the university research regulations.

Ethics approval and consent to participate

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application SOA 18/73. All participation is voluntary, and participants are able to opt out of any portion of the study at any time. Participants provide written consent before taking part in the study.

4.4 Discussion

Ethnicity

One potential limitation of this research is the possible role that ethnicity may play in bone density. Globally, the prevalence of CD is greatest in people of European descent. There is very limited research surrounding the demographic makeup of the population with CD in New Zealand, which is generally considered to be a very multicultural society. To the researchers' knowledge there has been no previous research to estimate the prevalence of CD in Māori and Pacific populations in New Zealand. However, research regarding the prevalence of the HLA serotypes commonly associated with CD suggests that this is not as common in Māori and Pacific populations (Edinur et al., 2013), and therefore the prevalence of CD in these groups may be lower than in people of European descent. Ethnicity in this study is self-identified and there is no assessment for the presence of the genetic predisposition, which is a potential limitation of the study.

Diagnostic criteria for CD

Diagnosis of CD requires a combination of tests including serology and biopsy sampling. Individuals are advised to continue eating gluten for at least 4-8 weeks prior to serology and biopsy tests (Coeliac New Zealand, n.d.) as removing gluten from the diet would lead to a negative result. Historically, it has been recommended to consume at least 10g/day for 6 weeks or more. However, recommended minimum intake varies and more recent studies have reported that

more than 3g of gluten per day for 2 weeks or more is sufficient to observe any morphological changes in the gut (Lebwohl et al., 2018). Regardless of the exact recommendation, the requirement to consume gluten prior to testing adds an additional burden, with symptoms ongoing over this period. In New Zealand, the timeframe from positive serology to the biopsy to confirm diagnosis can be long and drawn out. For many individuals who receive positive serology results, the requirement to continue consuming gluten for this period can be unrealistic and unachievable due to the symptoms they experience. Therefore, the requirement for CD to be confirmed via biopsy adds an additional limitation to the study as this may limit individuals with CD who are eligible to participate in this study.

Diagnostic criteria for NCGS

Another limitation of this study is the diagnostic criteria used for NCGS, due to it being a recently recognised condition. The pathogenesis of NCGS is not well understood, symptoms are nonspecific and vague, and there is a lack of consensus amongst medical professionals regarding diagnostic criteria (Ierardi et al., 2018). Some authorities question the existence of the condition or consider it to be psychosomatic (Branchi et al., 2015; Greuter et al., 2017). Despite this, NCGS offers an opportunity to investigate the effect of a rigorous GF diet on nutrient intake and bone health, in the absence of the gut-related inflammatory pathology distinctive of CD, which makes inclusion of this group invaluable for this research.

The collaborative group of researchers and the gastroenterology department at MidCentral Health have agreed that the Salerno Experts' Criteria (Catassi et al., 2015) are the most appropriate accepted method of diagnosis. This method should ensure that participants with undiagnosed CD are not incorrectly enrolled into this group. However, it should be noted that these criteria are not universally accepted by gastroenterologists, which may make it hard for individuals with apparent NCGS to meet the inclusion criteria.

4.5 List of abbreviations

CD: coeliac disease; NCGS: non-coeliac gluten sensitivity; GF: gluten-free; GFD: gluten-free diet; CTX: C-terminal telopeptide of type I collagen; tTG: tissue transglutaminase; EMA: endomysial antibodies; PTH: parathyroid hormone; hsCRP: high sensitivity C-reactive protein; QUS: Quantitative Ultrasound; DXA: Dual X-ray Absorptiometry; EDTA: ethylenediamine tetraacetic acid

4.6 Availability of data and materials

The datasets used and/or analysed during the current study will be made available from the corresponding author upon reasonable request.

4.7 Competing interests

The authors declare that they have no competing interests.

4.8 Funding

This study has received funding from Palmerston North Medical Research Fund and the School of Food & Advanced Technology, Massey University. The funding bodies played no role in the design of the study and collection, analysis, and interpretation of data or in writing the manuscript.

4.9 Authors' contributions

KS, MK, LB and JC conceived the study, acquired funding and ethics approval. KS will coordinate recruitment and participant management. KS and JC will collect data. All authors were involved in revising the manuscript and all read and approved the final manuscript. All authors will also be involved in future aspects of this study.

4.10 References

Bathrellou, E., Kontogianni, M. D., & Panagiotakos, D. B. (2018). Celiac disease and non-celiac gluten or wheat sensitivity and health in later life: A review.

Maturitas, *112*(January), 29–33. https://doi.org/10.1016/j.maturitas.2018.03.014

Biesiekierski, J. R., & Iven, J. (2015). Non-coeliac gluten sensitivity: Piecing the Puzzle together. *United European Gastroenterology Journal*, *3*(2), 160–165. https://doi.org/10.1177/2050640615578388

Bolland, M. J., Grey, A., & Rowbotham, D. S. (2016). Outcomes of bone density measurements in coeliac disease. *New Zealand Medical Journal*, *129*(1429), 40–44.

Branchi, F., Ferretti, F., Norsa, L., Roncoroni, L., Conte, D., Bardella, M. T., & Elli, L. (2015). Management of Nonceliac Gluten Sensitivity by Gastroenterology Specialists: Data from an Italian Survey. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/530136

Burkhardt, J. G., Chapa-Rodriguez, A., & Bahna, S. L. (2018). Gluten sensitivities and the allergist: Threshing the grain from the husks. *Allergy: European Journal of Allergy and Clinical Immunology*, *73*(7), 1359–1368. https://doi.org/10.1111/all.13354

Carroccio, A., Soresi, M., D'Alcamo, A., Sciumè, C., Iacono, G., Geraci, G., Brusca, I., Seidita, A., Adragna, F., Carta, M., & Mansueto, P. (2014). Risk of low bone mineral density and low body mass index in patients with non-celiac wheat-sensitivity: A prospective observation study. *BMC Medicine*, *12*(1), 1–8. https://doi.org/10.1186/s12916-014-0230-2

Catassi, C., Elli, L., Bonaz, B., Bouma, G., Carroccio, A., Castillejo, G., Cellier, C., Cristofori, F., de Magistris, L., Dolinsek, J., Dieterich, W., Francavilla, R., Hadjivassiliou, M., Holtmeier, W., Körner, U., Leffler, D. A., Lundin, K. E. A., Mazzarella, G., Mulder, C. J., … Fasano, A. (2015). Diagnosis of non-celiac gluten sensitivity (NCGS): The salerno experts' criteria. *Nutrients*, *7*(6), 4966–4977. https://doi.org/10.3390/nu7064966

Edinur, H. A., Dunn, P. P. J., Hammond, L., Selwyn, C., Brescia, P., Askar, M., Reville, P., Velickovic, Z. M., Lea, R. A., & Chambers, G. K. (2013). HLA and

MICA polymorphism in Polynesians and New Zealand Maori: Implications for ancestry and health. *Human Immunology*, *74*(9), 1119–1129. https://doi.org/10.1016/j.humimm.2013.06.011

Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods*, *39*(2), 175–191.

Greuter, T., Schmidlin, S., Lattmann, J., Stotz, M., Lehmann, R., Zeitz, J., Scharl, M., Misselwitz, B., Pohl, D., Fried, M., Tutuian, R., Fasano, A., Schoepfer, A. M., Rogler, G., Biedermann, L., & Vavricka, S. R. (2017). The perspective of celiac disease patients on emerging treatment options and non-celiac gluten sensitivity. *Digestive and Liver Disease*, *49*(3), 268–272. https://doi.org/10.1016/j.dld.2016.11.016

Høiberg, M. P., Rubin, K. H., Hermann, A. P., Brixen, K., & Abrahamsen, B. (2016). Diagnostic devices for osteoporosis in the general population: A systematic review. *Bone*, *92*, 58–69. https://doi.org/10.1016/j.bone.2016.08.011

Ierardi, E., Losurdo, G., Piscitelli, D., Giorgio, F., Amoruso, A., Iannone, A., Principi, M., & Leo, A. Di. (2018). Biological markers for non-celiac gluten sensitivity: a question awaiting for a convincing answer. *Gastroenterol Hepatol Bed Bench*, *11*(3), 203–208.

Kotze, L. M. S., Skare, T., Vinholi, A., Jurkonis, L., & Nisihara, R. (2016). Impact of a gluten-free diet on bone mineral density in celiac patients. *Revista Espanola de Enfermedades Digestivas*, *108*(2), 84–88. https://doi.org/10.17235/reed.2015.3953/2015

Krupa-Kozak, U. (2014). Pathologic bone alterations in celiac disease: Etiology, epidemiology, andtreatment. *Nutrition*, *30*(1), 16–24. https://doi.org/10.1016/j.nut.2013.05.027

Laszkowska, M., Mahadev, S., Sundström, J., Lebwohl, B., Green, P. H. R., Michaelsson, K., & Ludvigsson, J. F. (2018). Systematic review with meta-

analysis: the prevalence of coeliac disease in patients with osteoporosis. *Alimentary Pharmacology & Therapeutics*, *March*, 1–8. https://doi.org/10.1111/apt.14911

Lebwohl, B., Sanders, D. S., & Green, P. H. R. (2018). Coeliac disease. *The Lancet*, *391*, 70–81. https://doi.org/10.1016/S0140-6736(17)31796-8

Leonard, M. M., Sapone, A., Catassi, C., & Fasano, A. (2017). Celiac disease and nonceliac gluten sensitivity: A review. *JAMA - Journal of the American Medical Association*, *318*(7), 647–656. https://doi.org/10.1001/jama.2017.9730

Lerner, A., & Matthias, T. (2016). Gut-bone Cross Talks and Implications in Celiac Disease. *International Journal of Celiac Disease*, *4*(1), 19–23. https://doi.org/10.12691/ijcd-4-1-4

Newberry, C., McKnight, L., Sarav, M., & Pickett-Blakely, O. (2017). Going Gluten Free: the History and Nutritional Implications of Today's Most Popular Diet. *Current Gastroenterology Reports*, *19*(11). https://doi.org/10.1007/s11894-017-0597-2

Nielsen (2015) *We are what we eat: healthy eating trends around the world*. Nielsen Global Health and Wellness Report. The Nielsen Company. Available from: https://www.nielsen.com/wpcontent/uploads/sites/3/2019/04/Nielsen20Global20Health20and20Wellness20Report20-20January202015-1.pdf

Pantaleoni, S., Luchino, M., Adriani, A., Pellicano, R., Stradella, D., Ribaldone, D. G., Sapone, N., Isaia, G. C., Di Stefano, M., & Astegiano, M. (2014). Bone Mineral Density at Diagnosis of Celiac Disease and after 1 Year of Gluten-Free Diet. *The Scientific World Journal*, *2014*, 1–6. https://doi.org/10.1155/2014/173082

Pritchard, L., Wilson, S., Griffin, J., Pearce, G., & Lewis, S. J. (2017). Prevalence of low bone mineral density in adults with coeliac disease – Are we missing opportunities for detection in patients below 50 years of age? *Clinical Nutrition ESPEN*, *22*(12), 143–144. https://doi.org/10.1016/j.clnesp.2017.07.069

Schraders, K., Zatta, G., Kruger, M., Coad, J., Weber, J., Brough, L., & Thomson, J. (2019). Quantitative ultrasound and dual x-ray absorptiometry as indicators of bone mineral density in young women and nutritional factors affecting it. *Nutrients, 11*(10), 2336. https://doi.org/10.3390/nu12010030

Shim, J.-S., Oh, K., & Kim, H. C. (2014). Dietary assessment methods in epidemiologic studies. *Epidemiology and Health*, *36*. https://doi.org/10.4178/epih/e2014009

Singh, P., Arora, A., Strand, T. A., Leffler, D. A., Catassi, C., Green, P. H., Kelly, C. P., Ahuja, V., & Makharia, G. K. (2018). Global Prevalence of Celiac Disease: Systematic Review and Meta-analysis. *Clinical Gastroenterology and Hepatology*, *16*(6), 823-836.e2. https://doi.org/10.1016/j.cgh.2017.06.037

Szulc, P., Naylor, K., Hoyle, N. R., Eastell, R., & Leary, E. T. (2017). Use of CTX-I and PINP as bone turnover markers: National Bone Health Alliance recommendations to standardize sample handling and patient preparation to reduce pre-analytical variability. *Osteoporosis International*, *28*(9), 2541–2556. https://doi.org/10.1007/s00198-017-4082-4

Szymczak, J., Bohdanowicz-Pawlak, A., Waszczuk, E., & Jakubowska, J. (2012). Low bone mineral density in adult patients with coeliac disease. *Endokrynologia Polska*, *63*(4), 270–276.

Vici, G., Belli, L., Biondi, M., & Polzonetti, V. (2016). Gluten free diet and nutrient deficiencies: A review. *Clinical Nutrition*, *35*(6), 1236–1241. https://doi.org/10.1016/j.clnu.2016.05.002

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Katie Schraders
Name and title of main supervisor:	Professor Marlena C. Kruger
In which chapter is the manuscript/published work?	Chapter 5
What percentage of the manuscript/published work was contributed by the student?	85%

Describe the contribution that the student has made to the manuscript/published work:

Led the study design, recruited participants and collected data, completed DXA scans and analyses, processed blood samples, statistical analyses and wrote first manuscript draft and ethics protocol

Please select one of the following three options:

- ○ **The manuscript/published work is published or in press**
  Please provide the full reference of the research output:
- ○ **The manuscript is currently under review for publication**
  Please provide the name of the journal:
- ◉ **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:	Katie Schraders Digitally signed by Katie Schraders Date: 2022.08.28 18:37:37 +12'00'	Main supervisor's signature:	Marlena Kruger Digitally signed by Marlena Kruger DN: cn=Marlena Kruger, c=NZ, o=Massey University, ou=School of Health Sciences, email=m.c.kruger@massey.ac.nz Date: 2022.08.27 09:08:46 +12'00'

This form should be placed at the beginning of each relevant thesis chapter.

The findings of Chapter 5 follow on from the protocol presented in Chapter 4. The *Close to the Bone* study was adapted due to interference from the SARS-CoV-2 pandemic, with the non-coeliac gluten sensitive (NCGS) group removed from the population for assessment. The intended methods are described in the protocol in chapter 4, however, as many were altered from those initially presented in the protocol, these have been described in greater detail in chapter 5.

CHAPTER 5

Close to the Bone: Bone Health

5.1 Abstract:

Introduction: Low bone mineral density (BMD) is a common finding in adults with coeliac disease (CD), with BMD remaining low even in individuals strictly adhering to a gluten-free diet (GFD). Women lose BMD at a greater rate than men and are more likely to develop osteoporosis later in life, making women with CD at an even greater risk of developing osteoporosis due to pre-existing low BMD.

Materials and methods: BMD assessed through dual X-ray absorptiometry (DXA) and bone quality assessed through quantitative ultrasound (QUS) were investigated in 31 CD participants consuming a GFD, and 39 healthy controls from the Lower North Island, New Zealand. In addition, biomarkers of bone metabolism and nutrient status were assessed, and 4-day diet diaries (4DDD) were used to estimate nutrient intake.

Results: No statistically significant differences were found in BMD between the two groups at the hip, lumbar spine or forearm. However, parameters measured by the QUS were significantly lower in CD participants. Findings from the 4DDD indicated significantly lower intakes of energy, dietary fibre, magnesium and phosphorus, likely as a result of reduced intake of wholegrain foods. In addition, dietary data also suggest that both groups had inadequate intake of calcium. No significant differences were demonstrated in biochemical parameters.

Conclusions: BMD and biochemical parameters indicate no differences between coeliac and healthy women in New Zealand. However, findings from the QUS bring into question its suitability to assess bone and fracture risk in the coeliac population. Dietary data also suggest intake of calcium, although similar between CD and controls, is inadequate.

5.2 Introduction:

Coeliac disease (CD) in an immune-mediated disease that is triggered in response to the consumption of gluten in the diet (Singh et al., 2018), in individuals who are genetically predisposed and express the HLA-DQ2 and/or HLA-DQ8 genes (Krupa-Kozak, 2014). Diagnosis of the disease is confirmed by the presence of autoantibodies (anti-tissue transglutaminase, tTG and anti-endomysial antibodies, EMA), and evidence of intestinal damage assessed through endoscopic biopsy (Krupa-Kozak, 2014). In addition to intestinal symptoms, many individuals experience extra-intestinal symptoms resulting from both inflammation and malabsorption of essential nutrients (Leffler et al., 2015). Malabsorption of nutrients as a result of inflammation and villous atrophy is common, compromising the absorption of calcium, protein and other essential nutrients (Lerner & Matthias, 2016). As a result, bone mass is often affected (Laszkowska et al., 2018). Removal of gluten from the diet results in resolution of gut damage in most individuals with CD, with gut inflammation and nutrient absorption dramatically improved (Pantaleoni et al., 2014). However, even with rigorous compliance to a gluten-free diet (GFD) after diagnosis, full recovery of bone density is not always achieved in a significant proportion of individuals (Bathrellou et al., 2018; Pantaleoni et al., 2014). Low bone density is therefore a frequent finding in people with CD at the time of diagnosis, which may persist (Galli et al., 2018).

Despite New Zealand having one of the highest known prevalence of CD in the world at 1.2% (Burkhardt et al., 2018), with many still believed to be undiagnosed (Parzanese et al., 2017), very little research has been conducted investigating bone health in this population group. Previous research studies have assessed the prevalence of CD in individuals who have undergone a DXA scan for other reasons (Bolland et al., 2016). This approach only assesses patients who have been referred for DXA and does not accurately report on the prevalence of low bone density within the coeliac population. Osteoporosis more commonly affects

women (International Osteoporosis Foundation, 2017), with menopause and the associated decline in oestrogen resulting in a sharp increase in the rate of loss in bone density (Davis et al., 2015). As both the onset of menopause and the rate of loss in bone density can vary significantly between women, assessment of premenopausal women offers the opportunity to assess the risk of developing osteoporosis in later life and to potentially offer health advice to limit the deterioration of bone. As the prevalence of both CD and osteoporosis are greater in women than men (Cook et al., 2004), women with CD have a heightened health burden associated with osteoporosis. This study therefore aimed to investigate bone density, and factors affecting bone, in premenopausal women with CD compared to a matched healthy control group.

5.3 Materials and methods

5.3.1 Subjects

This cross-sectional study was undertaken at Massey University, Palmerston North campus. Participants visited the Human Nutrition Research Unit (HNRU), at Massey University, after fasting overnight but hydrated, where they had blood samples drawn, were provided with breakfast, completed questionnaires about their medical history and diet, and had BMD and bone quality assessed by DXA and QUS, respectively.

Participants were matched based predominantly on age and body mass index (BMI) (calculated from self-reported weight and height) reported in their responses to the pre-enrolment screening questionnaire. In addition, participants were asked about their self-identified ethnicity because ethnicity can affect BMD (Brown et al., 2007).

Exclusion criteria included diagnosis of any other known condition that affects bones or nutrient absorption including osteogenesis imperfecta, uncontrolled thyroid disease, chronic renal disease, inflammatory bowel disease, clinically significant liver disease and diagnosis of juvenile rheumatoid arthritis;

use of oral corticosteroids for 3 months or more. Coeliac participants were also excluded if they had not received a medical diagnosis through gastroenterology. Coeliac participants also needed to be diagnosed at least a year before participation and to report consuming a strict GFD at the time of their visit. All participants were fully informed about the requirements of the study, both prior to and at the start of their visit to the Human Nutrition Research Unit and gave written consent. The study was registered through Australian New Zealand Clinical Trials Registry (ANZCTR), Trial ID ACTRN12619001542189, and was approved by the Massey University Human Ethics Committee (Southern A), Reference Number SOA 18/73.

5.3.2 Dietary analysis

Participants were asked to complete a four-day estimated food record prior to their visit to the research unit. Participants were asked to record all food or beverages they consumed each day, on three weekdays and one weekend day. They were advised to be as accurate as possible, to use household measures to estimate intake, and to provide any recipes used or images or food packaging where appropriate. In addition, participants were asked to provide a separate list of all supplements currently being consumed, including the brand, quantity consumed and when these were taken. All food records were reviewed with participants at the time of their visit to the Human Nutrition Research Unit to address entry errors or missing values.

Dietary data were analysed using the New Zealand database available through FoodWorks 10 Professional (Xyris Software Pty, Brisbane, Australia). In addition, Australian and American databases and NZ Food Composition Tables were utilised where suitable options were unavailable through FoodWorks. Diets of participants were compared for average intake of nutrients which are commonly lacking (e.g., carbohydrate, dietary fibre, calcium, iron, phosphorous, magnesium

and zinc) or consumed in excess (e.g., fat and sodium) in the GFD (Melini & Melini, 2019).

The Goldberg cut-offs were used to assess validity of reporting of participants dietary intake (Banna et al., 2017; Black, 2000). A physical activity level of 1.55 (Black, 2000) was used to estimate under- and over-reporters. BMR was determined from weight, height and age using the Mifflin equations (Mifflin et al., 1990), with dietary data excluded where the EI:BMR ratio was outside of the 95% confidence interval (1.00-2.40) (Black, 2000).

5.3.3 Anthropometric measures

Participants' height was measured to the nearest 0.1 cm using a wall-mounted stadiometer (Seca Medical Measuring Systems, Chino, CA, USA), with weight measured to the nearest 0.1 kg using accurate electronic floor scales (Life Measurements Inc., Concord, CA, USA). BMI (weight (kg)/height^2 (m)) was calculated using these parameters. In addition, whole body composition (fat; lean tissue) was assessed using DXA Horizon A (Hologic Inc., Bedford, MA, USA).

5.3.4 Bone densitometry

DXA scans of the whole body, lumbar spine (L1-L4), left proximal femur (femur neck, Ward's triangle and trochanter) and left forearm (distal 1/3), as well as total body composition, were carried out by a qualified operator, using a Hologic Horizon series, fan beam X-ray Bone Densitometer, model A (Hologic Inc., Bedford, MA, USA) to determine bone mineral content (BMC, grams), bone mineral density (BMD, grams/centimetre^2) and z-scores for each participant. Calibration of the DXA was completed daily with a spine phantom and precision determined using the coefficient of variation of 0.45-0.54% for all measures, according to the manufacturer's instructions.

As participants in this study were premenopausal, z-scores were used to assess risk; these are more appropriate than t-scores due to being age-matched (Langdahl, 2017). Although commonly reported in other research studies, the

International Society for Clinical Densitometry (ISCD) cautions that the diagnosis of osteopenia and/or osteoporosis is not appropriate in premenopausal women (International Society for Clinical Densitometry, 2019); z-scores are used instead to classify whether bone density was within the expected range for age (z-score >-2.0) or below expected range for age (z-score <-2.0) (Shenoy et al., 2014).

5.3.5 Bone quality

In addition to assessment of BMD through DXA, bone quality was assessed through Quantitative Ultrasound, as this cheap and minimally invasive assessment tool has been used to assess bone quality in this type of subject group (Ballestero-Fernández et al., 2021; Potter et al., 2018), although primarily in children (Baroncelli et al., 2003; Hartman et al., 2004; Valerio et al., 2008).

An Achilles QUS ultrasonometer (Lunar Achilles Insight, GE Lunar Corporation Inc., Madison, WI, USA) was used to establish bone quality in the calcaneus (heel) of the non-dominant leg (to minimise variability). Calibration was completed on the morning of each visit according to the manufacturer's instructions. Broadband ultrasound attenuation (BUA) and speed of sound (SOS) were measured, with z-scores and stiffness index (SI) calculated to assess whether each participant's bone parameters were within the expected range for age (z-score >-1.0) (Hammad, 2013).

5.3.6 Blood parameters

Fasted venous blood samples were collected by a qualified phlebotomist between 8:30 and 9:30am using sterile flashback vacutainer needles; timing allowed for minimal variation in bone marker results as C-terminal telopeptide of type I collagen (CTX-I) has a circadian rhythm (Szulc et al., 2017). Serum samples were allowed to coagulate before being centrifuged.

Serum samples were used to measure 25-hydroxyvitamin D (vitamin D) and thyroglobulin (Tg). In addition, markers of CD, tissue transglutaminase (tTG) and endomysial antibodies (EMA), were analysed to assess adherence to a GFD in

coeliac participants and to exclude the possibility of undiagnosed CD in the healthy control group.

Plasma samples were collected in vacutainers containing EDTA anticoagulant to measure CTX-I and parathyroid hormone (PTH) and placed on ice to await processing. Additional plasma was collected in lithium-heparin containing vacutainers for the analysis of serum calcium (calcium), high sensitivity C-reactive protein (hsCRP), B12 and folate.

All samples were centrifuged at 3000g for 10 minutes at 4°C within 2 hours, after which they were aliquoted and frozen before being stored at -80°C to await further processing at the end of the study. At the conclusion of the study, collected samples were couriered on dry ice to Canterbury Health Laboratories, Christchurch, New Zealand for analysis.

Trained staff also collected a capillary blood sample from the finger using a lancet to measure whole blood haemoglobin concentration as a proxy for iron status using a point-of-care Hemocue Hb 201+ system. Prior to this procedure, participants were asked to wash their hands with warm water to remove any dirt and promote adequate blood flow in capillaries; the finger used for collection was also wiped with an alcohol wipe to reduce any further risk.

5.3.7 Physical activity

Physical activity was assessed using the internationally validated Global Physical Activity Questionnaire (GPAQ) developed by the World Health Organization (WHO) (World Health Organization, 2012). The questionnaire required participants to answer 16 questions regarding activity at work, travel to and from places, recreational activity and sedentary behaviour. Responses were then coded and used to produce Metabolic Equivalents of Task (METs), used for estimating metabolic expenditure (World Health Organization, 2012).

5.3.8 Questionnaires

Questionnaires were administered online using Qualtrics software (Qualtrics, Provo, Utah). Each participant's eligibility was assessed through the screening questionnaire, which was completed at home and clarified as required by an email from the research team to confirm eligibility. At the visit, participants were asked to complete a questionnaire regarding their health. This included questions about their diagnosis, symptoms (for participants with CD) and diet.

5.3.9 Statistical analysis

Statistical analysis was carried out using Statistical Analysis Software (SAS) (Version 9.4) (SAS Institute Inc., Cary, NC, USA). The Shapiro Wilkes test was used to test for normal distribution, with normally distributed results reported as mean ± standard deviation (SD) and non-parametric data presented as median with 25^{th} and 75^{th} percentile. Comparisons between the two participant groups were carried out using the Tukey-Kramer Method and associations between groups were assessed using Fisher's Exact Test. In addition, relationships between QUS and DXA were assessed using univariate linear regression, with the Fisher-Z transformation used to compare correlations. Statistical significance was set at $p<0.05$.

G* Power 3 software (Faul et al., 2007) was used to calculate a sample size of 39 women in each group was required to detect a difference in bone density between people with CD and healthy controls with an effect size 0.41 at power 0.95 and a probability of type 1 error 0.05 (Szymczak et al., 2012).

5.4 Results:

5.4.1 Participants characteristics

One hundred and ninety-seven women aged 18-40 years were screened for participation. Healthy controls were matched to coeliac participants, with 127 women excluded due to factors ranging from absence of definitive diagnosis of CD,

other pre-existing conditions and not adequately matching with coeliac participants. Seventy women aged 19-40 were recruited from the Manawatū and surrounding regions. This included 31 women with CD and 39 healthy controls who took part in the study between December 2019 and June 2021.

There was no statistically significant difference between the two groups based on these parameters or body fat percentage assessed through DXA (Table 5.1). In addition, no differences were found in education level between participant groups.

Table 5.1 Participant characteristics of premenopausal women with CD (n=31) compared to healthy control group (n=39)

	Coeliac Group n = 31	Healthy Control Group n = 39	*p* value
Age (years)	27.5 (24.0, 36.8)	25.0 (23.8, 31.5)	0.6259
Height	167.3 ± 6.5† (156.6 – 181.1)	168.1 ± 6.7† (154.1 – 182.0)	0.6092
Weight	67.5 ± 13.9† (38.0 – 102.4)	66.3 ± 8.4† (45.3 – 88.6)	0.6575
BMI	24.2 ± 5.2† (15.4 – 37.5)	23.5 ± 2.8† (17.7 – 29.9)	0.4714
Body Fat %	37.7 ± 6.8† (23.5 – 50.5)	35.8 ± 4.9† (27.1 – 46.6)	0.1829

BMI, body mass index. Characteristics presented are from anthropometric measures taken at participants visit (not those that were self-reported). Data presented as mean ± standard deviation † (Range). Non-parametric data presented as median (25th percentile, 75th percentile).

Participants primarily reported being of European descent with 77.4% (24/31) of coeliac participants and 66.7% (26/39) of healthy controls self-identifying as being New Zealand European. The remaining coeliac participants and a further 20.5% (8/39) of healthy controls identified as being partially of European descent; with the remaining 12.8% healthy controls identifying as Latin American (5/39). In addition, it should be noted that Māori and Polynesian groups were under-represented in the study population, with only 9.7% (3/31) coeliac participants and 2.6% (1/39) healthy controls identifying as Māori and 2.6% (1/39) healthy controls identifying as Polynesian. Although this does not provide an accurate representation of the New Zealand population, it is not unexpected for

the coeliac population, as the HLA haplotypes associated with CD are not commonly found in these ethnic groups (Edinur et al., 2013).

5.4.2 Bone density

Only 6.5% of coeliac participants (2/31) had previously had a DXA scan, compared with 12.8% healthy control participants (5/39). Six healthy controls also reported having had a previous QUS of which 2 were individuals who had also had a previous DXA; no coeliac participants had had a previous QUS.

The mean z-score for all sites were within expected range for age (z-score of >-2.0) (Shenoy et al., 2014), both overall and within the two participant groups (Table 5.2). Only two participants were identified as having bone density below the expected range for age for their whole-body scans; both participants were in the coeliac group. In addition, one coeliac participant had a lumbar spine z-score lower than expected for age. No other participants had DXA results outside the expected range at any site. However, the QUS identified four participants considered at risk based on a z-score of less than -1.0 (Hammad, 2013). These participants were not identified as being at any risk by DXA, at any site, with all results greater than -2.0 (Shenoy et al., 2014).

Table 5.2 Description of bone parameters for premenopausal women with CD compared to healthy control group

			Coeliac Disease	Healthy Control Group	*p* value
Hip			n=30	n=39	
	Total	BMD (g/cm2)	0.92 ± 0.09† (0.76 - 1.10)	0.96 ± 0.12† (0.77 - 1.16)	0.0765
		BMC	30.89 ± 4.56† (21.6 - 40.48)	32.09 ± 5.55† (22.15 - 45.19)	0.2423
		z-score	-0.13 ± 0.76† (-1.5 - 1.4)	0.19 ± 0.94† (-1.4 - 1.8)	0.0733
	FN	BMD (g/cm2)	0.81 ± 0.10† (0.67 - 0.99)	0.85 ± 0.12† (0.61 - 1.10)	0.1336
		BMC	3.99 ± 0.56† (2.92 - 5.05)	4.09 ± 0.69† (2.86 - 5.62)	0.3391
		z-score	-0.25 ± 0.87† (-1.6 - 1.5)	0.06 ± 1.08† (-1.9 - 2.3)	0.1172
Spine (L1-L4)			n=31	n=39	
	Total	BMD (g/cm2)	1.05 ± 0.11† (0.79 - 1.22)	1.06 ± 0.10† (0.88 - 1.25)	0.3816
		BMC	62.42 ± 9.31† (39.94 - 83.17)	64.67 ± 9.63† (47.44 - 86.69)	0.2242
		z-score	0.1 ± 1.02† (-2.4 - 1.7)	0.27 ± 0.93† (-1.5 - 1.9)	0.3295
Whole Body			n=31	n=39	
		BMD (g/cm2)	1.03 ± 0.06† (0.92 - 1.16)	1.07 ± 0.07† (0.95 - 1.22)	0.0274*
		BMC	2076.33 ± 248.95† (1464.58 - 2558.90)	2174.35 ± 257.18† (1696.61 - 2762.31)	0.0550
		z-score	-0.95 ± 0.77† (-2.4 - 0.5)	-0.48 ± 0.83† (-2.0 - 1.3)	0.0185*
Forearm			n=27	n=39	
	Distal 1/3	BMD (g/cm2)	0.69 ± 0.04† (0.63 - 0.77)	0.69 ± 0.03† (0.63 - 0.79)	0.9635
		BMC	1.76 ± 0.23† (1.35 - 2.21)	1.8 ± 0.17† (1.51 - 2.27)	0.3746
		z-score	0.1 ± 0.71† (-1.4 - 1.3)	0.11 ± 0.58† (-1.0 - 1.7)	0.9027

Data presented as mean ± standard deviation † (Range). FN, femoral neck; BMD, bone mineral density; BMC, bone mineral content

Assessment of DXA findings demonstrated a trend of lower BMD, BMC and z-score in coeliac participants at almost all sites, however, this difference was not statistically significant at the hip, spine or forearm. A significant difference between groups was demonstrated when considering only the whole-body BMD (p=0.0274) and z-score (p=0.0185).

5.4.3 Evaluation of QUS

The CD group had significantly lower SI (p=0.0069) and z-scores (p=0.0140) compared with healthy controls (Table 5.3).

Table 5.3 Summary of QUS Results for premenopausal women with CD (n=31) compared to healthy control group (n=39)

	Coeliac Disease n=31	**Healthy Control Group n=39**	***p* value**
Stiffness Index	102.0 ± 15.6† (77.0 – 135.0)	113.4 ± 18.4† (83.0 – 155.0)	0.0069*
z-score	0.20 ± 0.96† (-1.40 – 2.20)	0.87 ± 1.14† (-1.00 – 3.50)	0.0140*

Data presented as mean ± standard deviation † (Range)

A significant positive correlation was found between DXA and QUS (p<0.05) in healthy controls for all points excluding the forearm (Table 5.4). However, a significant positive correlation was only found in the coeliac group between SI and whole body DXA. In both groups the low correlation coefficient indicates that a linear regression would not be a suitable fit for this relationship. No significant difference was found between correlation coefficients of the two groups.

Table 5.4 Correlation between QUS and DXA of premenopausal women with CD (n=26) and healthy control group (n=39)

	Coeliac Disease n=26			Healthy Control Group n=39			
	Linear regression (R^2)	Correlation Coefficient (R)	*p* value for correlation	Linear regression (R^2)	Correlation Coefficient (R)	*p* value for correlation	Fisher z-score *p* value
SI vs. Hip BMD	0.1127	0.3357	0.0935	0.3404	0.5834	<0.0001	0.117
SI vs. LS BMD	0.071	0.2665	0.1883	0.1656	0.4069	0.0101	0.276
SI vs. FN BMD	0.1132	0.3365	0.0928	0.3554	0.5962	<0.0001	0.103
SI vs. Distal 1/3	0.0011	0.0332	0.8723	0.0859	0.2931	0.0701	0.157
SI vs. WB	0.2782	0.5274	0.0056*	0.4465	0.6682	<0.0001	0.204

n = 65 due to absence of some results for particular DXA points; SI, Stiffness Index; BMD, bone mineral density; LS, lumbar spine; FN, femoral neck; WB, whole body

5.4.4 Adherence

Biological testing for tissue transglutaminase (tTG) and endomysial antibodies (EMA) was used to assess adherence to the GFD in the CD group and identify possible undiagnosed CD in the healthy control group. Results detected no evidence of undiagnosed CD and a strong adherence to the GFD; all tTG results were <3 units indicating a negative result. One coeliac participant had a weak positive EMA result, however their tTG, although raised, was still <3 consistent with a negative result, with hsCRP within normal range.

hsCRP was raised in 6 coeliac participants and 2 healthy controls (hsCRP >5 indicating the presence of inflammation). However, there was no correlation with raised hsCRP and coeliac antibody results. All other hsCRP results were <5 indicating no signs of inflammation were present.

Consumption of oats was reported by 5/31 coeliac participants. However, none of these participants had raised antibodies (tTG or EMA) or hsCRP.

5.4.5 Coeliac participants

All coeliac participants were diagnosed by a gastroenterologist with all, but one, diagnosed through positive endoscopy. The average time since diagnosis ranged from 1 to 28.6 years with a median time of 8.0 years (3.1, 13.5). No relationship was found between bone parameters and time since diagnosis: femoral neck BMD (p=0.7692), total hip BMD (p=0.9657), lumbar spine (p=0.5186), distal 1/3 (p=0.5511).

5.4.6 Nutrient intake

The Goldberg cut-offs were used to assess validity of reporting of participants dietary intake. Based on EI:BMR ratio, dietary data from one coeliac participant and one healthy control were excluded due to being outside (under and over respectively) the 95% confidence interval (1.00-2.40).

The mean daily intake of calcium was 843 (± 307) mg and 927 (± 229) mg for coeliac and control groups respectively. However, only 12/25 (48.0%) coeliac participants met the Estimated Average Requirement (840 mg), while 20/31 (64.5%) of the healthy controls met this recommendation (Table 5.5).

In our study, 3/30 individuals (10.0%) with CD reported lactose intolerance, compared with 2/37 (5.4%) of healthy controls. Despite this, lactose intolerance did not appear to influence calcium intake in these participants (p=0.3409).

The average daily intake of protein for coeliac participants and healthy controls was 81.9g and 87.5g, respectively. There was no significant difference between these groups (p=0.4208). When using weight-based guidelines to assess protein intake (0.6g protein/kg body weight), the average intake was 1.26g/kg for coeliac participants and 1.30g/kg for the healthy control group. All but one participant met the EAR for protein (0.60g/kg); with one coeliac participant having an intake of 0.57g/kg, just below the EAR.

Table 5.5 Nutrient intake for premenopausal women with CD (n=25) compared to healthy control group (n=31)

	Coeliac Disease n=25	Healthy Control Group n=31	*p* value	Nutrient Reference Values (NRVs)
Macronutrients				
Energy Intake (kJ)	8005 ± 1648† (5890 - 12342)	8584 ± 1297† (6471 - 12172)	0.1466	
Protein (%EI)	16.0 (14.0, 19.3)	16.1 (14.7, 18.8)	0.8329	15-20[2]
Protein (g)	81.9 ± 29.6† (50.2 - 187.6)	87.5 ± 22.0† (50.7 - 137.6)	0.4208	37 (0.60g/kg)[1]
Fat (%EI)	38.9 ± 4.5† (27.3 - 47.3)	35.8 ± 4.8† (24.9 – 43.5)	0.0155*	20-35%[2]
Total Fat (g)	84.2 ± 19.8† (55.3 - 128.6)	83.0 ± 16.4† (58.1 - 123)	0.7876	
Saturated Fat (g)	31.3 ± 10.9† (16.2 – 56.1)	31.3 ± 8.6† (15.1 - 47.2)	0.9806	
Carbohydrate (%EI)	40.6 ± 7.0† (20.3 – 55.0)	44.5 ± 5.7† (33.6 – 58.6)	0.0267*	45-65%[2]
Carbohydrate (g)	193.5 ± 63.0† (73.8 - 399.5)	224.9 ± 47.1† (151.7 – 327.2)	0.0372*	
Sugars (g)	80.0 ± 34.2† (33.9 - 187.1)	84.7 ± 26.5† (31.5 – 141.3)	0.5642	
Dietary Fibre (g)	25.4 ± 6.9† (15.2 - 38.0)	30.3 ± 10.4† (12.7 - 51.8)	0.0474*	25[3]
Micronutrients				
Sodium (mg)	2502 ± 774† (1241 - 4530)	2539 ± 775† (1342 - 4313)	0.8591	460-920[3]
Potassium (mg)	2670 (2403, 3334)	3185 (2728, 3514)	0.4356	
Magnesium (mg)	290 (277, 369)	385 (302, 418)	0.0697	255 (19-30)[1] 265 (31-50)[1]
Calcium (mg)	843 ± 307† (349 - 1694)	927 ± 229† (558 - 1385)	0.2476	840[1]
Phosphorus (mg)	1245 (1152, 1456)	1405 (1215, 1629)	0.0992	580[1]
Iron (mg)	10.77 ± 3.42† (5.33 - 19.05)	13.53 ± 4.00† (6.79 – 21.94)	0.0089*	6[1]
Zinc (mg)	9.91 ± 3.15† (5.33 - 16.54)	10.04 ± 2.53† (6.13 - 17.22)	0.8628	12[1]

Normally distributed data presented as mean ± standard deviation † (Range). Non-parametric data presented as median (25th percentile, 75th percentile). NRVs, Nutrient Reference Values; EI, energy intake; kJ, kilojoule.
[1] Estimated Average Requirement (EAR), [2] Acceptable Macronutrient Distribution Range (AMDR), [3] Average Intake (AI) (National Health and Medical Research Council and New Zealand Ministry of Health, 2006)

Dietary analysis only included food and drinks, or supplements consumed as part of a food (i.e., protein powders). Additional supplementation in the form of tablets or capsules was considered separately. Nine coeliac participants and 9 healthy control participants consumed additional supplements. Of these individuals, only 1 healthy control and 1 coeliac participant reported consuming a supplement containing calcium, as part of a multi-vitamin. On average these multi-vitamins contained insignificant levels of calcium at roughly 50mg each. In addition, only one coeliac participant reported consuming a vitamin D supplement.

Although no significant differences were seen in total energy intake (p=0.1466), carbohydrate intake was significantly lower in the coeliac group (p=0.0372).

5.4.7 Blood parameters

Table 5.6 Comparison of blood parameters between premenopausal women with CD and healthy control group

	Coeliac Group	Healthy Control Group	*p* value	Reference Value
Haemoglobin g/L	n=31 131.0 ± 10.9† (117.0 - 155.0)	n=39 130.4 ± 10.3† (102.0 - 156.0)	0.7860	<70g/L severe anaemia[1] 80-109 g/L moderate[1] 110-119 g/L mild[1] >120 g/L non-anaemic[1]
B12 pmol/L	n=27 336.0 (267.0, 442.5)	n=38 249.0 (194.5, 373.0)	0.3477	130 – 650 pmol/L[2]
Folate nmol/L	20.3 ± 8.1† (9.0 - 37.0)	21.9 ± 8.5† (9.0 - 40.0)	0.4073	>8.0 nmol/L[2]
PTH nmol/L	3.23 ± 0.92† (1.40 - 5.20)	3.42 ± 1.08† (1.90 - 6.10)	0.4045	1.6 – 7.0 pmol/L[2]
Calcium mmol/L	2.40 (2.30, 2.40)	2.40 (2.30, 2.40)	0.1617	2.2 – 2.6 mmol/L[2]
CTX-I µg/L	0.44 ± 0.17† (0.15 - 0.74)	0.51 ± 0.22† (0.17 - 1.21)	0.2623	<0.75 µg/L[2]
Vitamin D nmol/L	n=28 75.3 ± 22.5† (46.0 - 130.0)	n=38 80.2 ± 25.4† (38.0 - 143.0)	0.4976	<25 nmol/L mod-severe deficiency[2] 25-50 nmol/L mild deficiency[2] 50-150 nmol/L optimal[2]

Data presented as mean ± standard deviation † (Range). Non-parametric data presented as median (25th percentile, 75th percentile). PTH, parathyroid hormone (corrected for albumin); CTX-I, C-terminal telopeptide of type I collagen; vitamin D, 25(OH)D, 25 hydroxyvitamin D. [1] World Health Organization, 2011. [2] Canterbury Health Laboratories, n.d.

No statistically significant difference was found between coeliac participants and healthy controls in any blood parameters measured (Table 5.6).

No participants were deficient in either B12 or folate, with only one coeliac participant having a PTH lower than the normal range. In addition, although no

participants were severely vitamin D deficient, 11 participants were mildly deficient: 6 coeliac participants (21.4%) and 5 healthy controls (13.2%), however, the difference between frequency was not significant (p=0.7294)

Four participants in the healthy control group had CTX-I greater than the reference value (0.75 µg/L); CTX-I was below 0.75 µg/L in all coeliac participants. The median age of participants over the reference range was 21.5 years (20.8, 22.5).

Consideration of a reference range accounting for oral contraceptive (de Papp et al., 2007) use resulted in an additional 8 participants (3 coeliacs and 5 healthy controls) being identified as having bone turnover greater than expected (>0.614 in oral contraceptive users and 0.675 in those not using them). The median age of participants over these reference ranges was 23.0 years (18.0, 25.0).

A significant negative correlation was demonstrated between CTX-I and BMD at the femoral neck (p=0.0231) and distal 1/3 (p=0.0310); however, no correlation was found with total hip or lumbar spine.

5.4.8 Physical activity

Table 5.7 Comparison of physical activity level between premenopausal women with CD (n=30) and healthy control group (n=36)

	Coeliac Group n=30	Healthy Control Group n=36	*p* value for correlation	Recommendation
Moderate Activity Minutes	111 (40, 381)	270 (115, 517.5)	0.1349	150 minutes
Vigorous Activity Minutes	90 (17.5, 240)	190 (25, 300)	0.4293	75 minutes
MET Minutes combined	1640 (470, 3190)	2400 (1000, 4980)	0.1622	600 MET minutes

Median (25th percentile, 75th percentile). MET, metabolic equivalent of task

There was no significant difference in physical activity level between groups; differences in both moderate and vigorous activity, as well as MET minutes were not statistically significant (table 5.7). Seven individuals with CD did not meet recommendations for MET minutes compared with 4 healthy controls.

5.5 Discussion:

5.5.1 Bone parameters

There were no significant differences found in bone density between coeliac women and healthy controls at the hip, lumbar spine or forearm. Although significant differences were found in whole body BMD and z-score it should be noted that risk identified through whole-body scans often differs from that found using more specific sites (Melton et al., 2005) and the ISCD does not recommend its use for assessment (International Society for Clinical Densitometry, 2019); the whole-body scan was primarily included to assess body composition in this cohort.

Previous studies have demonstrated significant differences between patients with CD when compared with matched healthy controls (Pantaleoni et al., 2014; Szymczak et al., 2012). However, much of the literature assessing bone density in adults with CD has investigated bone density at the time of diagnosis (Galli et al., 2018; Stein et al., 2015) and the extent to which it improves once a GFD is adopted (Ciacci et al., 2020). Evidence suggests adherence to a GFD results in initial improvements in BMD due to resolution of gut damage, however, very little increase is seen after the first year of adhering to the diet (Bathrellou et al., 2018; Kirsaçlioğlu et al., 2016). In addition, there are limited studies assessing BMD in CD patients who are premenopausal with most focusing on postmenopausal women or a wider age group including both pre and postmenopausal women. When comparing the bone density of the CD group in our research study to findings of other studies which have assessed BMD after adherence to the GFD for a year or more, our group had greater BMD.

A similar study in premenopausal women with CD by Sayar et al. (2021) reported a similar z-score of -0.3 at the femoral neck (-0.25 in the current study), but a much lower z-score of -0.9 at the lumbar spine (0.1 in the current study) in premenopausal women consuming a GFD for a minimum of a year. In addition, the proportion of z-scores suggestive of osteoporosis and osteopenia were greater than found in our study, with 39.1% of participants with z-scores indicating osteopenia and 2.1% indicating osteoporosis at the femoral neck, while 65.2% had z-scores consistent with osteopenia at the lumbar spine (Sayar et al., 2021).

In comparison after 1 year on a GFD, Szymczak (2012) reported a mean z-score at the femoral neck in premenopausal coeliac women of -0.23 ± 0.98, consistent with our findings (-0.25 ± 0.87), but z-scores at the lumbar spine (-0.14 ± 1.11), forearm (-1.23 ± 1.24) and whole body (-0.83 ± 0.92) were lower, both than in our group and that seen in their healthy control group (although these differences were not significant for femoral neck).

The lack of significant differences between groups in the current study could indicate that both groups had low BMD. However, mean z-scores for both hip (0.19 ± 0.94) and lumbar spine (0.27 ± 0.93) from the healthy control group in this study are comparable with other research by our group investigating bone density in healthy young women in the Manawatū region (0.19 ± 1.20 and -0.20 ± 1.13 respectively) (Schraders et al., 2019).

There was no significant difference found in CTX-I between the two groups, suggesting no significant differences in bone turnover. There are some inconsistencies regarding the appropriate range to use for premenopausal women with some authors suggesting different ranges dependent on the population assessed (Vasikaran et al., 2014). The reference range provided by Canterbury Health (Canterbury Health Laboratories, n.d.) for example, was for all adults and does not account for the impact of oestrogen on bone turnover. In a study by Eastell et al. (2012) CTX-I measured using an automated analyser (comparable to that used in the current study) in healthy premenopausal European women found

a mean CTX-I of 0.297 µg/L, much lower than in either of our study groups (CD 0.44 ± 0.17 µg/L; healthy 0.51 ± 0.22 µg/L). However, despite the higher mean CTX-I in the current study, the range in the coeliac population (0.15-0.74 µg/L) was similar to the normal range determined by Eastell's findings (0.111-0.791 µg/L); the healthy controls in the present study had a greater upper end of the range (0.17-1.21 µg/L), indicating participants with greater bone turnover. In addition, when considering the impact of oestrogen on bone turnover, the use of oral contraceptives must be taken into account, with participants using oral contraceptives having lower bone turnover represented through lower CTX-I results (He et al., 2022). When comparing findings of the current study with that of De Papp and colleagues (2007) in a similar age group, two different reference ranges in participants were proposed, dependent on whether they used oral contraceptives or not (0.08-0.614 µg/L and 0.113-0.675 µg/L respectively). Use of these reference ranges (with consideration of current oral contraceptive use) would result in an additional 8 participants (3 coeliacs and 5 healthy controls) being identified as having bone turnover greater than expected. However, of note in the current study is the average age of the participants with the greatest bone turnover. When considering those participants/individuals with CTX-I values above the reference range, either the range provided by Canterbury Health Laboratories (n.d.) or that identified in the De Papp research (de Papp et al., 2007), participants were at the younger end of the age range, with a median of 21.5 and 23.0 years respectively. This may indicate that bone turnover is high in this population as a result of ongoing ossification because peak bone mass has not yet been achieved (Stagi et al., 2013). As the current study did not measure P1NP to assess levels of bone formation, it is not possible to draw conclusions from these results regarding whether bone resorption is high in these participants. In addition, no consideration was made for the phase of the menstrual cycle and possible cyclic variations in CTX-I in participants taking oral contraceptives (Martin et al., 2021).

Findings from Quantitative Ultrasound

Despite no statistically significant difference between groups in bone parameters at almost all sites (excluding whole body) assessed through DXA (table 5.2), statistically significant differences were observed between groups for both SI and z-score assessed through QUS (table 5.3).

In addition, 3 coeliac participants were identified through QUS as having a z-score of less than -1 indicating bone quality below the expected range for age. However, all 3 participants had z-scores within the expected range for age (>-2.0) in their DXA assessment. Two coeliac participants had whole body z-scores of less than the expected range for age, with one coeliac lumbar spine z-score also being below the expected range for age. Despite this, all of these individuals had z-scores within the expected range for age for their QUS measurements.

Relationship between DXA and QUS

A statistically significant positive correlation between DXA and QUS was demonstrated in the healthy control group at all DXA sites except the forearm, consistent with findings from a previous study of women in the same region (Schraders et al., 2019). However, in the coeliac group this relationship was less clear, with QUS only showing a significant positive correlation with whole body DXA BMD. The weak correlation found in both groups suggest that QUS is not an appropriate method to assess the bone of an individual. In addition, these findings may indicate that the QUS of the calcaneus is not an appropriate tool to assess bone in populations with CD.

Prior research using QUS as a tool for assessment of bone is limited in adults with CD, however, the findings of one recent study by Ballestero-Fernández et al. (2021) are at odds with those of the current study, with no difference in bone quality between participants with CD and healthy controls demonstrated in premenopausal women.

The QUS used in the current research assesses the bone quality of a single site, the calcaneus, which is primarily trabecular bone and considered representative of the trabecular bone found in the hip (Rizzoli & Bonjour, 2004). However, research indicates that trabecular bone could be preferentially affected in individuals with CD who have low BMD, with Zanchetta et al. (2015) reporting a 26.4% lower trabecular density in premenopausal coeliac women compared with healthy controls. It has also been demonstrated that this lower trabecular density results in reduced bone strength in the tibia and radius (Stein et al., 2015) in this age group. With abnormal trabecular bone development also identified in children with CD (Pham-Short et al., 2019), further research is warranted to assess whether the use of QUS is appropriate for individuals with CD.

History of bone assessment

One finding of interest was the reporting of previous assessments of bone in participants. It should be noted that whereas only 2 individuals with CD had prior assessment of bone through DXA (6.5%), 9 healthy controls (24.3%) had a previous DXA (3/39), QUS (4/39) or both (2/39). As no investigation was completed into the reason for this prior investigation of participant bone health, we cannot rule out undisclosed bone-related health concerns in our healthy control population, which may have attracted them to take part in the study.

The low reports of previous DXA in individuals with CD in the current study is, although concerning, unsurprising based on findings from our other study (see Chapter 7).

5.5.2 Adherence

Coeliac participants adherence to a GFD as measured through tTG and EMA suggested that the participants adhered well to dietary recommendations. However, it should be noted that five coeliac participants reported consuming oats in their responses to the questionnaires. Consumption of oats as part of a GFD is a contested topic in New Zealand. In most countries, wheat-free oats (oats free of

contamination from wheat) are considered safe for individuals with CD (Aaltonen et al., 2017; Pinto-Sánchez et al., 2017), except in the small proportion (8%) who do not tolerate avenin found in oats (Hardy et al., 2015). However, the view of experts in New Zealand is that oats are not safe for people with CD and should be excluded from a GFD (Coeliac New Zealand, 2017). Despite this viewpoint, it should be noted that in the current study, tTG of <1 and negative EMA were detected in all participants consuming oats, consistent with no damage.

5.5.3 Nutrient intake

The GFD is characteristically low in dietary fibre, whilst being high in fat, sugar and sodium (Vici et al., 2016). When comparing groups, we found no significant difference between intakes (in grams) of protein, fat (both total and saturated) and sugar. However, significant differences in both carbohydrate and dietary fibre were observed with the healthy control group consuming significantly more of both nutrients (Table 5.5). The lower intake of dietary fibre is consistent with previous research which reported that the GFD is often a poor source of dietary fibre (Wünsche et al., 2018), primarily due to many common sources being excluded from the diet; this may be exacerbated in New Zealand where it is advised that oats should be excluded from a GFD and that oats are labelled as containing gluten (Food Standards Australia New Zealand, 2021). Although no significant differences were found in energy intake between groups, carbohydrate intake was significantly lower in the coeliac group (p=0.0372), which was also reflected in the contribution from carbohydrate to total energy intake (p=0.0267). Lower intake of carbohydrate fits with previous research findings which reported that individuals with CD have a low intake of carbohydrate (Cardo et al., 2021; Mijatov & Mičetić-Turk, 2016), particularly intake of complex carbohydrates. This may also explain the differences in intake of dietary fibre; although both groups consumed on average more than the average intake (AI), the coeliac group consumed significantly less than the healthy controls (p=0.0474).

In addition to assessment of macronutrients, mineral intake estimated from diet diaries was also compared. No statistically significant difference was found when comparing intake of potassium, calcium, magnesium, phosphorus and zinc. This differs from previous research that identifies the GFD as having low levels of certain grains and cereal products resulting in reduced intakes of nutrients such as magnesium and phosphorus (Melini & Melini, 2019).

Calcium intake in individuals with CD was lower than in healthy controls, (841 ± 303 and 927 ± 229 respectively) but the difference was not statistically significant (p=0.2271). Although fewer coeliac participants met the EAR than healthy controls (46.2% vs 64.5% respectively; p 0.2144), the difference was not statistically significant (p=0.2144). The median calcium intake of the coeliac group (819 mg) is still greater than that reported in the New Zealand Adult Nutrition Survey in 19–30-year-old women (704 mg) (University of Otago and Ministry of Health, 2011). The reported calcium intake in coeliac participants was also greater than previous research in 18–25-year-olds in the same region (784 mg) (Schraders et al., 2019). Calcium is commonly considered a nutrient of concern in the GFD, with research suggesting that individuals with CD have insufficient intake of calcium even if they have good adherence to a GFD which should mean calcium absorption is not compromised (Krupa-Kozak & Drabińska, 2016; Theethira et al., 2014). However, the low intake of calcium is often attributed to the comorbidity of lactose intolerance and exclusion of dairy foods. Lactose intolerance affects individuals with CD either prior to, or at time of, diagnosis. This is commonly due to a secondary insult resulting from the mucosal damage associated with gluten and consequent reduction in lactase production (Hall & Day, 2020). Although lactose can usually be safely reintroduced once damage to the intestinal villi has healed, many individuals are incorrectly advised to permanently avoid lactose (Alkalay, 2022). In addition, in some individuals the villi never fully recover and/or lactase production is never fully restored (Usai-Satta et al., 2022). In the current study, lactose intolerance did not appear to influence calcium intake (p=0.3409).

Due to incomplete analysis of some foods in the food composition databases, there were insufficient data to fully examine other micronutrients of concern such as vitamin B12, vitamin K and in particular vitamin D. However, as the diet is only a minor source of vitamin D, with most synthesized in the skin, serum levels were assessed.

Biochemical assessment of nutrient adequacy is of particular importance in this study as some individuals with CD never experience full recovery of their intestinal villi (Lebwohl et al., 2014). Lack of resolution of gut damage can result in ongoing malabsorption, which may be associated with nutrient deficiencies despite dietary data suggesting sufficient nutrients are being consumed (Malamut & Cellier, 2015). Individuals with CD in this study had been consuming a GFD for a minimum of 1 year prior to participation, which is considered an adequate time for the villi in the gut to be restored (Cardo et al., 2021), with coeliac antibodies reporting no presence of ongoing intestinal damage. Although, it is not possible to conclusively confirm resolution of gut damage without examination of biopsy specimens, it is likely this explains why no statistical difference in blood parameters was found.

5.5.4 Strengths and limitations

It is the researcher's belief that additional data regarding severity of gut damage, for example through Marsh scores at the time of diagnosis, may help to provide a better indication of the true cause of low bone density in patients with CD in future research. Marsh scores provide an assessment of the extent of damage found in the intestine during biopsy (Kaur et al., 2017). Although no correlation was found between time since diagnosis and BMD, Marsh scores would indicate the extent of the damage prior to diagnosis which has been shown to correlate with BMD (García-Manzanares et al., 2012).

Analysis of the diet through FoodWorks required some assumptions to be made and supplementary foods to be used, as unfortunately there were a

significant number of products consumed by the participants that are unavailable in these databases, particularly gluten-free (GF) products. Although the dietary analysis provides a rough indication of intake and how the diet may differ between groups, it may not be appropriate to consider an individual's risk factors based on this method particularly given the lack of robust data about the nutrient composition of GF foods. New Zealand would benefit greatly from a GF food composition database similar to that which has been established in Spain (Fajardo et al., 2020)

Additionally, it must be noted that the impact of the public health response to the SARS-CoV-2 virus may have influenced the participants' diets over this period of data collection. Although participants were requested not to complete their food records during lockdown periods, international research suggests SARS-CoV-2 infections and strategies to mitigate against these have resulted in significant dietary alterations which may persist after lockdowns have ended (Bascuñán et al., 2021). This is also further exacerbated for participants with CD, as New Zealand faced challenging supply issues for specialty food products, including GF foods (Mikocka-Walus et al., 2021) which persist two years later (Yeakle, 2021).

5.6 Conclusion:

Unexpectedly, we found no differences between BMD of individuals with CD compared with healthy controls. Findings from the QUS indicate further research may be needed to assess the effects of CD on trabecular bone and whether suitable sites are currently being used for assessment of both bone quality and density in these individuals. In addition, our findings support international research regarding inadequacies of the GFD and raise concerns that New Zealand women may have insufficient intakes of calcium.

5.7 References

Aaltonen, K., Laurikka, P., Huhtala, H., Mäki, M., Kaukinen, K., & Kurppa, K. (2017). The long-term consumption of oats in celiac disease patients is safe: A large cross-sectional study. *Nutrients*, *9*(6). https://doi.org/10.3390/nu9060611

Alkalay, M. J. (2022). Nutrition in Patients with Lactose Malabsorption, Celiac Disease, and Related Disorders. *Nutrients*, *14*(2), 1–17. https://doi.org/10.3390/nu14010002

Ballestero-Fernández, C., Varela-Moreiras, G., Úbeda, N., & Alonso-Aperte, E. (2021). Nutritional Status in Spanish Adults with Celiac Disease Following a Long-Term Gluten-Free Diet Is Similar to Non-Celiac. *Nutrients*, *13*(1626), 1–23. https://doi.org/10.3390/nu13051626

Banna, J. C., McCrory, M. A., Fialkowski, M. K., & Boushey, C. (2017). Examining Plausibility of Self-Reported Energy Intake Data: Considerations for Method Selection. *Frontiers in Nutrition*, *4*(45), 1–6. https://doi.org/10.3389/fnut.2017.00045

Baroncelli, G. I., Federico, G., Bertelloni, S., Sodini, F., De Terlizzi, F., Cadossi, R., & Saggese, G. (2003). Assessment of bone quality by quantitative ultrasound of proximal phalanges of the hand and fracture rate in children and adolescents with bone and mineral disorders. *Pediatric Research*, *54*(1), 125–136. https://doi.org/10.1203/01.PDR.0000069845.27657.EB

Bascuñán, K. A., Rodríguez, J. M., Osben, C., Fernández, A., Sepúlveda, C., & Araya, M. (2021). Pandemic effects and gluten-free diet: An adherence and mental health problem. *Nutrients*, *13*(6), 1–11. https://doi.org/10.3390/nu13061822

Bathrellou, E., Kontogianni, M. D., & Panagiotakos, D. B. (2018). Celiac disease and non-celiac gluten or wheat sensitivity and health in later life: A review. *Maturitas*, *112*(January), 29–33. https://doi.org/10.1016/j.maturitas.2018.03.014

Black, A. E. (2000). Critical evaluation of energy intake using the Goldberg cut-off for energy intake : basal metabolic rate . A practical guide to its calculation ,

use and limitations. *International Journal of Obesity*, *24*(9), 1119–1130. https://doi.org/10.1038/sj.ijo.0801376

Bolland, M. J., Grey, A., & Rowbotham, D. S. (2016). Outcomes of bone density measurements in coeliac disease. *New Zealand Medical Journal*, *129*(1429), 40–44.

Brown, P., McNeill, R., Radwan, E., & Willingale, J. (2007). The Burden of Osteoporosis in New Zealand: 2007-2020. In *Osteoporosis New Zealand Inc*. https://doi.org/10.1016/S0140-6736(07)61849-2

Burkhardt, J. G., Chapa-Rodriguez, A., & Bahna, S. L. (2018). Gluten sensitivities and the allergist: Threshing the grain from the husks. *Allergy: European Journal of Allergy and Clinical Immunology*, *73*(7), 1359–1368. https://doi.org/10.1111/all.13354

Canterbury Health Laboratories. (n.d.). *Laboratory Test Reference Guide: Canterbury Health Laboratories*. Available from: http://www.labnet.health.nz/testmanager/index.php

Cardo, A., Churruca, I., Lasa, A., Navarro, V., Vázquez-Polo, M., Perez-Junkera, G., & Larretxi, I. (2021). Nutritional imbalances in adult celiac patients following a gluten-free diet. *Nutrients*, *13*(8), 1–18. https://doi.org/10.3390/nu13082877

Ciacci, C., Bilancio, G., Russo, I., Iovino, P., Cavallo, P., Santonicola, A., Bucci, C., Cirillo, M., & Zingone, F. (2020). 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, and peripheral bone densitometry in adults with celiac disease. *Nutrients*, *12*(4), 1–11. https://doi.org/10.3390/nu12040929

Coeliac New Zealand. (2017). *What about Oats?* Available from: https://www.coeliac.org.nz/eating-gluten-free/what-about-oats.

Cook, B., Oxner, R., Chapman, B., Whitehead, M., & Burt, M. (2004). A thirty-year (1970–1999) study of coeliac disease in the Canterbury region of New Zealand. *The New Zealand Medical Journal*, *117*(1189), 8.

Davis, S. R., Lambrinoudaki, I., Lumsden, M., Mishra, G. D., Pal, L., Rees, M., Santoro, N., & Simoncini, T. (2015). Menopause. *Nature Reviews Disease*

Primers, *1*, 15004. https://doi.org/10.1038/nrdp.2015.4

de Papp, A. E., Bone, H. G., Caulfield, M. P., Kagan, R., Buinewicz, A., Chen, E., Rosenberg, E., & Reitz, R. E. (2007). A cross-sectional study of bone turnover markers in healthy premenopausal women. *Bone*, *40*(5), 1222–1230. https://doi.org/10.1016/j.bone.2007.01.008

Eastell, R., Garnero, P., Audebert, C., & Cahall, D. L. (2012). Reference intervals of bone turnover markers in healthy premenopausal women: Results from a cross-sectional European study. *Bone*, *50*(5), 1141–1147. https://doi.org/10.1016/j.bone.2012.02.003

Fajardo, V., González, M. P., Martínez, M., Samaniego-Vaesken, M. de L., Achón, M., Úbeda, N., & Alonso-Aperte, E. (2020). Updated food composition database for cereal-based gluten free products in Spain: Is reformulation moving on? *Nutrients*, *12*(8), 1–17. https://doi.org/10.3390/nu12082369

Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods*, *39*(2), 175–191.

Food Standards Australia New Zealand. (2022). *Australia New Zealand Food Standards Code: Standard 2.9.5. Food for special medical purposes*. Canberra, Australia: Australian Government. Available from: https://www.foodstandards.gov.au/code/Documents/2.9.5%20FSMPs%20v157.pdf

Galli, G., Lahner, E., Conti, L., Esposito, G., Sacchi, M. C., & Annibale, B. (2018). Risk factors associated with osteoporosis in a cohort of prospectively diagnosed adult coeliac patients. *United European Gastroenterology Journal*, *6*(8), 1161–1168. https://doi.org/10.1177/2050640618784340

García-Manzanares, Á., Tenias, J. M., & Lucendo, A. J. (2012). Bone mineral density directly correlates with duodenal Marsh stage in newly diagnosed adult celiac patients. *Scandinavian Journal of Gastroenterology*, *47*(8–9), 927–936. https://doi.org/10.3109/00365521.2012.688217

Hall, S. W., & Day, A. S. (2020). An Overview of International Guidelines Focusing

on the Long-Term Management of Coeliac Disease. *Gastrointestinal Disorders*, *2*(2), 152–163. https://doi.org/10.3390/gidisord2020016

Hammad, L. F. (2013). Quantitative Ultrasound Measurements Of Stiffness Index In Young Adult Females. *Archives of Medicine*, *5*(1), 1–5. https://doi.org/10.3823/107

Hardy, M. Y., Tye-Din, J. A., Stewart, J. A., Schmitz, F., Dudek, N. L., Hanchapola, I., Purcell, A. W., & Anderson, R. P. (2015). Ingestion of oats and barley in patients with celiac disease mobilizes cross-reactive T cells activated by avenin peptides and immuno-dominant hordein peptides. *Journal of Autoimmunity*, *56*, 56–65. https://doi.org/10.1016/j.jaut.2014.10.003

Hartman, C., Hino, B., Lerner, A., Eshach-Adiv, O., Berkowitz, D., Shaoul, R., Pacht, A., Rozenthal, E., Tamir, A., Shamaly, H., & Shamir, R. (2004). Bone Quantitative Ultrasound and Bone Mineral Density in Children with Celiac Disease. *Journal of Pediatric Gastroenterology and Nutrition*, *39*(5), 504–510. https://doi.org/10.1097/00005176-200411000-00011

He, W., Dam, T. V., Thøgersen, R., Hansen, M., & Bertram, H. C. (2022). Fluctuations in Metabolites and Bone Markers Across the Menstrual Cycle in Eumenorrheic Women and Oral Contraceptive Users. *The Journal of Clinical Endocrinology & Metabolism*, *107*(6), 1577–1588. https://doi.org/10.1210/clinem/dgac112

International Osteoporosis Foundation. (2017). *Facts and Statistics*. Available from: https://www.iofbonehealth.org/facts-statistics#category-26

Kaur, A., Shimoni, O., & Wallach, M. (2017). Celiac disease: from etiological factors to evolving diagnostic approaches. *Journal of Gastroenterology*, *52*(9), 1001–1012. https://doi.org/10.1007/s00535-017-1357-7

Kirsaçlioğlu, C. T., Kuloğlu, Z., Tanca, A., Küçük, N. Ö., Aycan, Z., Öcal, G., Ensari, A., Kalayci, A. G., & Girgin, N. (2016). Bone mineral density and growth in children with coeliac disease on a gluten free-diet. *Turkish Journal of Medical Sciences*, *46*(6), 1816–1821. https://doi.org/10.3906/sag-1508-52

Krupa-Kozak, U. (2014). Pathologic bone alterations in celiac disease: Etiology, epidemiology, and treatment. *Nutrition*, *30*(1), 16–24. https://doi.org/10.1016/j.nut.2013.05.027

Krupa-Kozak, U., & Drabińska, N. (2016). Calcium in gluten-free life: Health-related and nutritional implications. *Foods*, *5*(3), 1–11. https://doi.org/10.3390/foods5030051

Langdahl, B. L. (2017). Osteoporosis in premenopausal women. *Current Opinion in Rheumatology*, *29*(4), 410–415. https://doi.org/10.1097/BOR.0000000000000400

Laszkowska, M., Mahadev, S., Sundström, J., Lebwohl, B., Green, P. H. R., Michaelsson, K., & Ludvigsson, J. F. (2018). Systematic review with meta-analysis: the prevalence of coeliac disease in patients with osteoporosis. *Alimentary Pharmacology & Therapeutics*, *March*, 1–8. https://doi.org/10.1111/apt.14911

Lebwohl, B., Murray, J. A., Rubio-Tapia, A., Green, P. H. R., & Ludvigsson, J. (2014). Predictors of persistent villous atrophy in coeliac disease: A population-based study. *Alimentary Pharmacology & Therapeutics*, *39*(5), 488–495. https://doi.org/10.1111/apt.12621

Leffler, D. A., Green, P. H. R., & Fasano, A. (2015). Extraintestinal manifestations of coeliac disease. *Nature Reviews Gastroenterology and Hepatology*, *12*(10), 561–571. https://doi.org/10.1038/nrgastro.2015.131

Lerner, A., & Matthias, T. (2016). Gut-bone Cross Talks and Implications in Celiac Disease. *International Journal of Celiac Disease*, *4*(1), 19–23. https://doi.org/10.12691/ijcd-4-1-4

Malamut, G., & Cellier, C. (2015). Complications of coeliac disease. *Best Practice and Research: Clinical Gastroenterology*, *29*(3), 451–458. https://doi.org/10.1016/j.bpg.2015.05.005

Martin, D., Cooper, S. B., Tang, J. C. Y., Fraser, W. D., Sale, C., & Elliott-Sale, K. J. (2021). Bone metabolic marker concentrations across the menstrual cycle and

phases of combined oral contraceptive use. *Bone*, *145*(January), 115864. https://doi.org/10.1016/j.bone.2021.115864

Melini, V., & Melini, F. (2019). Gluten-Free Diet: Gaps and Needs for a Healthier Diet. *Nutrients*, *11*(1), 170. https://doi.org/10.3390/nu11010170

Melton, L. J., Looker, A. C., Shepherd, J. A., O'Connor, M. K., Achenbach, S. J., Riggs, B. L., & Khosla, S. (2005). Osteoporosis assessment by whole body region vs. site-specific DXA. *Osteoporosis International*, *16*(12), 1558–1564. https://doi.org/10.1007/s00198-005-1871-y

Mifflin, M. D., St Jeor, S. T., Hill, L. A., Scott, B. J., Daugherty, S. A., & Koh, Y. O. (1990). A new predictive equation for resting energy expenditure in healthy individuals. *American Journal of Clinical Nutrition, 51*(2), 241–247. https://doi.org/10.1093/ajcn/51.2.241

Mijatov, M. A. K., & Mičetić-Turk, D. (2016). Dietary intake in adult female coeliac disease patients in Slovenia. *Zdravstveno Varstvo*, *55*(2), 86–93. https://doi.org/10.1515/sjph-2016-0013

Mikocka-Walus, A., Skvarc, D., van Tilburg, M. A. L., Barreiro-de Acosta, M., Bennebroek Evertsz, F., Bernstein, C. N., Burisch, J., Ferreira, N., Gearry, R. B., Graff, L. A., Jedel, S., Mokrowiecka, A., Stengel, A., & Knowles, S. (2021). COVID-19–related personal product shortages are associated with psychological distress in people living with gastrointestinal disorders: A cross-sectional survey. *Neurogastroenterology and Motility*, *34*(3), 1–13. https://doi.org/10.1111/nmo.14198

National Health and Medical Research Council and New Zealand Ministry of Health. (2006). *Nutrient Reference Values for Australia and New Zealand including recommended dietary intakes*. National Health and Medical Research Council. Available from: https://www.nhmrc.gov.au/about-us/publications/nutrient-reference-values-australia-and-new-zealand-including-recommended-dietary-intakes

Pantaleoni, S., Luchino, M., Adriani, A., Pellicano, R., Stradella, D., Ribaldone, D.

G., Sapone, N., Isaia, G. C., Di Stefano, M., & Astegiano, M. (2014). Bone Mineral Density at Diagnosis of Celiac Disease and after 1 Year of Gluten-Free Diet. *The Scientific World Journal*, *2014*, 1–6. https://doi.org/10.1155/2014/173082

Parzanese, I., Qehajaj, D., Patrinicola, F., Aralica, M., Chiriva-Internati, M., Stifter, S., Elli, L., & Grizzi, F. (2017). Celiac disease: From pathophysiology to treatment. *World Journal of Gastrointestinal Pathophysiology*, *8*(2), 27–38. https://doi.org/10.4291/wjgp.v8.i2.27

Pham-Short, A., Donaghue, K. C., Ambler, G., Briody, J., Garnett, S., Munns, C. F., & Craig, M. E. (2019). Abnormal cortical and trabecular bone in youth with type 1 diabetes and celiac disease. *Diabetes Care*, *42*(8), 1489–1495. https://doi.org/10.2337/dc18-2376

Pinto-Sánchez, M. I., Causada-Calo, N., Bercik, P., Ford, A. C., Murray, J. A., Armstrong, D., Semrad, C., Kupfer, S. S., Alaedini, A., Moayyedi, P., Leffler, D. A., Verdú, E. F., & Green, P. (2017). Safety of Adding Oats to a Gluten-Free Diet for Patients With Celiac Disease: Systematic Review and Meta-analysis of Clinical and Observational Studies. *Gastroenterology*, *153*(2), 395-409.e3. https://doi.org/10.1053/j.gastro.2017.04.009

Potter, M. D. E., Walker, M. M., Hancock, S., Holliday, E., Brogan, G., Jones, M., McEvoy, M., Boyle, M., Talley, N. J., & Attia, J. (2018). A serological diagnosis of coeliac disease is associated with osteoporosis in older Australian adults. *Nutrients*, *10*(7), 1–12. https://doi.org/10.3390/nu10070849

Rizzoli, R., & Bonjour, J.-P. (2004). Dietary Protein and Bone Health. *Journal of Bone and Mineral Research*, *19*(4), 527–531. https://doi.org/10.1359/JBMR.040204

Sayar, S., Aykut, H., Kaya, Ö., Kürbüz, K., Ak, Ç., Gökçen, P., Bilgiç, N. M., Adalı, G., Kahraman, R., Doganay, L., & Özdil, K. (2021). Bone mineral density screening and the frequency of osteopenia/osteoporosis in Turkish adult patients with celiac disease. *Turkish Journal of Gastroenterology*, *32*(7), 600-

607. https://doi.org/10.5152/tjg.2021.20313

Schraders, K., Zatta, G., Kruger, M., Coad, J., Weber, J., Brough, L., & Thomson, J. (2019). Quantitative ultrasound and dual x-ray absorptiometry as indicators of bone mineral density in young women and nutritional factors affecting it. *Nutrients, 11*(10), 2336. https://doi.org/10.3390/nu12010030

Shenoy, S., Chawla, J. K., & Sandhu, J. S. (2014). Multisite quantitative ultrasound: It's comparison with dual energy X-ray absorptiometry in the diagnosis of osteoporosis. *Journal of Orthapedics and Allied Sciences*, *2*(2), 40–44. https://doi.org/10.4103/2319-2585.145598

Singh, P., Arora, A., Strand, T. A., Leffler, D. A., Catassi, C., Green, P. H., Kelly, C. P., Ahuja, V., & Makharia, G. K. (2018). Global Prevalence of Celiac Disease: Systematic Review and Meta-analysis. *Clinical Gastroenterology and Hepatology*, *16*(6), 823-836.e2. https://doi.org/10.1016/j.cgh.2017.06.037

Stagi, S., Cavalli, L., Iurato, C., Seminara, S., Brandi, M. L., & Martino, M. De. (2013). Bone metabolism in children and adolescents: Main characteristics of the determinants of peak bone mass. *Clinical Cases in Mineral and Bone Metabolism*, *10*(3), 172–179.

Stein, E. M., Rogers, H., Leib, A., McMahon, D. J., Young, P., Nishiyama, K., Guo, X. E., Lewis, S., Green, P. H., & Shane, E. (2015). Abnormal skeletal strength and microarchitecture in women with celiac disease. *Journal of Clinical Endocrinology and Metabolism*, *100*(6), 2347–2353. https://doi.org/10.1210/jc.2015-1392

Szulc, P., Naylor, K., Hoyle, N. R., Eastell, R., & Leary, E. T. (2017). Use of CTX-I and PINP as bone turnover markers: National Bone Health Alliance recommendations to standardize sample handling and patient preparation to reduce pre-analytical variability. *Osteoporosis International*, *28*(9), 2541–2556. https://doi.org/10.1007/s00198-017-4082-4

Szymczak, J., Bohdanowicz-Pawlak, A., Waszczuk, E., & Jakubowska, J. (2012). Low bone mineral density in adult patients with coeliac disease. *Endokrynologia*

Polska, *63*(4), 270–276.

International Society for Clinical Densitometry (ISCD). (2019). *Official Position Statement 2019, Adult*. Available from: https://iscd.org/learn/official-positions/adult-positions/

Theethira, T. G., Dennis, M., & Leffler, D. A. (2014). Nutritional consequences of celiac disease and the gluten-free diet. *Expert Review of Gastroenterology and Hepatology*, *8*(2), 123–129. https://doi.org/10.1586/17474124.2014.876360

University of Otago and Ministry of Health. (2011). *A Focus on Nutrition: Key findings of the 2008/09 New Zealand Adult Nutrition Survey*. Ministry of Health

Usai-Satta, P., Lai, M., & Oppia, F. (2022). Lactose Malabsorption and Presumed Related Disorders: A Review of Current Evidence. *Nutrients*, *14*(3), 1–7. https://doi.org/10.3390/nu14030584

Valerio, G., Spadaro, R., Iafusco, D., Lombardi, F., Del Puente, A., Esposito, A., De Terlizzi, F., Prisco, F., Troncone, R., & Franzese, A. (2008). The influence of gluten free diet on quantitative ultrasound of proximal phalanxes in children and adolescents with type 1 diabetes mellitus and celiac disease. *Bone*, *43*(2), 322–326. https://doi.org/10.1016/j.bone.2008.04.004

Vasikaran, S. D., Chubb, SA. P., Ebeling, P. R., Jenkins, N., Jones, G. R. D., Kotowicz, M. A., Morris, H. A., Schneider, H-G., Seibel, M. J., & Ward, G. (2014). Harmonised Australian Reference Intervals for Serum PINP and CTX in Adults. *Clin Biochem Rev, 35*(4), 237-242

Vici, G., Belli, L., Biondi, M., & Polzonetti, V. (2016). Gluten free diet and nutrient deficiencies: A review. *Clinical Nutrition*, *35*(6), 1236–1241. https://doi.org/10.1016/j.clnu.2016.05.002

World Health Organization. (2011). *Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity* (No. WHO/NMH/NHD/MNM/11.1). World Health Organization. Available from: https://apps.who.int/iris/bitstream/handle/10665/85839/WHO_NMH_NHD_MNM_11.1_eng.pdf

World Health Organization. (2012). Global physical activity questionnaire (GPAQ)

analysis guide. *Geneva: World Health Organization*, 1-22. Available from: https://cdn.who.int/media/docs/default-source/ncds/ncd-surveillance/gpaq-analysis-guide.pdf?sfvrsn=1e83d571_2

Wünsche, J., Lambert, C., Gola, U., & Biesalski, H. K. (2018). Consumption of gluten free products increases heavy metal intake. *NFS Journal*, *12*, 11–15. https://doi.org/10.1016/j.nfs.2018.06.001

Yeakle, L. (2021). The latest on Gluten Allergy and Sensitivity. *Food Safety Magazine*. June/July 2021. Available from: https://www.food-safety.com/articles/7194-the-latest-on-gluten-allergy-and-sensitivity

Zanchetta, M. B., Costa, F., Longobardi, V., Longarini, G., Mazure, R. M., Moreno, M. L., Vázquez, H., Silveira, F., Niveloni, S., Smecuol, E., Temprano, M. D. L. P., Hwang, H. J., González, A., Mauriño, E. C., Bogado, C., Zanchetta, J. R., & Bai, J. C. (2015). Significant bone microarchitecture impairment in premenopausal women with active celiac disease. *Bone*, *76*, 149–157. https://doi.org/10.1016 /j.bone.2015.03.005

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Katie Schraders
Name and title of main supervisor:	Professor Marlena C. Kruger
In which chapter is the manuscript/published work?	Chapter 6
What percentage of the manuscript/published work was contributed by the student?	85%

Describe the contribution that the student has made to the manuscript/published work:

Led the study design, recruited participants and collected data, processed blood samples, statistical analyses and wrote first manuscript draft and ethics protocol

Please select one of the following three options:

- ○ **The manuscript/published work is published or in press**
  Please provide the full reference of the research output:
- ○ **The manuscript is currently under review for publication**
  Please provide the name of the journal:
- ◉ **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:	Katie Schraders Digitally signed by Katie Schraders Date: 2022.08.28 18:38:13 +12'00'	Main supervisor's signature:	Marlena Kruger Digitally signed by Marlena Kruger DN: cn=Marlena Kruger, c=NZ, o=Massey University, ou=School of Health Sciences, email=m.c.kruger@massey.ac.nz Date: 2022.08.27 09:09:04 +12'00'

This form should be placed at the beginning of each relevant thesis chapter.

In addition to the assessment and comparison of bone mineral density (BMD) in individuals with coeliac disease (CD) compared with healthy controls, the *Close to the Bone* study also allowed the opportunity to investigate iodine in this population group. Iodine is a nutrient of concern in New Zealand, particularly in pre-menopausal women of child-bearing age. The primary target for promotion of iodine intake in New Zealand uses bread as a vector for iodine supplementation, a source which may not be a sufficient vector in individuals with CD due to low bread intake in this group. Although the Ministry of Health has previously identified individuals with CD as a group who may not receive the projected benefit from supplementation, there has been no research to investigate whether this population are at greater risk in New Zealand or whether the fortification practices are sufficient in these individuals.

This aspect of the study was included due to the previously mentioned alterations to the *Close the bone* study as a result of the SARS-CoV-2 pandemic.

CHAPTER 6

Close to the Bone: Thyroid function and Nutritional status

6.1 Abstract:

Introduction: Mandatory fortification levels in New Zealand are based on assumptions that New Zealanders consume enough bread, fortified with iodised salt, however recent research demonstrates that intake of bread in young women of reproductive age, who require optimal preconceptual iodine falls below this target. Premenopausal women consuming a gluten-free diet (GFD) are at additional risk as iodine deficiency is common in this group, with the diet often lacking sufficient iodine intake.

Materials and methods: This study aimed to investigate bread consumption and iodine status in premenopausal (18–40-year-old) coeliac women (n=31) compared to healthy age matched controls (n=39) from the lower North Island of New Zealand. Urinary iodine concentration (UIC) was determined from spot samples, while thyroid function was assessed through blood tests. Four-day diet diaries (4DDD) were used to estimate iodine intake (excluding iodised salt), along with food frequency questionnaires (FFQ) to assess average intakes of iodine rich foods.

Results: Median UIC was significantly lower in coeliac participants with 45 µg/L, compared with 72.0 µg/L in healthy controls (p=0.0227). Estimated iodine intake was also significantly lower in coeliac participants with 73 µg consumed per day, compared with 101 µg/day in healthy controls (p=0.0041). In addition, both groups had low intake of bread, with a median intake of 0.64 slices per day in coeliac participants and 0.86 slices per day in healthy controls (p=0.1939). No significant differences were found between markers of thyroid function.

Conclusions: Further research is needed to investigate iodine status in individuals with coeliac disease in New Zealand.

6.2 Introduction:

Iodine is an essential micronutrient required for the synthesis of thyroid hormones, thyroxine (T4) and 3,5,3′ triiodothyronine (T3), which are essential for protein synthesis, neuronal/nervous system development, growth and other metabolic processes (Zimmermann & Boelaert, 2015). In New Zealand, iodine is present in low levels in the soil, which is reflected in foods grown in New Zealand and consequently in the composition of the diet (Brough & Skeaff, 2020). Iodine deficiency, even when mild, can result in iodine deficiency disorders (IDDs), inter-related clinical abnormalities including hypertrophy of the thyroid gland which can be manifest as goitre. In pregnancy, iodine deficiency increases the risk of stillbirth, spontaneous abortion, birth defects and impaired cognitive development. As 40% of all pregnancies in New Zealand are unplanned (Morton et al., 2022), preconceptual nutrition is often not a priority, making the iodine status of women of childbearing age particularly important. To combat the rising levels of deficiency and the threat of re-emergence of goitre that became evident at the end of the 20th century, the New Zealand government introduced mandatory fortification of bread with iodised salt in 2009 (Food Standards Australia New Zealand, 2015). Since its introduction, there has been improvement in iodine status (Ministry for Primary Industries and Ministry of Health, 2016), however recent research suggests, improvements in iodine status in adults have not met predictions (Edmonds et al., 2016).

One proposed explanation is the reduction in the consumption of bread (Lockyer & Spiro, 2020), possibly as a result of the common, but unfounded, belief that bread is associated with weight gain (O'Connor, 2012). The level at which mandatory fortification was introduced was modelled on the assumption that women of childbearing age consumed on average 3-4 slices of bread per day (Food Standards Australia New Zealand, 2006). However, it has since been established that bread intake in New Zealand, particularly in premenopausal women, is declining. A 2012 study by Mallard et al. reported an average intake of 2 slices/day

in premenopausal women, which was supported by a more recent study by Edmonds et al. (2016). In addition, changes to the formulation of bread products over the past 15 years may have resulted in a decrease in the level of sodium added to bread (Wang et al., 2022) and a consequential drop in the level of iodine in these products as iodised salt is used in bread manufacture as the fortification vehicle.

People with coeliac disease (CD) were identified as a group of concern by experts during the implementation of mandatory fortification (Food Standards Australia New Zealand, 2006) due to their lower intakes of bread. However, despite this, to the author's knowledge there has been no research done to assess iodine intake in people with CD living in New Zealand. One such study investigating nutrient intake in premenopausal women in Spain, identified inadequate iodine intake in 94% of premenopausal women with CD (Churruca et al., 2015). In addition, in children with CD, iodine deficiency has shown to be a common finding at diagnosis, probably due to malabsorption because of villous atrophy. Although some improvements occurred once gluten was avoided, iodine absorption was still impaired after a year on a gluten-free diet (GFD) (Delvecchio et al., 2021).

Individuals with CD have an increased likelihood of having concurrent autoimmune thyroid disease (AITD, also known as autoimmune thyroiditis). AITD is estimated to affect up to 4-fold more people with CD than in the general population (Baharvand et al., 2020). AITD covers a spectrum of conditions from hypothyroidism, such as Hashimoto thyroiditis (HT), to hyperthyroidism, such as Graves' disease (GD). HT is characterised by the presence of antibodies to thyroid peroxidase (TPO), the enzyme involved in thyroid hormone production, and also antibodies to thyroglobulin (Tg) are raised, whereas GD is characterised by the presence of antibodies to thyroid stimulating hormone (TSH). Among the shared risk factors for CD and AITD are genetic susceptibility (HLA antigens DQ2 and *DQ8)* and environmental factors including micronutrient malnutrition (Rayman,

2019). Paradoxically, HT is more likely at both low and high iodine intakes. The self-antigen Tg is unusual as it undergoes post translational modification depending on the supply of iodine so both low and high intakes increase the likelihood of new epitopes of Tg being formed which may be recognised by the immune system. It should be noted that increased circulating antibodies to Tg and TPO are common and whilst they may not indicate current thyroid disease, it is possible that they may predict it (Effraimidis, 2019).

The interaction between sodium and gluten, which is associated with the stabilisation of the gluten structure affecting its rheological properties, means that some of the sodium in gluten-containing foods is not available to interact with sodium taste receptors (Tuhumury et al., 2014). However, a comparison of the sodium content of gluten-free (GF) and gluten-containing bread available in New Zealand from the Nutrition Information Panels indicates that the manufacturers add a similar amount of sodium, presumably as iodised salt, to both types of bread (appendix C). The cost per serving is markedly different with GF bread costing about 3 times as much per serving (appendix C). Despite the level of sodium added to bread being at a similar level, young women with CD are at risk of compromised iodine-related thyroid function because the gut damage associated with CD can adversely affect the absorption of other nutrients required for thyroid function as well as iodine. The absorption and utilisation of iodine may also be limited if there are concurrent issues with the intake or absorption of iron, zinc or selenium (O'Kane et al., 2018).

6.3 Materials and methods

6.3.1 Subjects

This was a sub-study of the *Close to the Bone* Study, with premenopausal women with CD and healthy controls recruited from the lower North Island, New Zealand. The full methodology is described in Chapter 5.

The study was approved by the Massey University Human Ethics Committee (Southern A), Reference Number SOA 18/73 and registered with the Australian New Zealand Clinical Trials Registry (ANZCTR), Trial ID ACTRN12619001542189. All participants were fully informed about the requirements of the study and provided written consent before participation.

Participants consisted of two groups of premenopausal women, those with CD and a healthy age- and BMI-matched control group. Participants attended one visit to the Human Nutrition Research Unit (HNRU) at Massey University, Palmerston North, where they had fasted blood samples collected, anthropometric measurements taken and provided a urine sample (in addition to other testing described in chapter 5 for assessment of bone parameters).

6.3.2 Dietary analysis

Full details of food record collection are described in chapter 5. For this sub-section of the study, food records were used to assess iodine intake. Although participants were asked about use of iodised salt, due to many participants being unaware of the type of salt used, iodised salt was excluded from food records for the analysis. In addition, not all commercially made bread has been assessed in New Zealand and there is limited information available regarding the iodine content of GF breads. Iodine concentrations for categories of gluten-containing bread were used from the assessment of iodine in bread sampled by the Ministry of Primary Industries (Ministry for Primary Industries, 2014). However, this assessment did not consider GF breads, so iodine concentrations for categories of GF bread were taken from the New Zealand Food Composition Tables 10th edition (New Zealand Institute of Plant and Food Research, 2013) which reports iodine content of GF bread sampled in 2012 (post mandatory fortification).

The number of servings of foods consumed which are considered good sources of iodine (bread and dairy) was also calculated from the Food Frequency Questionnaire (FFQ) provided to participants as part of the questionnaires

completed at their visit to the HNRU. Serving sizes were determined using standard definitions provided by the Ministry of Health (Ministry of Health, 2003) that were the current recommendations when the study took place. One serving of bread was one bread roll or one slice of bread, with a dairy serving consisting of a glass (250mL) of milk, a pottle of yoghurt (150g), or 2 slices of cheese (40g). Unfortunately, not all sources of fish/seafood were assessed as part of the FFQ. Although participants were asked separately about their intake of both, this only allows for assessment of frequency of intake but not quantity consumed.

6.3.3 Urine sample collection

Iodine status was determined from spot urine samples (mid-stream). Samples were stored at -20°C prior to analysis. Iodine concentration was determined by an accredited commercial laboratory (Hills Laboratories, Hamilton, New Zealand) using inductively-coupled plasma mass spectrometry (Flores et al., 2020). Quality control procedures included analysis of blanks, analytical repeats and certified reference material (CRM) to ensure accuracy and precision. Each batch of twenty-five urine samples was analysed together with an external reference standard (Seronorm Trace Elements Urine, L-2) giving a mean iodine concentration of 567 (SD 11) µg/l (published value: 565 µg/l) with a CV of 2.0 % (*n*3).

Calibration standards and checks were undertaken on every run; the limit of detection for iodine was 0.001 mg/kg. To correct for hydration status, creatinine was measured in the same samples by Massey University Nutrition Laboratory using the Jaffe Method Flexor E (Vital Scientific NV, 6956 AV Spankeren/Dieren, The Netherlands).

6.3.4 Blood parameters

Fasted venous blood samples were collected by a qualified phlebotomist using sterile flashback vacutainer needles. Serum samples were allowed to coagulate, before being centrifuged. Serum samples were used to measure

thyroglobulin (Tg) and thyroglobulin antibodies (TgAb), while plasma samples were collected in vacutainers containing lithium-heparin for the analysis of thyroid stimulating hormone (TSH), thyroxine (T4), free triiodothyronine (free T3) and anti-thyroid peroxidase antibodies (Anti-TPO).

All samples were centrifuged at 3000g for 10 minutes at 4°C within 2 hours, after which they were aliquoted and frozen before being stored at -80°C to await further processing at the end of the study. At the conclusion of the study, collected samples were couriered on dry ice to Canterbury Health Laboratories, Christchurch, New Zealand for analysis.

6.3.5 Questionnaires

Questionnaires were administered online through Qualtrics software (Qualtrics, Provo, Utah). Participant's eligibility was assessed through the screening questionnaire, which was completed at home, with any clarifications followed up via email by the research team to confirm eligibility. At the visit participants were asked to complete a questionnaire regarding their health. Questions related to this sub-study included a FFQ about iodine sources, types of dairy products consumed (or plant-based alternatives and salt use).

6.3.6 Statistical analysis

Statistical analysis was carried out using Statistical Analysis Software (SAS) (Version 9.4) (SAS Institute Inc., Cary, NC, USA). The Shapiro Wilkes test was used to test for normal distribution, with normally distributed results reported as mean ± standard deviation (SD) and non-parametric data presented as median with 25^{th} and 75^{th} percentile. Comparisons between the two participant groups were carried out using the Tukey-Kramer Method and associations between groups were assessed using Fisher's Exact Test. Statistical significance was set at $p<0.05$.

6.4 Results:

A total of 70 premenopausal women were recruited for the original study, consisting of 31 coeliac women and 39 age and BMI matched healthy controls. All 80 women took part in aspects of this sub-study. Participant characteristics for this group are described in Table 6.1 (note this table is a duplicate of table 5.1 from chapter 5 but is repeated here for ease of reading).

Table 6.1 Participant characteristics of premenopausal women with CD (n=31) compared to healthy control group (n=39)

	Coeliac Group n = 31	**Healthy Control Group n = 39**	***p* value**
Age (years)	27.5 (24.0, 36.8)	25.0 (23.8, 31.5)	0.6259
Height	167.3 ± 6.5 (156.6 – 181.1)	168.1 ± 6.7 (154.1 – 182.0)	0.6092
Weight	67.5 ± 13.9 (38.0 – 102.4)	66.3 ± 8.4 (45.3 – 88.6)	0.6575
BMI	24.2 ± 5.2 (15.4 – 37.5)	23.5 ± 2.8 (17.7 – 29.9)	0.4714
Body Fat %	37.7 ± 6.8 (23.5 – 50.5)	35.8 ± 4.9 (27.1 – 46.6)	0.1829

BMI, body mass index. Characteristics presented are from anthropometric measures taken at participants visit (not those that were self-reported). Data presented as mean ± standard deviation (Range). Non-parametric data presented as median (25th percentile, 75th percentile).

One coeliac participant was unable to provide sufficient urine for analysis of iodine. In addition, one coeliac participant's urinary iodine results were excluded from analysis due to unexplained extremely high iodine excretion (2300 µg/L); the participant did not report use of iodine supplementation indicating a potentially inaccurate reading.

Table 6.2 Comparison of biomarkers between premenopausal women with coeliac disease and healthy controls

	Coeliac Group	Healthy Control Group	*p* value	Reference Value
	n=31	n=39		
Haemoglobin (g/L)	131.0 ± 10.9† (117.0 - 155.0)	130.4 ± 10.3† (102.0 - 156.0)	0.7860	115-155 g/L[1]
	n=29	n=39		
UIC (µg/L)	45.0 (28.0, 76.0)	72.0 (32.5, 95.0)	0.0227*	
Urinary Creatinine (g/L)	0.44 (0.26, 1.02)	0.72 (0.40, 1.14)	0.3949	
Iodine: creatinine (µg/g)	67.0 (54.3, 86.2)	79.6 (59.7, 127.8)	0.0337*	
Estimated 24-hour UIE (µg/day)	85.1 (61.3, 106.1)	101.3 (72.8, 140.3)	0.0240*	
	n=28	n=38		
Anti-TPO (IU/mL)	1.0 (0.0, 1.0)	1.0 (1.0, 2.0)	0.3953	<10
Free T4 (pmol/L)	13.0 (12.0, 14.0)	13.0 (12.0, 13.0)	0.2293	8-20
Free T3 (pmol/L)	4.40 (4.05, 4.90)	4.35 (4.20, 4.80)	0.7270	3.3-6.8
TSH (mIU/L)	1.28 (1.06, 1.78)	1.40 (1.02, 2.13)	0.4980	0.4-5.3
	n=27	n=38		
Tg (µg/L)	14.5 (9.7, 24.5)	13.2 (9.4, 23.7)	0.4317	<0.1-58
TgAb (IU/mL)	23.5 (22.0, 35.3)	23.0 (22.3, 26.0)	0.9479	
	n=28	n=38		<25 mod-severe deficiency[2]
Vitamin D nmol/L	75.3 ± 22.5† (46.0 - 130.0)	80.2 ± 25.4† (38.0 - 143.0)	0.4976	25-50 mild deficiency[2] 50-150 optimal[2]

Normally distributed data presented as mean ± standard deviation † (Range). Non-parametric data presented as median (25th percentile, 75th percentile). UIC, Urinary Iodine Concentration; UIE, Urinary Iodine Excretion; Anti-TPO, anti-thyroid peroxidase antibodies; free T4, thyroxine; free t3,

free triiodothyronine; TSH, thyroid stimulating hormone; Tg, thyroglobulin; TgAb, thyroglobulin antibodies. UIE is estimated for using 24 creatinine estimates by Mage et al. (2008).

The WHO guidelines (as above) advise that a population has sufficient iodine if the median UIC in school-aged children is >100 μg/L and if not more than 20% has a UIC less than 50 μg/L, (World Health Organization, 2007). In the current study the median UIC for the coeliac participants and healthy controls was 45.0 μg/L and 72.0 μg/L respectively; 55.2% percent of coeliac participants and 35.9% healthy controls had a UIC below 50 μg/L, indicating mild iodine deficiency. Using the WHO cut-offs would suggest both groups in the current study have an inadequate intake.

No relationship was found between iodine to creatinine ratio and bone mineral density (BMD) (reported in chapter 5) at the forearm (p=0.7398), total hip (p=0.1520), neck (p=0.5425) or lumbar spine (p=0.5425).

In addition, no relationship was found between iodine to creatinine ratio and anti-TPO (p=0.5538), T4 (p=0.5093), free T3 (p=0.8349), TSH (p=0.3183), Tg (p=0.4090) or TgAb (p=0.4951). No relationship was found between iodine to creatinine ratio and haemoglobin (p=0.3598) or vitamin D (p=0.6127).

Use of iodised salt was reported in 13 (43.3%) of CD participants compared with 25 (64.1%) of healthy controls. The remaining participants either did not know whether the salt they used was iodised (3 (10%) coeliac participants), did not use iodised salt (3 (10%) coeliac participants; 4 (10.3%) healthy controls) or did not provide this information (11 (36.7%) coeliac participants; 10 (25.6%) healthy controls). As such, all estimates in table 6.3 have iodised salt excluded as it is difficult to determine which participants were using this.

Table 6.3 Estimated iodine intake based on dietary assessment

	Coeliac Group n=26	Healthy Control Group n=32	*p* value
Estimated intake with fortified bakery products excluded; µg/day	67 (49, 89)	68 (54, 88)	0.7564
Estimated intake based on concentration of Iodine reported in NZ breads; µg/day	73[1] (53, 90)	101[2] (77, 115)	0.0041*

All estimates have iodised salt excluded. Estimates are calculated from diet diaries. [1] New Zealand Institute of Plant and Food Research, 2013, [2] Ministry for Primary Industries, 2014

No significant difference was found in intake of iodine from non-fortified sources (i.e., with exclusion of iodised salt and bread products) (p=0.7564). However, inclusion of bread products fortified to the same level reported in previous research for gluten-containing (Ministry for Primary Industries, 2014) and GF breads (New Zealand Institute of Plant and Food Research, 2013) resulted in a significant difference in iodine intake between groups (p=0.0041).

In comparison, a theoretical increase in the iodine content of GF bread to the same level found in gluten-containing breads (per 100g) (Ministry for Primary Industries, 2014) would result in a significant increase in iodine intake for coeliac participants from 73 µg/day to 91 µg/day (p=0.0001). Although this increase in fortification would not increase iodine intake to the same level found in gluten-containing breads, intake between groups would not be significantly different (p=0.4651).

Twenty-seven coeliac participants and 33 healthy controls provided complete food records. After assessment for validity of reporting using the Goldberg cut-offs, dietary data from one coeliac participant and one healthy control were excluded due to being outside (under and over respectively) the 95% confidence interval (1.00-2.40) for the ratio.

No correlation was found between the Iodine to Creatinine ratio and estimated intake with fortified bakery products excluded (p=0.7170) or estimated intake based on GF bread (p=0.4728).

Six participants reported consuming no bread, of which 4 had CD. In addition, 17 participants with CD (53.7%) and 20 healthy controls (54.1%) reported consuming less than 1 slice of bread per day. The median intake of bread reported per day was 0.64 slices (0.29, 2.00) for coeliac participants and 0.86 slices (0.29, 2.00) for healthy controls (p=0.1939).

Twenty-seven CD participants (90.0%) reported consuming fish compared with 29 healthy controls (78.4%) (p=0.3212). However, only 10 coeliac participants (33.3%) and 14 healthy controls (37.8%) reported consuming fish at least once per week. In addition, 14 coeliac participants (46.7%) and 18 healthy controls (48.6%) reported consuming other seafoods/shellfish (p=0.5897), however, all participants consuming seafood/shellfish reported consuming it less than once per week.

Eighteen CD participants reported consuming dairy-based milk (60.0%), of which 3 consumed lactose-free dairy milk products (10.0%). In comparison, 24 healthy controls reported consuming dairy-based milk products (64.9%), with no consumption of lactose free dairy products. Of the remaining participants, 8 coeliacs (26.7%) and 10 healthy controls (27.0%) consumed plant-based milks, while 4 coeliacs (13.3%) and 3 healthy controls (8.1%) consumed no milk products. No association was found between reported consumption of milk products and disease status (p=0.1975).

Three coeliac participants and three healthy controls had been medically diagnosed with lactose intolerance. Of these participants 4/6 reported consuming plant-based milks while two reported not consuming any type of milk products.

Median intake of dairy milk products reported through FFQ appeared lower in CD participants at 1.56 servings/day (0.98, 2.14) compared with healthy controls 1.93 servings/day (1.37, 2.68). However, these differences were not found to be

statistically significant (p=0.0603), and no relationship was found between intake of dairy milk servings and iodine to creatine ratio (p=0.0853).

Assessment of the relationship between all milk products (inclusion of plant-based consumers) and iodine to creatine ratio also showed no correlation (p=0.0705). No differences in all milk product servings (including plant-based consumers) were found between the two groups (p=0.9520).

No statistically significant difference in iodine to creatinine ratio was found between dairy milk product consumers and those who consumed non-dairy products (p=0.4215).

6.5 Discussion:

The median UIC in the coeliac women (45 µg/L) was significantly lower than that found in the healthy age-matched control population in the current study (72 µg/L) (p=0.0227). It was also similar to the results of the 2008/9 New Zealand Health Survey (NZHS), which reported a median UIC of 48 µg/L for women of childbearing age (16-44 years) collected prior to the introduction of mandatory fortification of bread (Ministry for Primary Industries and Ministry of Health, 2016). The NZHS report also indicated significant improvements in iodine status post-fortification with a median UIC of 104 µg/L for this same population (16–44-year-old women) in 2014/2015, suggesting adequate intake. The findings from this most recent NZHS report also fit with the Iodine Global Network (IGN) Global Scorecard which defines New Zealand as having an adequate intake of iodine, with a reported median intake of 116 µg/L in school age children (Iodine Global Network, 2021). However, it should be noted that the median intake reported by the IGN Global Iodine Scorecard is based on a singular study assessing iodine intake in New Zealand children (Jones et al., 2016) and therefore has no bearing on the iodine status of childbearing women; a concern which has been raised previously (Brough et al., 2016).

Comparison of the current findings with a more relevant population of premenopausal women in the same region (post-mandatory fortification) indicates a higher median UIC in healthy controls (72 μg/L), with the study by Shukri et al. (2014) reporting a median UIC of only 65 μg/L. However, although lower than the findings of the healthy control group in the current study, this previous research still found a greater UIC than seen in our coeliac participants.

Iodine status for both groups was lower than the WHO definition of sufficiency in school aged children which advocated a median UIC of >100μg/L with no more than 20% of samples <50μg/L (World Health Organization, 2007). In both groups in the current study the median fell below this, with 55.2% of coeliacs and 35.9% of healthy controls having a median UIC below 50 μg/L, which would indicate iodine deficiency.

The small numbers in this study mean that application of the WHO cut-offs cannot conclusively define the iodine status of either of the 2 groups. Furthermore, there is some debate about the UIC cut-offs for defining mild, moderate and severe deficiency within the population being assessed. In addition, the WHO cut-off of 100 μg/L was based on goitre affecting less than 10% of the population when urinary iodine excretion (UIE) was greater than 100 μg/day in school aged children School aged children typically produce 1L of urine per day and hence UIC and UIE are interchangeable. However, UIC and UIE are not interchangeable in adults as they produce and excrete a larger urine volume, with UIC only representing approximately 60-65% of iodine excreted in a 24-hour period. As such Zimmermann and Andersson (2012), advocate that for adults a UIC cut-off of about 60-70 μg/L would be more appropriate. When considering this lower cut-off iodine status is still inadequate for the CD participants in the current study, but adequate for the healthy controls. These findings suggest that despite mandatory fortification of bread, women with CD are still iodine deficient.

There has been no previous research to assess iodine status in adults with CD. However, a study by Finlayson et al. (2019), in low bread consuming (<1

slice/day) women aged 40-63 years in New Zealand reported UIC similar to that found in the CD group in the current study with a median UIC of 49 μg/L (35, 78). In addition, a pilot study by Delvecchio et al. (2021) in Italy assessed median UIC among children with CD, however this is not possible to compare with the current study as it was done in children. The authors of this Italian study reported a lower median UIC in coeliac children (median age of 8.5 years) of 89 μg/L after one year on a strict GFD compared with the median found in a larger Italian study (125 μg/L) of 11–13-year-old school children from the general population (Delvecchio et al., 2021). The authors highlight the differences in age between the two population groups, however, it still indicates a trend toward a lower iodine intake in children with CD compared with the general population.

In the present study after correction for hydration, the iodine to creatinine ratio still demonstrated a significant difference between the groups (p=0.0337), demonstrating that the difference found was not related to hydration status.

Although in this current study no relationship was found between iodine status and BMD, the low iodine status is still of concern as this group of the women in the current study are premenopausal and thus of reproductive age. It is estimated that approximately 40% of all pregnancies in New Zealand are unplanned (so preconception iodine supplementation is unlikely) (Morton et al., 2022) and only just over half of pregnant women (52%) report taking iodine supplements during pregnancy (Reynolds & Skeaff, 2018). The implications for fetal development if these findings are representative of the population, particularly those with CD, could be substantial.

The median bread intake for both groups in the current study was low, with a median intake of less than one slice of bread consumed each day (0.64 and 0.86 slices respectively; p 0.1939). This is considerably lower than the modelled bread intake used to establish the fortification requirements for New Zealanders (3-4 slices per day for women) (Food Standards Australia New Zealand, 2006). In addition, the intake of bread in healthy control participants in this study is lower

than reported in previous research, with Shukri et al. (2014) reporting an average intake of 2.5 slices per day. However, similar low bread consumption was reported in the *A Gut Feeling* study performed by our group, where 538 adult participants with CD reported a median intake of 0.57 slices (0.29, 2.00) per day (Chapter 7). The median intake reported in CD participants in chapter 7 was similar suggesting that the intake reported in the current study may be representative of the general coeliac population.

Comparison of fortification practices with Australia, suggest that changes to the New Zealand fortification level to bring this in-line with Australia may result in significant improvements in iodine status of premenopausal women, including those with CD. Although Australia and New Zealand had similar pre-fortification mean iodine content of bread of <2 µg/100g, this level had increased to a greater extent in Australian bread with recent publication by the Australian Institute of Health and Welfare (2016) reporting a mean of 53-70 µg/100g post-fortification, resulting in only 9% of women aged 16-44 estimated to have intakes below EAR. In comparison, the same report indicated that post-fortification New Zealand has a mean iodine in bread of 28-49 µg/100g, with an estimated 39% of 18–44-year-old women consuming less than EAR (Australian Institute of Health and Welfare, 2016).

Consideration of iodine in the GFD is harder to assess, as many of the brands of GF bread available on the market and consumed by participants in this study have not undergone recent independent assessment of nutrient composition. However, Plant and Food Research assessed the iodine content of some existing GF breads in 2012, after the implementation of mandatory fortification of breads in New Zealand; these are the values used to estimate intake in table 6.3 (New Zealand Institute of Plant and Food Research, 2013). Samples for this assessment were collected from Palmerston North and Lower Hutt, the same locations as participants from the current study. With the exception of Vogel's 6 seed bread (31 µg/100g) and Burgen Ancient Grain and Seeds (7 µg/100g), all

samples of GF bread assessed were found to contain only traces of iodine (New Zealand Institute of Plant and Food Research, 2013). These samples were collected in 2012 and may not be representative of the current fortification practices for GF bread; there are insufficient recent data to assess whether the situation has changed over time. In comparison, a recent monitoring report assessing 16 GF bread products in Australia found a mean iodine of 125 µg/100g GF bread (Food Standards Australia & New Zealand, 2016). Even with consumption consistent with that found in this study (<1 slice/day), fortification at the level reported in this Australian audit would have a significant impact on iodine status in GF bread consumers.

As no significant difference was found in the bread intake of women with CD compared with healthy controls, it suggests that increases in fortification practices to bring GF products to the level of fortification found in non-GF products reported in New Zealand are likely to result in substantial improvements in the iodine status in coeliac women (table 6.3) (p=0.0001). Consideration must also be given to the discrepancies in size between a slice of gluten-containing bread and its GF counterpart. GF bread is often significantly smaller and therefore lighter than a normal slice of regular gluten-containing breads (Horstmann et al., 2017). Regardless of the lack of difference between the number of slices consumed, as iodine concentration is calculated per 100g, the variation in slice size would result in a difference in intake of iodine from bread even if GF breads were fortified to the same level per 100g.

Dairy foods have been demonstrated to be another major contributor to iodine intake in the New Zealand diet, contributing approximately 21% of iodine intake in New Zealand adults (post-fortification) (Ministry for Primary Industries, 2014). In comparison, in the study by Shukri et al. (2014) dairy products were found to be the main contributor of iodine in the diet in premenopausal women. However, the findings of our study did not demonstrate a correlation between consumption of dairy milk products and iodine corrected for hydration (p=0.0603).

In addition, no significant difference was found in iodine corrected for hydration between consumers of dairy milk products and those participants consuming plant-based alternatives (p=0.4215). This contradicts previous research with a recent review reporting plant-based diets result in inadequate intakes of iodine (Neufingerl & Eilander, 2022). In addition, a recent cross-sectional study assessing the quality of the vegan diet found a significantly lower UIC in vegan participants when compared with healthy controls (p<0.0001) (Menzel et al., 2021). The only published research to compare iodine intake in vegetarians and omnivores, in New Zealand, found no significant difference in iodine intake between groups (Ong, 2019). However, as this research was limited to adolescent females, did not consider UIC (relying on results from dietary data alone), and did not distinguish between vegan and vegetarian participants (the latter commonly consume dairy products), it is not possible to draw conclusions about how exclusion of dairy products influences iodine status in New Zealand. It must also be noted that the Ministry of Health updated the recommendations of serving sizes in 2020, after the study had started (Ministry of Health, 2020). As such the serving sizes reported by participants for yoghurt are less than the current recommendations; 150g vs. 200g. However, as many of the participants had already completed their FFQs at the time of this update, the research was not updated to reflect this change.

It is possible that the differences in iodine identified in the current study between individuals with CD and healthy controls fit with previous reports in children with CD, that iodine absorption is impaired even after resolution of gut damage once a GF diet is adopted (Delvecchio et al., 2021). Assessment of CD-related antibodies (reported in chapter 5) indicate good adherence to the GFD and there is no indication of obvious ongoing intestinal damage. Haemoglobin was also assessed as an additional marker of malabsorption because some individuals with CD have ongoing malabsorption of iron (Stefanelli et al., 2020), but no statistically significant differences were found between participant groups (p=0.7860). However, as iodine is not absorbed by the same transporter as iron, lack of

difference in haemoglobin between groups in the current study has no implications for iodine absorption as it suggests gut recovery. As proposed by Delvecchio (2021), iodine malabsorption in people with CD may still offer a potential explanation for the differences in urinary iodine concentration between groups.

In addition, it is unclear from prior research assessing iodine status in CD, whether requirements for iodine are the same in this group. As mentioned previously, it is not uncommon for inflammation to continue even with strict adherence to a GFD (Stamnaes et al., 2021). As iodine has roles in the immune system and has been demonstrated to have anti-inflammatory properties (Aceves et al., 2021), it is possible that low UIC in individuals with CD are not simply a result of inadequate intake or absorption but may perhaps be due to differences in utilisation and therefore a greater requirement in this group.

Further research is required to assess both iodine absorption and utilisation in individuals with CD and whether these findings of malabsorption can be translated to adults, particularly women of childbearing age.

In addition, the reduction in the consumption of bread should be considered in terms of the level of iodine required to be added in the fortification process as it is possible that it is no longer having the desired impact on iodine status. However, as autoimmune thyroiditis is more commonly seen because of excess iodine, particularly in those who are genetically susceptible such as individuals with CD (Rayman, 2019), a cautious approach should be taken with increasing iodine fortification of GF products. It is suggested that sudden changes to iodine intake may contribute to the onset of autoimmune thyroiditis, indicating a gradual increase in fortification levels may be the most appropriate approach in these products (Farebrother et al., 2019).

The low consumption of bread reported in this study may also have repercussions for the planned introduction of mandatory fortification of folic acid to bread. Although findings from the *Close to the Bone* study (reported in

Chapter 5) suggest that folate levels do not differ between women with CD and healthy controls, this is another nutrient of concern in women of childbearing age including those with CD (Obeid et al., 2019). This is particularly important in New Zealand where until July 2022 all government funded folic acid supplements for pregnant women contained gluten and were therefore unavailable to the coeliac population (Coeliac New Zealand, 2022). Although the introduction of mandatory fortification of folic acid to wheat flour used for bread manufacturing started in mid-2021 and all manufacturers are required to comply by mid-2023 (Ministry for Primary Industries, 2021a), little consideration has been given to people with CD or who choose to consume a GFD; no mandatory fortification of these products is required (Ministry for Primary Industries, 2021b). The Ministry of Primary Industries have advised that manufacturers of GF bread can continue voluntary addition of folic acid and that women on a GFD should be encouraged to follow guidance regarding folic acid use for pregnancy (Ministry for Primary Industries, 2021b). However, the current recommendation from the Ministry of Health is to consume folic acid supplements for at least 4 weeks prior to conception (Ministry of Health, 2006), a recommendation which is difficult to follow in unplanned pregnancies (Morton et al., 2022). In addition, the Growing Up in New Zealand study reported that only 29.6% of pregnant women used folic acid supplements during their pregnancy (Teixeira et al., 2018), suggesting that promotion of folic acid supplements (even once GF alternatives are readily available) is an inadequate approach to protect against neural tube defects in coeliac women. It must also be mentioned, that despite the introduction of voluntary folic acid fortification of bread in 2009, in 2022 we were unable to identify any GF bread products with folic acid added, suggesting that continuation of voluntary fortification will do very little in the way of increasing intake for those on a GFD. The Ministry of Primary Industries agreed that people with CD should not be at a disadvantage (Ministry for Primary Industries, 2021b) however, it is clear that further research is needed as

the current situation promotes health inequities between coeliac women and the general population.

One major limitation of this study is the use of single spot urine samples. It has previously been indicated that there is significant intra-subject variation in UIC (Samidurai et al., 2015), so it is suggested that 10 spot samples should be collected to accurately account for variation in women (König et al., 2011). As such, further research is required in a larger cohort, with multiple samples collected to reduce risk of intra-subject variation and confirm the disparity in iodine status between coeliac women and the general public.

There are also limitations in the assessment of iodine intake. Iodine intake is notoriously difficult to assess, but in New Zealand where both iodised and non-iodised table salt are readily available, there are additional challenges. In addition, assessment of discretionary use of salt is difficult to precisely quantify because consumers are often unable to assess or report what type of salt they purchase or how much salt they consume (McLean, 2014).

Finally, as mentioned in chapter 5, participation in this study began just prior to the start of the SARS-CoV-2 pandemic. During the time of participation there were a number of challenges in finding specialty products including GF food products (Mikocka-Walus et al., 2021), in addition to shortages in wheat bread and flour. Although participants were asked not to complete their food records during lockdown periods, it is not possible to rule out impacts of these shortages on dietary intake; some of the challenges regarding availability of GF products, including bread, persist two years later (Yeakle, 2021).

Further research investigating iodine knowledge in the coeliac population would also prove beneficial. Additional research by our group (Chapter 7, table 7.9) found that 91.3% of adults (aged 16+) with CD reported having never received advice about iodine-related nutrition from any source. It is possible this lack of advice may reflect difficulty interpreting the question related to a low level of understanding about iodine.

6.6 Conclusion:

This is the first study to assess iodine status in premenopausal women with CD in New Zealand. Findings from this study suggest that iodine status may be inadequate in this population. Further research is needed to assess the iodine status of the coeliac population in New Zealand and the possible malabsorption of this nutrient in this group.

6.7 References

Aceves, C., Mendieta, I., Anguiano, B., & Delgado-González, E. (2021). Molecular iodine has extrathyroidal effects as an antioxidant, differentiator, and immunomodulator. *International Journal of Molecular Sciences*, *22*(3), 1–15. https://doi.org/10.3390/ijms22031228

Australian Institute of Health and Welfare. (2016). Folic acid & iodine fortification. Cat. no. WEB 134. Canberra: AIHW. Available from: https://www.aihw.gov.au/reports/food-nutrition/folic-acid-iodine-fortifica tion

Baharvand, P., Hormozi, M., & Aaliehpour, A. (2020). Comparison of thyroid disease prevalence in patients with celiac disease and controls. *Gastroenterology and Hepatology from Bed to Bench*, *13*(1), 44–49. https://doi.org/10.22037/ghfbb.v13i1.1743

Brough, L., & Skeaff, S. (2020). Iodine status in New Zealand. In P. A. C. Richards, *Iodophor: Tamed Iodine: The Status of Iodine in Tasmania*. Gatekeeper Press

Brough, L., Thomson, B. M., & Skeaff, S. A. (2016). Revisiting the Iodine Global Network's definition of iodine status by country. *British Journal of Nutrition*, *115*(2), 374–376. https://doi.org/10.1017/S0007114515004389

Churruca, I., Miranda, J., Lasa, A., Bustamante, M., Larretxi, I., & Simon, E. (2015). Analysis of body composition and food habits of Spanish celiac women. *Nutrients*, *7*(7), 5515–5531. https://doi.org/10.3390/nu7075234

Coeliac New Zealand. (2022). *Thriving with Coeliac Disease*. Available from: https://coeliac.org.nz/thriving-with-coeliac-disease/

Delvecchio, M., Bizzoco, F., Lapolla, R., Gentile, A., Carrozza, C., Barone, M., Simonetti, S., Giordano, P., Dargenio, V. N., Cristofori, F., & Francavilla, R. (2021). Iodine absorption in celiac children: A longitudinal pilot study. *Nutrients*, *13*(3), 1–10. https://doi.org/10.3390/nu13030808

Edmonds, J. C., McLean, R. M., Williams, S. M., & Skeaff, S. A. (2016). Urinary iodine concentration of New Zealand adults improves with mandatory fortification of bread with iodised salt but not to predicted levels. *European Journal of Nutrition*, *55*(3), 1201–1212. https://doi.org/10.1007/s00394-015-0933-y

Effraimidis, G. (2019). Predictive scores in autoimmune thyroid disease: Are they useful? *European Journal of Endocrinology*, *181*(3), R119–R131. https://doi.org/10.1530/EJE-19-0234

Farebrother, J., Zimmermann, M. B., & Andersson, M. (2019). Excess iodine intake: sources, assessment, and effects on thyroid function. *Annals of the New York Academy of Sciences*, *1446*(1), 44–65. https://doi.org/10.1111/nyas.14041

Finlayson, J., Hurst, P. Von, & Brough, L. (2019). Iodine Intake and Status of Mid-Life Women in Auckland, with Low Bread Intakes. *Proceedings*, 3390. https://doi.org/10.3390/proceedings2019037015

Flores, E. M. M., Mello, P. A., Krzyzaniak, S. R., Cauduro, V. H., & Picoloto, R. S. (2020). Challenges and trends for halogen determination by inductively coupled plasma mass spectrometry: A review. *Rapid Communications in Mass Spectrometry*, *34*(S3). https://doi.org/10.1002/rcm.8727

Food Standards Australia & New Zealand. (2016). *Monitoring the Australian Population's Intake of Dietary Iodine Before and After Mandatory Fortification*. Available from: https://www.foodstandards.gov.au/publications/Documents/Iodine%20Fortification%20Monitoring%20Report.pdf

Food Standards Australia New Zealand. (2006). *Final Assessment Report: Proposal P295. Consideration of mandatory fortification with folic acid*. Available from: http://www.foodstandards.govt.nz/code/proposals/documents/FAR_P295_Folic_Acid_Fortification_ Attachs_1_6.pdf

Food Standards Australia New Zealand. (2015). *Australia New Zealand Food Standards Code: Standard 2.1.1. Cereal and cereal products.* Canberra, Australia: Australian Government. Available from: https://www.legislation.gov.au/Details/F2015L00420/Download

Horstmann, S. W., Foschia, M., & Arendt, E. K. (2017). Correlation analysis of protein quality characteristics with gluten-free bread properties. Food and Function, 8(7), 2465–2474. https://doi.org/10.1039/c7fo00415j

Iodine Global Network. (2021). Global scorecard of iodine nutrition in 2021 in the general population based on school-age children (SAC). Ottawa (Canada): Iodine Global Network. Available from: https://www.ign.org/scorecard.htm.

Jones, E., McLean, R., Davies, B., Hawkins, R., Meiklejohn, E., Ma, Z. F., & Skeaff, S. (2016). Adequate iodine status in New Zealand school children post-fortification of bread with iodised salt. *Nutrients*, *8*(5), 1–9. https://doi.org/10.3390/nu8050298

König, F., Andersson, M., Hotz, K., Aeberli, I., & Zimmermann, M. B. (2011). Ten repeat collections for urinary iodine from spot samples or 24-hour samples are needed to reliably estimate individual iodine status in women. *Journal of Nutrition*, *141*(11), 2049–2054. https://doi.org/10.3945/jn.111.144071

Lockyer, S., & Spiro, A. (2020). The role of bread in the UK diet: An update. *Nutrition Bulletin*, *45*(2), 133–164. https://doi.org/10.1111/nbu.12435

Mage, D. T., Allen, R. H., & Kodali, A. (2008). Creatinine corrections for estimating children's and adult's pesticide intake doses in equilibrium with urinary pesticide and creatinine concentrations. *Journal of Exposure Science and Environmental Epidemiology*, *18*(4), 360–368. https://doi.org/10.1038/sj.jes.7500614

McLean, R. M. (2014). Measuring population sodium intake: A review of methods. *Nutrients*, *6*(11), 4651–4662. https://doi.org/10.3390/nu6114651

Menzel, J., Abraham, K., Stangl, G. I., Ueland, P. M., Obeid, R., Schulze, M. B., Herter-Aeberli, I., Schwerdtle, T., & Weikert, C. (2021). Vegan diet and bone

health—results from the cross-sectional rbvd study. *Nutrients*, *13*(2), 1–16. https://doi.org/10.3390/nu13020685

Mikocka-Walus, A., Skvarc, D., van Tilburg, M. A. L., Barreiro-de Acosta, M., Bennebroek Evertsz, F., Bernstein, C. N., Burisch, J., Ferreira, N., Gearry, R. B., Graff, L. A., Jedel, S., Mokrowiecka, A., Stengel, A., & Knowles, S. (2021). COVID-19–related personal product shortages are associated with psychological distress in people living with gastrointestinal disorders: A cross-sectional survey. *Neurogastroenterology and Motility*, *34*(3), 1–13. https://doi.org/10.1111/nmo.14198

Ministry for Primary Industries (MPI). (2014). *Update report on the dietary iodine intake of New Zealand children following fortification of bread with iodine*. MPI Technical Paper No: 2014/21 Ministry for Primary Industries.

Ministry for Primary Industries (MPI). (2021a). *Folate and the addition of folic acid to food*. Available from: https://www.mpi.govt.nz/food-safety-home/nutrients-added-food/folate-and-the-addition-of-folic-acid-to-food/#:~:text=The%20Government%20has%20decided%20that,except%20for%20some%20exported%20products.

Ministry for Primary Industries (MPI). (2021b). *Folic acid fortification: Summary and analysis of submissions on New Zealand Food Safety Discussion Paper No: 2019/08*. Ministry for Primary Industries Paper No. 2021/03. (Issue ISSN No: 2253-394X). Available from: https://www.mpi.govt.nz/dmsdocument/45838-Folic-acid-fortification-Summary-and-analysis-of-submissions-on-New-Zealand-Food-Safety-Discussion-Paper

Ministry for Primary Industries and Ministry of Health. (2016). *Mandatory iodine fortification in New Zealand: supplement to the Australian Institute of Health and Welfare 2016 report - Monitoring the health impacts of mandatory folic acid and iodine fortification*. Ministry for Primary Industries Technical Paper No: 2016/32. Available from: https://www.mpi.govt.nz/dmsdocument/12786/sitemap

Ministry of Health. (2003). *Food and Nutrition Guidelines for Healthy Adults: A background paper*. Wellington. Ministry of Health. Available from: https://www.nzihf.co.nz/media-resources-1/articles/personal%20training-nutrition-guidelines-adults

Ministry of Health. (2006). *Food and Nutrition Guidelines for Healthy Pregnant and Breastfeeding Women: A background paper*. Available from: https://www.health.govt.nz/publication/food-and-nutrition-guidelines-healthy-pregnant-and-breastfeeding-women-background-paper

Morton, S. M. B., Napier, C., Morar, M., Waldie, K., Peterson, E., Atatoa Carr, P., Meissel, K., Paine, S. J., Grant, C. C., Bullen, P., Fenaughty, J., Bird, A., Underwood, L., Wall, C., Exeter, D., Prickett, K., Kingi, T. K., Liang, R., Fa'alili-Fidow, J., … Cha, J. (2022). Mind the gap–unequal from the start: evidence from the early years of the Growing Up in New Zealand longitudinal study. *Journal of the Royal Society of New Zealand*, 1–21. https://doi.org/10.1080/03036758.2022.2058026

Neufingerl, N., & Eilander, A. (2022). Nutrient intake and status in adults consuming plant-based diets compared to meat-eaters: A systematic review. *Nutrients*, *14*(1). https://doi.org/10.3390/nu14010029

New Zealand Institute of Plant and Food Research. (2013). *The Concise New Zealand Food Composition Tables. 10th edition.* Palmerston North: New Zealand Institute of Plant and Food Research and The New Zealand Ministry of Health. Available from: www.foodcomposition.co.nz

O'Connor, Á. (2012). Bread consumption in the UK: What are the main attitudinal factors affecting current intake and its place in a healthy diet? *Nutrition Bulletin*, *37*(4), 368–379. https://doi.org/10.1111/j.1467-3010.2012.01989.x

O'Kane, S. M., Mulhern, M. S., Pourshahidi, L. K., Strain, J. J., & Yeates, A. J. (2018). Micronutrients, iodine status and concentrations of thyroid hormones: A systematic review. *Nutrition Reviews*, *76*(6), 418–431. https://doi.org/10.1093/nutrit/nuy008

Ong, Y. S. (2019). *Iodine intakes of vegetarian and non-vegetarian adolescent females*. (Thesis, Master of Dietetics). University of Otago. Available from: http://hdl.handle.net/10523/9980

Rayman, M. P. (2019). Multiple nutritional factors and thyroid disease, with particular reference to autoimmune thyroid disease. *Proceedings of the Nutrition Society*, *78*, 34–44. https://doi.org/10.1017/S0029665118001192

Reynolds, A. N., & Skeaff, S. A. (2018). Maternal adherence with recommendations for folic acid and iodine supplements: A cross-sectional survey. *Australian and New Zealand Journal of Obstetrics and Gynaecology*, *58*(1), 125–127. https://doi.org/10.1111/ajo.12719

Samidurai, A. J., Ware, R. S., & Davies, P. S. W. (2015). Advantages of collecting multiple urinary iodine concentrations when assessing iodine status of a population. *Acta Paediatrica, International Journal of Paediatrics*, *104*(11), e524–e529. https://doi.org/10.1111/apa.13168

Shukri, N. H., Coad, J., Weber, J., Jin, Y., & Brough, L. (2014). *Iodine and Selenium Intake in a Sample of Women of Childbearing Age in Palmerston North , New Zealand after Mandatory Fortification of Bread with Iodised Salt*. *2014*(February), 382–389.

Stamnaes, J., Stray, D., Stensland, M., Sarna, V. K., Nyman, T. A., Lundin, K. E. A., & Sollid, L. M. (2021). In Well-Treated Celiac Patients Low-Level Mucosal Inflammation Predicts Response to 14-day Gluten Challenge. *Advanced Science*, *8*(4), 1–7. https://doi.org/10.1002/advs.202003526

Stefanelli, G., Viscido, A., Longo, S., Magistroni, M., & Latella, G. (2020). Persistent iron deficiency anemia in patients with celiac disease despite a gluten-free diet. *Nutrients*, *12*(8), 1–19. https://doi.org/10.3390/nu12082176

Teixeira, J. A., Castro, T. G., Wall, C. R., Marchioni, D. M., Berry, S., Morton, S. M. B., & Grant, C. C. (2018). Determinants of folic acid supplement use outside national recommendations for pregnant women: Results from the Growing Up in New Zealand cohort study. *Public Health Nutrition*, *21*(12), 2183–2192.

https://doi.org/10.1017/S1368980018000836

Tuhumury, H. C. D., Small, D. M., & Day, L. (2014). The effect of sodium chloride on gluten network formation and rheology. *Journal of Cereal Science*, *60*(1), 229–237. https://doi.org/10.1016/j.jcs.2014.03.004

Wang, N. X., Skeaff, S., Cameron, C., Fleming, E., & McLean, R. M. (2022). Sodium in the New Zealand diet: proposed voluntary food reformulation targets will not meet the WHO goal of a 30% reduction in total sodium intake. *European Journal of Nutrition*, *0123456789*. https://doi.org/10.1007/s00394-022-02864-5

World Health Organization. (2007). *Assessment of iodine deficiency disorders and monitoring their elimination: a guide for programme managers* (3rd ed.) World Health Organization. Available from: https://apps/who.int/iris/handle/10665/43781

Yeakle, L. (2021). The latest on Gluten Allergy and Sensitivity. *Food Safety Magazine*. June/July 2021. Available from: https://www.food-safety.com/articles/7194-the-latest-on-gluten-allergy-and-sensitivity

Zimmermann, M. B., & Andersson, M. (2012). Assessment of iodine nutrition in populations: Past, present, and future. *Nutrition Reviews*, *70*(10), 553–570. https://doi.org/10.1111/j.1753-4887.2012.00528.x

Zimmermann, M. B., & Boelaert, K. (2015). Iodine deficiency and thyroid disorders. *The Lancet Diabetes and Endocrinology*, *3*(4), 286–295. https://doi.org/10.1016/S2213-8587(14)70225-6

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Katie Schraders
Name and title of main supervisor:	Professor Marlena C. Kruger
In which chapter is the manuscript/published work?	Chapter 7
What percentage of the manuscript/published work was contributed by the student?	90%

Describe the contribution that the student has made to the manuscript/published work:

Led the study design, recruited participants and collected data, statistical analyses and wrote first manuscript draft and ethics protocol

Please select one of the following three options:

- ○ **The manuscript/published work is published or in press**
  Please provide the full reference of the research output:
- ○ **The manuscript is currently under review for publication**
  Please provide the name of the journal:
- ◉ **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:	Katie Schraders Digitally signed by Katie Schraders Date: 2022.08.28 18:38:50 +12'00'	Main supervisor's signature:	Marlena Kruger Digitally signed by Marlena Kruger DN: cn=Marlena Kruger, c=NZ, o=Massey University, ou=School of Health Sciences, email=m.c.kruger@massey.ac.nz Date: 2022.08.27 09:09:30 +12'00'

This form should be placed at the beginning of each relevant thesis chapter.

A final study was added as a result of the SARS-CoV-2 pandemic and concerns regarding what aspects of the existing research would go ahead. The *A Gut Feeling* study was introduced to allow for a "covid-resilient" study, where data would be collected entirely through an online questionnaire should lockdowns and a pause of in-person research continue. The study aimed to incorporate aspects of the *Close to the Bone* study including questions about bone and iodine, while also assessing sources of advice where individuals had received information about these areas. This incorporated a broad area of topics but was designed to allow for follow-up should further online research be needed for the thesis. The study aimed to investigate these topics in anyone over the age of 16 years who consumed a gluten-free diet (GFD) regardless of the reason.

Findings from individuals consuming a GFD due to a biopsy confirmed diagnosis of coeliac disease are presented in chapter 7.

CHAPTER 7

A Gut Feeling: Nationwide Gluten-free Diet Questionnaire Study

7.1 Abstract

Introduction: Patients diagnosed with coeliac disease (CD) are required to strictly adhere to a lifelong gluten-free diet (GFD). Although perceived as a simple solution, adherence to this diet can be challenging and has been shown to be greatly improved with evidence-based advice from medical professionals including dietitians. However, recent research indicates that over a third of newly diagnosed patients with CD in New Zealand do not receive advice from a dietitian. So, this preliminary study aimed to investigate what advice individuals with CD received and where this was sourced. A particular focus was placed on iodine intake and bone density which have been previously identified as risk factors for individuals with CD.

Materials and methods: Self-selected individuals who reported consuming a GFD took part in an 88-item online questionnaire, comprising of the Biagi adherence score questions which assess adherence to a GFD, together with questions about their diagnosis, dietary intake of iodine, bone related health, a food frequency questionnaire assessing dietary behaviours and sociodemographic questions.

Results: Of the 538 participants who completed the questionnaire and reported a medical diagnosis of CD, 91.4% were female, 83.8% of participants were diagnosed after a positive biopsy and 91% had a Biagi adherence score indicating good adherence to the GFD. Beneficial advice was most commonly reported as coming from a nutritionist or dietitian (40.3%), but 14.3% of participants also reported receiving contradictory or misleading advice from this source. Over half of participants reported not having received advice about bone related health (58.3%). DXA scans did not appear to be common practice with only 29.7% participants reporting a past DXA scan, although 69.4% of these were referred due

to diagnosis of CD. Reported advice about iodine was not common with 91.3% participants indicating they had never received advice from any source about iodine. Although use of iodised salt was reported by 60.4% participants during cooking or baking, intake of bread was low, with a median intake of 0.57 slices per day, suggesting further research is needed to investigate iodine intake in this group.

Conclusions: Despite apparent good adherence to the GFD, it appears that there is some confusion about foods which are safe to eat by participants and their sources of information. Further research investigating the quality of nutritional advice and how it is interpreted is warranted.

7.2 Introduction

The prevalence of coeliac disease (CD) in New Zealand is estimated to be roughly 1.1% (Cook et al., 2004), one of the highest prevalence in the world (Burkhardt et al., 2018). With global trends showing increasing incidence, it is likely that New Zealand's high prevalence will also continue to increase (Zhu et al., 2022). Despite the high prevalence, there has been limited research into CD in New Zealand, with very little investigation into the advice provided to patients about the gluten-free diet (GFD), or comorbidities such as low bone mineral density (BMD) or inadequate iodine intake.

CD is an autoimmune disease triggered by ingestion of gluten in genetically predisposed individuals that leads to intestinal damage, inflammation and subsequent malabsorption (Hardy & Tye-Din, 2016). Strict adherence to a GFD must be maintained to ensure gut recovery and that these issues do not recur. However, adherence is difficult to maintain, with factors such as the high cost of gluten-free (GF) foods, the lack of understanding of what foods are considered safe and how to identify these foods. These issues are considered to be significant barriers to adherence for individuals with CD (Abu-Janb & Jaana, 2020; Al-Sunaid et al., 2021; Arias-Gastelum et al., 2018). Receiving advice from a nutritionist or

dietitian has been shown to improve knowledge and understanding of the GFD and interpretation of food labels, resulting in great adherence to the diet (Gładyś et al., 2021; Moore et al., 2018). However, recent research in New Zealand and Australia demonstrated that over a third of patients with CD do not see a dietitian or nutritionist when they are diagnosed (Halmos et al., 2018); it is unclear where participants are acquiring their information and the implications this may have on dietary adherence. Poor adherence to the diet is likely to result in re-emergence of inflammation and intestinal damage leading to malabsorption of nutrients.

Nutrients of concern in New Zealand include iodine, which is found in low levels in the soil resulting in inadequate levels in the food supply (Brough & Skeaff, 2020). The introduction of mandatory fortification of most breads with iodised salt in 2009 resulted in improvements in iodine status of many New Zealanders (Ministry for Primary Industries and Ministry of Health, 2016), however, these improvements are below what was predicted (Edmonds et al., 2016) and no research has investigated how these changes have affected individuals on a GFD. Assessment of iodine in bread after the introduction of mandatory fortification suggests that iodine levels may be much lower in GF breads than gluten-containing breads (New Zealand Institute of Plant and Food Research, 2013), indicating that the effect of mandatory fortification may not have had such a positive impact for those individuals consuming a GFD. In addition, recent research suggests that iodine absorption may be impaired in individuals with CD even after intestinal damage appears to be resolved (Delvecchio et al., 2021) which further exacerbates concerns about intake in this group.

In addition, malabsorption of protein, calcium, iodine and other essential minerals may compromise BMD in people with CD, with up to 75% of adults with CD estimated to have reduced BMD (osteopenia or osteoporosis) at the time of diagnosis (Bianchi & Bardella, 2008; Galli et al., 2018). Although adherence to a GFD results in significant improvements in inflammation and absorption in most individuals (Pantaleoni et al., 2014), low BMD persists in many individuals (Galli et

al., 2018). The Coeliac Society of New Zealand (Coeliac NZ), which promotes the welfare of individuals in New Zealand who have been diagnosed with CD and provides resources to health professionals, advises that all adult patients newly diagnosed with CD should have a discussion with a health professional and request a DXA scan to assess bone density (Coeliac New Zealand, n.d.-d). Despite this, research suggests that referral practices differ across New Zealand, with many practitioners not routinely referring patients for BMD assessment (Kenrick, 2018).

This preliminary study aimed to ascertain where individuals with CD source their advice and what advice they have received regarding adherence to a GFD, iodine intake and bone-related health.

7.3 Materials and methods

7.3.1 Participants

Self-selected individuals consuming a GFD completed an open online questionnaire. The questionnaire was promoted online through social media advertising and posted to social media support groups for people consuming a GFD, including those aimed specifically at people with CD.

The following inclusion criteria were used: (1) individuals living in New Zealand who consented to participate in the study, (2) over 16 years of age, and (3) self-reported to be consuming a GFD. Participants who had previously adhered to a GFD but were consuming gluten at the time of participation for medical reasons related to imminent testing requirements, were also included. Participant duplicates were automatically identified by Qualtrics software; however, participant IP address, name and email (when provided) were used to confirm identification of duplicates, with all correctly identified duplicates excluded from analysis.

7.3.2 Survey design

The 88-item questionnaire was administered online using Qualtrics software (Qualtrics, Provo, Utah) and was adapted from the questionnaire used for the *Close to the Bone* study (Chapter 5 & 6). Prior to circulation, the questionnaire was pre-tested by members of the Massey University CD Stakeholder group, including individuals habitually consuming a GFD, to ensure appropriate structure and interpretation of the questions. The final questionnaire consisted of closed, open, and multi-choice questions organised into 11 sections. As some sections of questions were dependent on previous responses, each participant answered no more than 76 questions.

Participants were initially presented with background information about the study including details of the research group, the purpose and aims of the study and a link to download the full information sheet (pdf file) (appendix D). Participants were then advised of the privacy statement and their rights should they choose to participate, followed by two questions to confirm their consent and their eligibility.

The following question determined the main reason for the participant choosing to follow a GFD; Q4 "what is your reasoning for consuming a gluten-free diet?". Depending on their response to this question, the participant answered a different set of 6 questions probing their full reasons for consuming a GFD, their symptoms and diagnosis (if applicable).

These pathways then converged, with all participants being asked to respond to the same questions about advice they had received related to adopting a GFD (up to 13 questions), the characteristics of their GFD (including assessment of their adherence to the GFD) (up to 19 questions), dietary intake of iodine (9 questions), dietary behaviours including a food frequency questionnaire (FFQ; up to 6 questions), bone related health (up to 11 questions) and sociodemographic questions (6 questions). In addition, a further 3 questions were asked about the

participant's interest in being invited to complete a follow-up questionnaire and, should they be interested, requested them to provide their contact email.

7.3.3 Assessment of adherence to a gluten-free diet

Adherence to a GFD is commonly assessed by one of three standardised validated methods (Gładyś et al., 2020) which allow comparison between studies. The Standardised Dietitian Evaluation (SDE) involves an in-depth interview, analysis of a food diary or 24-hour recall and a quiz-based assessment of abilities to identify gluten in ingredients and read food labels (Wieser et al., 2021). The test is lengthy and has to be carried out by an experienced dietitian. The Celiac Dietary Adherence Test (CDAT) is faster to administer and has 7 questions; it is used alone or in conjunction with serological assessment (Leffler et al., 2009). It is often recommended that the CDAT tool is used in conjunction with the SDE together with assessment of biological markers (Gładyś et al., 2020). The Biagi adherence score (Biagi et al., 2009) is also validated; it is simpler and can be self-administered which makes it suitable for online assessment of adherence.

As part of the question suite used in this study regarding the GFD, participants were asked to indicate their adherence to the GFD using the Biagi questionnaire with wording adapted for a New Zealand audience. The adherence assessment consisted of 4 questions regarding dietary intake of gluten (Do you eat gluten voluntarily? When you eat out, do you tell the person why you need your food to be gluten-free? Do you check labels of packaged foods for the presence of gluten? Do you only eat packaged foods with a clear label claim e.g., Labelled as gluten-free, guaranteed by the Coeliac Society or marked with a cross grain logo?) which can be used to calculate an adherence score of 0-4. Participant responses were then grouped, with scores of 3 or 4 indicating good adherence, and scores of 2 (suggesting dietary errors) and 0 or 1 (suggesting the diet is not being adhered to) indicating poor adherence. In addition to the Biagi questions, additional

questions were included about behaviour related to a GFD based on specific situations identified from social media queries on Facebook support groups.

7.3.4 Statistical and ethical issues

Questionnaire results were exported from Qualtrics to Microsoft Excel, and data were cleaned and checked for repeat respondents and errors, with only data from participants who answered some demographic questions being analysed.

The Shapiro Wilkes test was used to test for normal distribution; normally distributed results are reported as mean ± standard deviation (SD) and non-parametric data are presented as median with 25^{th} and 75^{th} percentile. Pearson's Chi-squared test or Fisher's exact test were used to examine sex differences in dietary adherence, biopsy rates, symptoms experienced, and advice received. In addition, the Tukey-Kramer method was used to assess gender differences in dietary intake reported by the food frequency questionnaire. All analyses were performed using Statistical Analysis Software (SAS) (Version 9.4) (SAS Institute Inc., Cary, NC, USA).

Qualitative data were organised through Microsoft Excel (MSO 2016 version), with the response to each open-ended question grouped by theme (Burnard et al., 2008). Quotes were selected that represent these themes and are presented in the results section.

7.4 Results

This chapter will describe the responses of individuals who reported that they adopted a GFD following a medical diagnosis of CD.

7.4.1 Participant characteristics

The questionnaire was available for participants to complete between January 2021 and April 2021. Of the 1,736 participant responses, results from 831 participants indicated they were following a GFD because they had been diagnosed with CD. Of these participants, 4 responses were excluded as they were identified

as having been a second response from a participant who had already responded to the questionnaire.

A further 256 respondents were excluded due to incomplete responses to the questionnaire (e.g., the participant provided no demographic data). In addition, a further 33 participants were excluded because they reported CD was self-diagnosed with no medical assessment (19 responses) or diagnosed solely through genetic testing (14 responses). Although some of these individuals may have CD, because 30-40% of the population carry a susceptibility gene, genetic testing is considered a test of exclusion and is not considered adequate to confirm diagnosis without further testing (Horan et al., 2018; Lebwohl et al., 2018). The results of the remaining 538 participants are reported below.

Table 7.1 Age distribution of participants

Age (years)	Total n=538	Male n=36	Female n=492	Other/Unanswered[1] n=10
16-24	78 (13.8%)	4 (11.3%)	74 (15.0%)	
25-30	82 (14.5%)	3 (8.3%)	79 (16.1%)	
31-40	134 (23.7%)	9 (25.0%)	122 (24.8%)	3 (30.0%)
41-50	109 (19.3%)	8 (22.2%)	99 (20.1%)	2 (20.0%)
51-60	78 (13.8%)	6 (16.7%)	70 (14.2%)	2 (20.0%)
61-70	35 (6.2%)	4 (11.1%)	31 (6.3%)	
71-80	9 (1.6%)	1 (2.8%)	7 (1.4%)	1 (10.0%)
81+	1 (0.2%)		1 (0.2%)	
Age not disclosed	12 (2.1%)	1 (2.8%)	9 (1.8%)	2 (10.0%)

[1] Other/Unanswered consists of 3 participants who identified themselves as non-binary and 7 participants who did not indicate their sex; to maintain anonymity of these participants these groups were combined.

A total of 538 participants responded to the questionnaire, of which 91.4% were female and 6.7% were male; a ratio of 13.7:1. Of the remaining participants, 3 (0.6%) identified themselves as non-binary and 7 (1.3%) did not respond to this question. As the number of respondents identifying as non-binary was small, for the purpose of the analysis, this group was combined with respondents who did not provide their sex to maintain some anonymity for participants in both groups (table 7.1).

The majority of participants self-identified as being New Zealand European (table 7.2) and were fairly evenly distributed throughout the country (table 7.3).

Table 7.2 Participants' self-identified ethnicity

Ethnicity	Number of Responses (%)
Māori	2 (0.4%)
New Zealand European	464 (86.2%)
New Zealand European/Māori	29 (5.4%)
New Zealand European/Other	14 (2.6%)
Other European	21 (3.9%)
Other[1]	8 (1.5%)

[1]Other included South American, Latin American, Middle Eastern, Pakistani and Korean participants

Table 7.3 Regional distribution of participants

Region	Total n=538	Male n=36	Female n=492	Other/Unanswered[1] n=10
Auckland	136 (25.3%)	9 (25.0%)	124 (25.2%)	3 (30.0%)
Bay of Plenty	31 (5.8%)		30 (6.1%)	1 (10.0%)
Canterbury	83 (15.4%)	3 (8.3%)	80 (16.3%)	
Gisborne	2 (0.4%)		2 (0.4%)	
Hawkes Bay	15 (2.8%)		15 (3.0%)	
Marlborough	5 (0.9%)	1 (2.8%)	4 (0.8%)	
Nelson-Tasman	10 (1.9%)		10 (2.0%)	
Northland	12 (2.2%)	4 (11.1%)	8 (1.6%)	
Otago	32 (5.9%)	4 (11.1%)	28 (5.7%)	
Southland	15 (2.8%)	1 (2.8%)	14 (2.8%)	
Taranaki	11 (2.0%)		11 (2.2%)	
Waikato	61 (11.3%)	4 (11.1%)	54 (11.0%)	3 (30.0%)
Wellington	79 (14.7%)	8 (22.2%)	70 (14.2%)	1 (10.0%)
West Coast	2 (0.4%)	1 (2.8%)	1 (0.2%)	
Whanganui-Manawatū	41 (7.6%)	1 (2.8%)	39 (7.9%)	1 (10.0%)
No location given	3 (0.6%)		2 (0.4%)	1 (10.0%)

[1] Other/Unanswered consists of 3 participants who identified themselves as non-binary and 7 participants who did not indicate their sex; to maintain anonymity of these participants these groups were combined.

7.4.2 Participants' diagnosis of Coeliac Disease

Most participants (83.8%) were diagnosed by a positive biopsy (table 7.4). Only 16.2% of participants were diagnosed despite not having a biopsy result to confirm the diagnosis.

Table 7.4 Method of diagnosis of Coeliac Disease in participants

Diagnosis		Number of Responses (%)
Biopsy		451 (83.8%)
	No other additional test reported	128* (23.8%)
	Genetic testing (blood test)	34 (6.3%)
	Antibody testing (tTG and/or EMA)	148 (27.5%)
	Blood tests but unsure which tests	141 (26.2%)
No Biopsy		87 (16.2%)
	Genetic testing (blood test) and other blood tests, but unsure which test	3 (0.6%)
	Antibody testing (tTG and/or EMA)	32 (5.9%)
	Blood tests but unsure which tests	52 (9.7%)

* Based on New Zealand diagnostic practices, it is unlikely that participants would have been diagnosed through biopsy alone without serology tests unless the biopsy was being done for another purpose. tTG, tissue transglutaminase; EMA, endomysial antibodies.

There was no difference in biopsy rates between genders (p=0.6639) or location (p=0.9223). Participants were diagnosed on average 9.3 years ± 7.0 years prior to participating in the study. Average age at diagnosis is not possible to determine as participants identified their age range at the time they participated, rather than exact age. However, using the median for extremes in each age group,

an estimated age of diagnosis of between 25-34 (16-23 – 37-46) can be determined. This means that between 367 (68.2%) and 472 (87.7%) participants were 18 years or older at the time of diagnosis.

Almost all participants reported experiencing symptoms prior to diagnosis (537/538, 97.8%), with 80.3% of participants reporting classical intestinal/gut symptoms of abdominal pain, bloating, constipation, diarrhoea, flatulence, or nausea (432/538). Over half of participants (394/538; 73.2%) reported ongoing symptoms despite adhering to a GFD. This included 253 participants (47.0%) who still experienced persistent classical symptoms. No association was found between method of diagnosis and reported symptoms prior to diagnosis (classical, non-classical or asymptomatic) (p=0.7349).

Table 7.5 Comparison of symptoms prior to diagnosis with those experienced while following a GFD or consuming gluten post-diagnosis

	Symptoms present prior to diagnosis	Symptoms experienced while on a strict GFD	Symptoms upon consumption of gluten*
Abdominal pain[1]	309 (57.4%)	77 (14.3%)	395 (73.4%)
Bloating[1]	294 (54.6%)	152 (28.3%)	331 (61.5%)
Constipation[1]	177 (32.9%)	97 (18.0%)	126 (23.4%)
Dermatitis Herpetiformis	108 (20.1%)	55 (10.2%)	98 (18.2%)
Depression	120 (22.3%)	72 (13.4%)	79 (14.7%)
Diarrhoea[1]	250 (46.5%)	63 (11.7%)	340 (63.2%)
Fatigue	377 (70.1%)	192 (35.7%)	326 (60.6%)
Flatulence[1]	210 (39.0%)	92 (17.1%)	250 (46.5%)
Headaches	188 (34.9%)	83 (15.4%)	216 (40.1%)
Indigestion or acid reflux	136 (25.3%)	72 (13.4%)	125 (23.2%)
Iron Deficiency	285 (52.9%)	109 (20.3%)	60 (11.2%)
Mouth ulcers	121 (22.5%)	37 (6.9%)	60 (11.2%)
Muscle cramps	89 (16.5%)	40 (7.4%)	98 (18.2%)
Nausea[1]	147 (27.3%)	43 (8.0%)	267 (49.6%)
Weakness	125 (23.2%)	32 (5.9%)	150 (27.9%)
Weight loss	115 (21.4%)	5 (0.9%)	24 (4.5%)
Vomiting	67 (12.5%)	1 (0.2%)	196 (36.4%)
No symptoms	12 (2.2%)	91 (16.9%)	14 (2.6%)

* Consumption of gluten includes both accidental and intentional intake. [1.] Classical intestinal/gut symptoms. Participants were able to select more than one symptom.

7.4.3 Adherence to the gluten-free diet

Most participants (490/538; 91%) had a Biagi adherence score of 3 or 4 indicating good adherence to the GFD (table 7.6). There was no statistically significant difference in the Biagi adherence score and gender (p=0.2758) or diagnosis method (p=0.6111). In addition, no association was found between the adherence score and education level (p=0.2547).

No association was found between ongoing symptoms and Biagi adherence scores (p=0.5089). Of participants with an adherence score of 3 or 4, which indicates good adherence to the diet (Table 7.6), 230 (46.9%) reported ongoing intestinal/gut symptoms, compared with 23 (47.9%) of those who scored a 0,1 or 2 (p=0.8966). In addition, 83 of participants scoring a 3 or 4 reported no ongoing symptoms (16.9%), compared with 8 (16.7%) who scored a 0,1 or 2 (p=0.9617).

Table 7.6 Biagi Score of participants indicating adherence to the GFD

Adherence Score	Total n=538	Male n=36	Female n=492	Other/unanswered[1] n=10
0	15 (2.8%)		15 (3.0%)	
1	14 (2.6%)	1 (2.8%)	12 (2.4%)	1 (10.0%)
2	19 (3.5%)	1 (2.8%)	17 (3.5%)	1 (10.0%)
3	410 (76.2%)	31 (86.1%)	374 (76.0%)	5 (50.0%)
4	80 (14.9%)	3 (8.3%)	74 (15.0%)	3 (30.0%)

[1] Other/Unanswered consists of 3 participants who identified themselves as non-binary and 7 participants who did not indicate their sex; to maintain anonymity of these participants these groups were combined.

When asked if they "check the labels of packaged foods for the presence of gluten", 516 (95.9%) participants indicated that they always checked packaged

foods, with the remaining 22 (4.1%) participants reporting that they check sometimes. In addition, as part of a follow-up question, 29.0% of participants said they checked food labels regardless of whether they had purchased the item before, while 50.7% reported they only checked the labels of products they had purchased previously if the packaging appeared to have changed and 20.3% indicated they only checked the labels of new foods.

Most participants reported being wary about cross contamination from cooking equipment; 75.8% (408/538), 80.4% (432/537) and 80.3% (431/537) participants said they would try to avoid or not consume food that might have gluten cross-contamination from a non-dedicated toaster, serving tongs or deep fryer, respectively (table 7.7). Additionally, roughly half of participants were concerned about cross contamination risk from food production lines; 54.0% (290/537) and 54.5% (291/534).

In addition, despite being defined as GF, 80.8% (433/536) participants reported they would avoid products containing caramel from wheat, while 50.0% participants (258/538) reported they would avoid products containing glucose syrup from wheat. No association was found between Biagi adherence score and responses from participants regarding whether they would consume products containing caramel from wheat (p=0.1259) or glucose syrup from wheat (p=0.2281).

Table 7.7 Reported behaviours associated with dietary restriction

	I would eat it	I would try to avoid this	I would not consume this
… gluten-free bread that has been toasted in a toaster which is not dedicated gluten-free (n = 538)	130 (24.2%)	158 (29.4%)	250 (46.5%)
… a gluten-free cake that was next to a gluten-containing cake and was removed from the cabinet using the same tongs (n=537)	105 (19.6%)	165 (30.7%)	267 (49.7%)
… food that has been fried in a deep fryer which is not dedicated gluten-free (n=537)	106 (19.7%)	151 (28.1%)	280 (52.1%)
… gluten-containing cake at a friend's wedding (n=535)	4 (0.7%)	16 (3.0%)	515 (96.3%)
… packaged food which states that it may contain traces of gluten (n=537)	247 (46.0%)	180 (33.5%)	110 (20.4%)
… packaged food which states that it is made on the same line as gluten-containing products (n=534)	243 (45.5%)	208 (39.0%)	83 (15.5%)
… products containing caramel from wheat (n=536)	103 (19.2%)	133 (24.8%)	300 (56.0%)
… products containing glucose syrup from wheat (n=538)	280 (52.0%)	86 (16.0%)	172 (32.0%)

The majority of participants (90.1%) reported that they exclude oats from their diet (table 7.8). The main reason reported for avoiding oats was that participants had been advised not to consume these with 75.1% of participants who did not eat oats reporting this as their reason.

Table 7.8 Reported consumption of oats

Consumption of oats; Do you consume oats?	
Yes	23 4.3%
Yes, but only those labelled as wheat free	28 5.2%
No	485 90.1%
Unanswered	2 0.4%
Reason for avoiding oats*	
I don't like oats	26 5.4%
Consuming oats makes me unwell	86 17.7%
I was advised to avoid/not consume oats	364 75.1%
Other	83 17.1%

* Participants were able to select more than one reason for avoiding oats.

Of participants who selected other for their reason for avoiding oats, 64 (77.1%) indicated that this was due to a similar protein found in oats, with some participants highlighting a need for biopsy to confirm if they reacted to this protein. The remaining participants highlighted confusion about the topic and inaccessibility of 'GF oats' as their reason for avoiding these. Biagi adherence scores were negatively correlated with consumption of oats, with a greater proportion of those with a low score (<3) reporting consuming oats (37.0%), than in those with a good score (≥3) (8.7%) (p=0.0002). No association was found between gender and reported consumption of oats (p=0.4922).

7.4.4 Nutritional advice received

Doctors were the most common source of advice about the GFD, followed by the internet or social media and a nutritionist or dietitian; 67.3% (358/532), 66.4% (353/532) and 63.9% (340/532) respectively (table 7.9). The most common source of advice for reading food labels was the internet or social media (289/524; 55.2%), followed by a nutritionist or dietitian (221/524; 42.2%). The internet or social media was also the most common source of advice when it came to the topic of oats, with 51.9% (264/509) of participants sourcing their advice from here, compared to only 23.8% (121/509) of participants who reported sourcing their advice about oats from a nutritionist or dietitian.

Table 7.9 Sources of nutrition advice on various aspects of diet

	Advice about:					
Source of advice	**GFD n=532**	**Food Labels n=524**	**Oats n=509**	**Dairy n=516**	**Bone n=518**	**Iodine n=505**
Doctor i.e., GP	358 (67.3%)	60 (11.5%)	76 (14.9%)	58 (11.2%)	155 (29.9%)	14 (2.8%)
Nutritionist or Dietitian	340 (63.9%)	221 (42.2%)	121 (23.8%)	93 (18.0%)	41 (7.9%)	11 (2.2%)
Naturopath etc	31 (5.8%)	4 (0.8%)	8 (1.6%)	16 (3.1%)	6 (1.2%)	9 (1.8%)
Whanau/Partner	140 (26.3%)	97 (18.5%)	37 (7.3%)	26 (5.0%)	13 (2.5%)	4 (0.8%)
Internet/social media	353 (66.4%)	289 (55.2%)	264 (51.9%)	111 (21.5%)	62 (12.0%)	14 (2.8%)
Other	37 (7.0%)	51 (9.7%)	36 (7.1%)	12 (2.3%)	7 (1.4%)	5 (1.0%)
No advice received	11 (2.1%)	76 (14.5%)	129 (25.3%)	299 (57.9%)	302 (58.3%)	461 (91.3%)

Participants were able to select more than one source of advice for each topic. Unanswered responses were removed for analysis

A follow-up question about the advice participants received from these sources revealed that 395 (74.2%) participants perceived they received beneficial or

useful advice regarding consuming a GFD from one or more source. Of these participants 99 (25.1%) reported receiving this from a medical professional (GP, gastroenterologist, nurse etc.), 79 (20.0%) reported finding this on the internet, 95 (24.1%) reported sourcing this through Coeliac NZ, with 159 (40.3%) reporting beneficial advice from a nutritionist or dietitian and 10 (2.5%) from a naturopath. In addition, 66 (16.7%) participants reported receiving advice they considered to be beneficial or useful from social media support groups and 49 (12.4%) from family or friends, several of whom themselves had previously been diagnosed with CD. However, with the exception of Coeliac NZ, all sources were also reported by other participants as being sources of misleading or contradictory information.

In addition, 322 (60.5%) reported receiving advice about the GFD which they perceived as being misleading or contradictory. Of these participants, 59 (18.3%) reported this was from a medical professional, 52 (16.1%) reported finding this on the internet, 46 (14.3%) from a nutritionist or dietitian, 66 (20.5%) from social media support groups and 39 (12.1%) from family or friends. Of the 395 participants who reported receiving beneficial or useful advice, 46 (11.6%) reported also receiving misleading or contradictory advice from the same source. The main sources included nutritionists or dietitians (15/46; 32.6%), Facebook or social media groups (13/46; 28.3%) and the internet (16/46; 34.8%). Of note, participants highlighted that a nutritionist or dietitian taught them to read food labels and identify gluten but had lacked knowledge in some areas such as cross contamination risk or had only provided rudimentary advice about the general characteristics of a GFD:

"*There were also lots of things that the dietician didn't know when I spoke to her, past giving me a booklet about the diet*"

"*My dietician said that cross contamination is not an issue for all coeliacs but I always thought you had to be very strict*"

Very few participants provided details about the information they found beneficial or contradictory or identified the specific sources of this. However, 274 participants (50.9%) identified oats as a topic that they were given advice about that they found contradictory or misleading. In addition to oats, several participants reported being confused about other foods which are considered GF in other countries, but not in New Zealand, due to differing cut-off levels; these included products like malt vinegar and beer, where information on the internet may vary depending on the host site's location. Thirty-one participants (5.8%) also highlighted "may contain" statements as a source of confusion, with a further 7 participants identifying some companies who specifically state their products contain gluten when they are GF by ingredient as an additional source of confusion:

"That some companies write "contains gluten" on products to cover themselves but there is no gluten in their products."

"... an example – [company name] reasonably recently started a new line of potato chips designated as Gluten Free. These are about twice as expensive as the normal chips. Reading the label of ordinary chips - some types do not contain gluten in ingredient but they state that they contain gluten. I wrote to them and they said that they were made in a factory where gluten also processed - not the same (in my mind) as "containing". Possible contamination only."

In addition, 7 participants reported being given incorrect advice regarding testing requirements for a biopsy, with doctors and nurses advising them to cut-out gluten immediately after positive serology before an intestinal biopsy was taken:

"A GP advised that I stop eating gluten before referral for biopsy, which meant I was required to reintroduce gluten for biopsy purposes which proved impossible to do due to severity of reaction. Which means I only have positive bloods and not a full diagnosis."

Two hundred and ninety-two participants with diagnosis confirmed by a biopsy reported receiving advice from a nutritionist or dietitian about following a GFD (64.8%), compared with 48 participants who were diagnosed without a biopsy confirmation (55.2%) (p=0.0901).

Of participants with an adherence score of 3 or 4, 63.5% had received advice about the GFD, compared with 60.4% of those who scored a 0, 1 or 2 (p=0.6752). Of participants who scored a 3 or 4, 41.4% had received advice about reading food labels from a nutritionist or dietitian, compared with 37.5% of those who scored a 0,1 or 2 (p=0.5975).

7.4.5 Bone health

One hundred and sixty participants (29.7%) reported having had a DXA scan (Table 7.10). These consisted of 10/36 males (27.8%) compared with 148/492 females (30.1%) (p=0.7711). The majority of participants who reported a DXA scan were over the age of 40 (111/160, 69.3%). In addition, DXA scans were more common in older age groups with more than 50% participants over the age of 50 reporting having a past DXA scan (table 7.10).

Table 7.10 Age distribution of participants who reported a DXA scan n=160

Age group	# DXA scans reported	% People reporting DXA scans in each age group
16-24	2	2.6%
25-30	19	23.2%
31-40	28	20.9%
41-50	41	37.6%
51-60	41	52.6%
61-70	20	57.1%
71-80	5	55.6%
Undisclosed	4	33.3%

One hundred and eleven of the participants who reported having had a DXA scan (69.4%) indicated this was recommended because they had been diagnosed with CD; 3 participants reported having had a DXA scan while they were overseas. Of the participants who received a DXA following diagnosis of CD in New Zealand, 64/108 (59.3%) were referred for a DXA through the public health system.

In addition, 3 participants indicated that although they had been referred for a DXA scan by a gastroenterologist, their request for a scan was turned down by their DHB or GP:

"*My GP told me that some celiacs have issues with bone density. I was referred for a bone scan, just to check but this was denied by the DHB as I have a high enough BMI and no history of bone issues*"

Fifty-one of the participants who reported having had a DXA scan (51/160; 31.9%), stated that they had been medically diagnosed with either osteoporosis or osteopenia, of which 34 (34/111; 30.6%) had been referred for a DXA scan solely due to diagnosis of CD. An additional 3 participants reported a history of stress fractures, of whom only 1 was referred for a DXA scan due to diagnosis of CD.

7.4.6 Iodine

Most participants (325, 60.4%) reported the main salt they used in cooking/baking is iodised, with 73 participants (13.6%) reporting they did not use iodised salt in cooking/baking and 140 participants (26.0%) reporting being unsure whether they used iodised salt in cooking/baking. In comparison, only 251 participants (46.7%) reported knowing that the main salt they used at the table was iodised, with 117 participants (21.7%) reporting it was not iodised and 170 participants (31.6%) reporting they were unsure whether the salt they used at the table was iodised.

The median number of slices of bread consumed per day was 0.57 (0.29, 2.00). Men consumed significantly more bread than women with a median intake of 1.71 (0.43, 2.00) slices per day compared to 0.57 (0.21, 2.00) slices per day in women (p=0.0140).

The majority of participants reported consuming dairy products 416 (77.3%). Of the remaining participants, 110 (20.4%) reported consuming plant-based alternatives. In addition, a further 12 (2.2%) participants excluded all dairy products from their diet (with no alternatives consumed).

Avoidance of lactose was reported by 43 (8.0%) participants. Of participants avoiding lactose, 11 participants (11/43; 25.6%) consumed plant-based milk alternatives, making up 10.0% of total plant-based alternative consumers. Of the remaining participants, 28 (65.1%) reported consuming lactose-free products and 2 (4.7%) reported consuming regular milk in small quantities. In addition, of dairy consumers, 62 (14.9%) participants reported that they only consumed dairy in small quantities due to dairy products upsetting their gut.

The median intake of dairy products reported was 2.2 serves per day (1.4, 3.5) (excluding participants who reported consumption of plant-based alternatives or no dairy products). Intake of dairy products was not statistically different between women and men, with a median of 2.2 servings (1.4, 3.4) per day and 2.4 servings (1.7, 3.9) per day respectively (p=0.9967).

7.5 Discussion

7.5.1 Advice

Only 63.9% of participants in the current study reported receiving advice about the GFD from a nutritionist or dietitian, despite referral to a nutritionist or dietitian post diagnosis being considered the best practice for patients newly diagnosed with CD (Ben Houmich & Admou, 2021). In comparison, a previous New Zealand study found that 81.6% of respondents reported seeing a dietitian for education about a GFD after their diagnosis with CD (Sharp et al., 2014). However,

the findings of the current study are similar to those reported in the study by Halmos et al. (2018), which assessed dietary adherence in adults with CD from Australia and New Zealand and reported that 37% of those diagnosed with CD had not seen a dietitian after diagnosis.

In the current study, no association was found between Biagi adherence scores and advice about either the GFD (p=0.6752) or food labels (p=0.5975) from a nutritionist or dietitian. This is similar to the findings from research by Mahadev (2013), which despite reporting a greater proportion of participants having received advice from a dietitian (79%) than in the current study, found no association between dietitian involvement and adherence scores, symptom severity or quality of life (QoL).

Research indicates that receiving advice from a dietitian can significantly improve adherence to the GFD (Gładyś et al., 2021). One such study reported improvements in adherence in newly diagnosed CD patients, assessed after 1 month on a GFD and again at 6 months, with the percentage of those with good adherence to the diet increasing from 64.8% to 96.3% after dietitian involvement (Rajpoot et al., 2015). The study also assessed improvements in adherence after dietitian involvement in those already consuming a GFD, with 53.2% initially having good adherence to a GFD, increasing to 92.4% after 6 months. The authors also noted significant improvements in some areas affected QoL, including mental health.

In New Zealand, referrals to dietitians usually occur soon after a person is given their diagnosis, however, in practice it may be weeks to months later that a patient actually receives their appointment due to dietitian shortages (New Zealand Institute of Economic Research (NZIER), 2021). This delay often results in patients finding their own information from other sources and beginning their journey of consuming a GFD without professional support.

It should be noted that in the current study, participants were not asked about their reason for seeing a dietitian or nutritionist and we cannot exclude

referral for other conditions or draw conclusions regarding the timeframe for referral and how this influences dietary adherence.

It is possible that benefit from dietary intervention (consultation with a dietitian or nutritionist after diagnosis) may depend on the quality of other available sources of information as well as the experience of the dietitian in supporting an individual with a change to the GFD; New Zealand has a low dietitian count for the size of the population, limiting the ability for dietitians to specialise (New Zealand Institute of Economic Research (NZIER), 2021). Coeliac NZ provides both free online information services, with further and more extensive support for paid members (Coeliac New Zealand, n.d.-c). This organisation was mentioned by 24.1% of participants who reported receiving advice about the GFD and was the only source which was not also identified as a source of misleading or contradictory information.

Inadequate nutritional advice at the outset of an individual adopting a GFD may result in significant setbacks that could delay an individual rigorously adopting a GFD: adherence has been shown to be much greater in individuals who can accurately interpret food labels and select safe products (Muhammad et al., 2017).

In addition, although quality of the advice provided was hard to assess in the current study, because few participants provided specific information about what advice they had received or where they received this from, of participants who reported receiving beneficial or useful advice, only 159 (40.3%) reported receiving this from a dietitian or nutritionist. Concerningly, 46 (14.3%) participants who perceived they had been given misleading or contradictory advice reported receiving this from a dietitian or nutritionist. Although this is only a small proportion of participants, it raises concerns about the quality of advice given. In addition, 15 participants reported that despite receiving beneficial advice from a dietitian or nutritionist, some of the advice given was contradictory or misleading. It is possible that this may stem from the difference in cut-off for acceptable gluten

levels in New Zealand (and Australia and Chile) with detection of gluten down to 3 parts per million (ppm), compared to other countries which tend to have more leniency for ingestion of gluten (Cohen et al., 2019; Food Standards Australia New Zealand, 2016); if the advice from a New Zealand dietitian differs from the advice found from sources perceived as being reputable on the internet, it may be perceived as poor advice despite being appropriate. Further research is needed to investigate the quality of advice provided to newly diagnosed CD cases in New Zealand and whether this could be improved.

Other sources of information that were identified as being particularly contradictory or confusing for participants included social media support groups and the internet. Social media support groups have been demonstrated to be a useful place for people with health conditions to garner more information about their condition while also gaining social support (Chung, 2014). However, health research suggests that despite this, health misinformation is highly prevalent in social media (Suarez-Lledo & Alvarez-Galvez, 2021), making this an often-unreliable source of information. In addition, given the differences in regulations in New Zealand compared with other countries and New Zealand's much lower acceptable threshold for gluten in products (Coeliac New Zealand, n.d.-a) it is unsurprising that the internet was identified by multiple participants as a source of contradictory or misleading advice.

7.5.2 Adherence to the gluten-free diet

The majority of participants (91.1%) reported strict adherence to the GFD as assessed by the Biagi Questionnaire (Biagi et al., 2009), with adherence scores of ≥3. This is higher than reported in two other recent studies carried out in adults in Italy and Israel which also assessed adherence with the Biagi questionnaire and found 82-87% of participants strictly adhered to the GFD (Canova et al., 2021; Dana et al., 2020).

A previous study investigating adherence in New Zealand and Australia found only 61% of coeliac patients over 13 years old had scores indicating excellent or very good adherence (Halmos et al., 2018). However, the majority (80%) of these participants were based in Australia and dietary adherence was assessed by the 7-item Celiac Disease Adherence Test (CDAT) (Leffler et al., 2009). The questions in the CDAT emphasise symptoms (e.g., experiencing low energy level and headaches) and psychological aspects (e.g., experiencing feelings of failure and considering the consequences before an action) rather than being practical aspects of behaviour related to consumption of gluten which are the focus of the shorter Biagi questionnaire. Although, CDAT is a commonly used approach to assess adherence, as a number of people with CD continue to experience symptoms despite strict adherence to a GFD (Vuolle et al., 2022), the emphasis on ongoing symptoms in this tool may result in an underestimation of dietary adherence.

The Biagi adherence score in the current study were calculated using a questionnaire validated against serum antibody results (Biagi et al., 2009), the validation was completed in Italy and although it has been shown to correlate with serology in a number of other studies (Biagi et al., 2012; Lau et al., 2018; Marsilio et al., 2020), no assessment of its validity for the New Zealand population has been undertaken. In addition, although serology is often used to validate adherence assessments, the only way to accurately assess adherence is assessing duodenal biopsies (Freeman, 2018). This method is invasive, expensive and has a high respondent burden so validation of the scoring system through this approach is difficult to justify.

Although the Biagi questionnaire has been used to assess adherence to a GFD in studies carried out in a number of European countries, the question regarding selection of products certified as GF may not be appropriate for a New Zealand audience. Manufacturers in New Zealand pay to have their products analysed to certify them as GF and to be permitted to make a ‘gluten-free’ claim, with testing requiring no more than 3 ppm gluten be detected (Cohen et al., 2019),

which limits the number of companies whose products carry this claim. Products without this claim often carry no greater risk of contamination so consumption of these does not necessarily indicate poorer adherence to the diet. Assessment of understanding of food labels may provide greater insight into true adherence, something that the Biagi questionnaire is not designed to do (Gładyś et al., 2020). Understanding food labels has been demonstrated to be poor both in the general population in New Zealand (Mhurchu & Gorton, 2007) and in individuals with CD (Gutowski et al., 2020).

Concerns regarding the ability of individuals with CD to interpret the information presented on food labels and how to accurately identify GF products have been raised in a number of studies (Muhammad et al., 2019; Muhammad et al., 2017; Xhakollari et al., 2019). In the current study, 95.9% of participants reported that they always check the labels of packaged foods for the presence of gluten. However, this study did not assess participants ability to accurately determine if a product is GF, so it is difficult to determine their proficiency of food label reading. Nevertheless, participants responses to questions about foods they would avoid indicate a possible lack of education or understanding about what foods are considered safe, with most participants tending to be cautious in their approach.

Responses to questions about products that Coeliac NZ define as GF, such as caramel from wheat and glucose syrup, demonstrated that respondents were not certain whether these products were regarded as safe. Just over half of participants (52.0%) said they would consume glucose derived from wheat, while only 19.2% reported they would consume caramel derived from wheat. Both products are heavily processed and have been found to contain no detectable gluten (or other protein) and are therefore defined by Coeliac NZ as being GF (Coeliac New Zealand, n.d.-b). In addition, over half of participants indicated they would try to avoid (33.5%) or would not consume (20.4%) products which displayed a ‘may contain traces of gluten’ warning and over half of participants

indicated they would avoid (39.0%) or not consume (15.5%) items made on the same production line as gluten-containing products. The use of these statements on products is voluntary and indicates no additional risk of gluten being present when compared to products which have no warning (Food Standards Australia New Zealand, 2020). As this study only assessed behaviour, it is unclear from these findings whether this is due to lack of knowledge about these products being GF or the participant's personal preference to avoid these products.

Advice from a nutritionist or dietitian has been demonstrated to improve patients understanding of both the GFD (Gładyś et al., 2021) and food labels (Moore et al., 2018). However, it should be noted that when comparing findings in individuals who received advice about the GFD or food labelling and those who did not, there was no difference in adherence scores or the percentage of participants who reported they would consume caramel from wheat or glucose syrup from wheat.

These findings suggest that current nutrition education strategies for individuals with CD may not adequately cover certain aspects of the GFD or provide skills to interpret food labels. Food labelling of products in New Zealand requires manufacturers to indicate where allergens are present either in a "contains" statement or by marking these allergens in bold in the ingredients list (Ministry for Primary Industries, 2022). As wheat can be an allergen independent of its contribution to gluten intake, it is marked in the ingredient list regardless of whether the product also contains gluten. This can cause confusion in individuals with CD, as with the exception of a few processed items where proteins (including gluten) have been removed, wheat should usually be avoided because of the presence of gluten (Coeliac New Zealand, n.d.-b). Although avoiding these products would not cause physical harm to participants, uncertainty about what is considered a safe product or avoidance of products which list a "may contain" statement may lead to participants unnecessarily restricting an already restricted diet. Previous research conducted in both Australia and New Zealand that

identified that poor food knowledge was associated with excessively restricted diets in individuals with CD.

In addition, restricting the diet to foods specifically certificated as GF may have financial implications for people on a GFD as the additional cost to manufacturers of certifying these products is often reflected in the cost of the product. Internationally, GF food has been demonstrated to cost significantly more than comparable gluten-containing products (White et al., 2016); some items are reported to be more than five times more expensive than their gluten-containing equivalent products. Although a recent study by Lee et al. (2019) reported that prices of GF products have started to decline in parallel with the increased popularity of the GFD, with less disparity between these and non-GF product pricing, these were still upwards of 1.8 times the price of equivalent non-GF products. A price comparison made by Coeliac NZ found even greater differences in price, reporting that GF alternatives are between 1.7 to 5 times more expensive (Edmonds, 2016). Inaccessibility of GF products due to high cost has been identified to be one of the leading causes of poor adherence to a GFD (Al-Sunaid et al., 2021; Arias-Gastelum et al., 2018; Villafuerte-Galvez et al., 2015).

7.5.3 Iodine

Most participants (461, 91.3%) reported not having received advice about iodine. This is a statistic of considerable concern given both the history of iodine deficiency in New Zealand and the proportion of women in this study (91.4%). Even mild to moderate iodine deficiency in pregnant and breastfeeding women can have a significant impact on neurological development of the fetus and child (Eastman et al., 2019). The New Zealand diet is naturally low in iodine due to low levels of the mineral in soil (Brough & Skeaff, 2020). This led to the New Zealand government introducing mandatory iodine fortification of bread in 2009 to help combat the rising incidence of iodine deficiency (Food Standards Australia New Zealand, 2015). Although there have been improvements in iodine status since its

introduction (Ministry for Primary Industries and Ministry of Health, 2016), minimal research has been done to investigate the effects on the coeliac population (see chapter 6).

However, despite low reports of advice about iodine, most participants (60.4%) reported using iodised salt for cooking or baking, with just under half (46.7%) reporting they used iodised salt at the table. Use of discretionary salt is hard to quantify (McLean, 2014) and although assessment of sodium excretion estimates intake to be significantly higher than recommendations in New Zealand, with 77% participants having a sodium excretion greater than the upper limit of intake (2300mg/day) (McLean et al., 2015), much of this sodium comes from processed foods which are unlikely to contain iodised salt. In addition, as we did not assess knowledge about iodine in the current study, it is not possible to determine whether participants were aware of the importance of consuming iodised salt regardless of their behaviour.

A report by Plant and Food Research assessing iodine content of bread products in 2012 (post introduction of mandatory fortification) found that all GF breads analysed, although iodine fortified, were not fortified to the same extent as their gluten-containing counterparts; with only trace levels of iodine detected in most GF breads (New Zealand Institute of Plant and Food Research, 2013). The findings of the current study only further exacerbate concerns that the coeliac population is not benefiting from bread fortification. Intake of bread in both men and women in the current study was low (1.71 slice and 0.57 slices respectively), with the intake in women significantly lower than men (p=0.0140). In comparison, when mandatory iodine fortification was introduced, the modelling was based on an intake of 3-4 slices of bread per day in women of childbearing age (Food Standards Australia New Zealand, 2006); considerably higher than that reported in the current study.

Although there are currently no published data regarding iodine or bread intake in adults with CD in New Zealand, these findings suggest that further

research is warranted to investigate iodine status in this group and confirm the extent to which fortified GF bread contributes to this.

7.5.4 Bone

Findings from the current study identified low referral rates, with only 160 participants (29.7%) reporting they had received a DXA scan. It is extensively reported internationally that individuals with CD are at a greater risk of low BMD (Bianchi & Bardella, 2008; Galli et al., 2018), with multiple studies reporting low BMD in adults with CD (Pantaleoni et al., 2014; Szymczak et al., 2012). Although improvements in BMD are to be expected after a period (generally a year or more) of consuming a GFD (Bathrellou et al., 2018), these improvements often do not result in full restoration of BMD (Bathrellou et al., 2018; Pantaleoni et al., 2014). Despite having one of the highest prevalence rates of CD in the world (Burkhardt et al., 2018), research on the relationship between CD and low BMD in New Zealand is limited. There is also no consensus on recommendations for DXA screening in individuals with CD with referral patterns being inconsistent (Kenrick, 2018).

It should be noted that people have DXA scans for various reasons and only 104 participants (19.3%) in the current study indicated that they were referred for a DXA scan specifically because of their diagnosis of CD. As we did not request the dates of either the DXA scan or diagnosis of CD, it is unclear whether the individuals who either did not report a reason for a DXA scan or reported another reason for this, had these scans prior to or post diagnosis. It should also be noted that some participants may have been referred for a DXA scan because of their diagnosis of CD but may not have been explicitly aware of the reason for referral for a scan.

Although there is no official position statement in New Zealand regarding what medical practitioners should do, the recommendation by Coeliac NZ is that gastroenterologists or GPs should discuss the need for a DXA scan with patients at

the time of diagnosis of CD (Coeliac New Zealand, 2019). Coeliac New Zealand also provides learning resources through an online course for physicians and dietitians, developed by the Division for Paediatric Gastroenterology and Hepatology, Munich, Germany, which advises that everyone diagnosed with CD as an adult, who have other risk factors for osteoporosis, or are unable to maintain strict adherence to the GFD, should have BMD assessed (Karla et al., 2021).

Based on the reported time period since diagnosis, between 367 (68.2%) and 472 (87.7%) participants were 18 years or older at the time of diagnosis, indicating that the proportion (29.7%) of participants who received a DXA scan is well below recommended practice. This is supported by findings from a previous study in New Zealand investigating health practitioner referral practices which reported that 48.7% of the 150 practitioners questioned did not routinely refer coeliac patients for a DXA scan (Kenrick, 2018).

Of the 378 participants in the current study who did not receive a DXA scan (70.3%), 3 reported that they had been referred for a DXA scan by their gastroenterologist or GP but added that the referral had been declined by their DHB. Although it is not possible to determine the true reason for these referrals being declined, these findings fit with practitioners' reports that some DHBs do not cover funding of DXA scans in patients with CD (Kenrick, 2018).

Although 31.9% of participants who received DXA scans reported diagnosis of either osteoporosis or osteopenia, because we have no data regarding either the exact age that participants were diagnosed or whether there were concurrent medical conditions, we are unable to project how these numbers may apply to the coeliac population in New Zealand. However, these findings suggest that further research is warranted to evaluate the true prevalence of low BMD in New Zealand adults with CD.

In addition to reporting whether they had a DXA scan, participants were also asked about advice they had been given about bone. Over half of participants (58.3%) stated they had not received any advice about bone-related health, while

29.9% reported receiving advice about bone from a doctor and 7.9% reported receiving advice about bone from a nutritionist or dietitian. More than a fifth of the participants in the current study reported consuming plant-based alternatives to dairy products or excluding dairy from the diet entirely. Although many plant-based alternative products are now fortified with calcium (Chalupa-Krebzdak et al., 2018; Zhang et al., 2020), this is not the case for all products and further research is warranted to investigate how this may impact calcium intake in this group who may be at greater risk of low bone density.

It is evident from the findings of this study that referral for assessment of bone density is not the common experience for individuals with CD in New Zealand. Although this may be in part due to financial constraints limiting access to publicly funded DXA scans, it is also clear that most individuals with CD have received little advice about bone-related health. Strategies to promote bone-related health in these individuals, even in the absence of assessment of bone density, are needed to ensure the long term economic and QoL implications of low BMD are minimised.

7.5.5 Strengths and limitations

One major limitation of the current study was the recruitment method, with advertisements through both Facebook-targeted advertising and Facebook support groups. The open access questionnaire meant that participants self-selected to take part, so it is not possible to determine if this sample group is representative of the coeliac population in New Zealand. There are limited data regarding the composition of this group, including age and regional distribution. In addition, a much higher proportion of women responded to this questionnaire than men, with a ratio of almost 14:1. Although CD is more common in women, this ratio indicates responses disproportionately came from women; with a recent systematic review suggesting a gender ratio of roughly 2.2:1 (King et al., 2020), similar to the ratio previously reported in New Zealand of 2.1:1 (Cook et al., 2004).

The current study lacked assessment of participants' knowledge about the GFD. Our study focused on participants' behaviours and the advice they received but did not assess their knowledge. Future studies should consider inclusion of a New Zealand based knowledge questionnaire similar to that used in the research study carried out in Italy by Paganizza et al. (2019). Assessment of knowledge of foods that are safe to include in a GFD would increase insight into the participants' understanding of the diet and whether further dietary education should be targeted at this population group.

In addition, collecting data regarding the region or DHB where participants were diagnosed would have allowed for greater insight into the composition of this population in New Zealand, along with assessment of regional variation in referral practices both for DXA scans and dietary advice from dietitians. Further investigation is warranted to investigate regional variation.

7.6 Conclusions

This preliminary study identified several areas where future research is warranted. Although findings indicate that individuals with CD in New Zealand have good adherence to the GFD, it appears there is still some confusion about foods that are safe to eat. Participants also reported inconsistencies in advice, with sources of advice generally perceived as helpful being also identified by some as inconsistent or contradictory. Additional research is needed to investigate areas of advice that are perceived as contradictory. In addition, assessment of bone density and iodine status in individuals in New Zealand with CD is needed, with investigation into approaches to promote nutrition strategies that enhance the intake of iodine and other nutrients required for bone in these individuals.

7.7 References

Abu-Janb, N., & Jaana, M. (2020). Facilitators and barriers to adherence to gluten-free diet among adults with celiac disease: a systematic review. *Journal of Human Nutrition and Dietetics*, *33*(6), 786–810. https://doi.org/10.1111/jhn.1

2754

Al-Sunaid, F. F., Al-Homidi, M. M., Al-Qahtani, R. M., Al-Ashwal, R. A., Mudhish, G. A., Hanbazaza, M. A., & Al-Zaben, A. S. (2021). The influence of a gluten-free diet on health-related quality of life in individuals with celiac disease. *BMC Gastroenterology*, *21*(1), 1–9. https://doi.org/10.1186/s12876-021-01908-0

Arias-Gastelum, M., Cabrera-Chávez, F., Vergara-Jiménez, M. de J., & Ontiveros, N. (2018). The gluten-free diet: access and economic aspects and impact on lifestyle. *Nutrition and Dietary Supplements*, *Volume 10*, 27–34. https://doi.org/10.2147/nds.s143404

Bathrellou, E., Kontogianni, M. D., & Panagiotakos, D. B. (2018). Celiac disease and non-celiac gluten or wheat sensitivity and health in later life: A review. *Maturitas*, *112*(January), 29–33. https://doi.org/10.1016/j.maturitas.2018.03.014

Ben Houmich, T., & Admou, B. (2021). Celiac disease: Understandings in diagnostic, nutritional, and medicinal aspects. *International Journal of Immunopathology and Pharmacology*, *35*. https://doi.org/10.1177/20587384211008709

Biagi, F., Andrealli, A., Bianchi, P. I., Marchese, A., Klersy, C., & Corazza, G. R. (2009). A gluten-free diet score to evaluate dietary compliance in patients with coeliac disease. *British Journal of Nutrition*, *102*(6), 882–887. https://doi.org/10.1017/S0007114509301579

Biagi, F., Bianchi, P. I., Marchese, A., Trotta, L., Vattiato, C., Balduzzi, D., Brusco, G., Andrealli, A., Cisaro, F., Astegiano, M., Pellegrino, S., Magazzu, G., Klersy, C., & Corazza, G. R. (2012). A score that verifies adherence to a gluten-free diet: A cross-sectional, multicentre validation in real clinical life. *British Journal of Nutrition*, *108*(10), 1884–1888. https://doi.org/10.1017/S0007114511007367

Bianchi, M. L., & Bardella, M. T. (2008). Bone in celiac disease. *Osteoporosis International*, *19*(12), 1705–1716. https://doi.org/10.1007/s00198-008-0624-0

Brough, L., & Skeaff, S. (2020). Iodine status in New Zealand. In P. A. C. Richards,

Iodophor: Tamed Iodine: The Status of Iodine in Tasmania. Gatekeeper Press

Burkhardt, J. G., Chapa-Rodriguez, A., & Bahna, S. L. (2018). Gluten sensitivities and the allergist: Threshing the grain from the husks. *Allergy: European Journal of Allergy and Clinical Immunology*, *73*(7), 1359–1368. https://doi.org/10.1111/all.13354

Burnard, P., Gill, P., Stewart, K., Treasure, E., & Chadwick, B. (2008). Analysing and presenting qualitative data. *British Dental Journal*, *204*(8), 429–432. https://doi.org/10.1038/sj.bdj.2008.292

Canova, C., Rosato, I., Marsilio, I., Valiante, F., Zorzetto, V., Cataudella, G., D'odorico, A., & Zingone, F. (2021). Quality of life and psychological disorders in coeliac disease: A prospective multicentre study. *Nutrients*, *13*(9), 1–12. https://doi.org/10.3390/nu13093233

Chalupa-Krebzdak, S., Long, C. J., & Bohrer, B. M. (2018). Nutrient density and nutritional value of milk and plant-based milk alternatives. *International Dairy Journal*, *87*, 84–92. https://doi.org/10.1016/j.idairyj.2018.07.018

Chung, J. E. (2014). Social networking in online support groups for health: How online social networking benefits patients. *Journal of Health Communication*, *19*(6), 639–659. https://doi.org/10.1080/10810730.2012.757396

Coeliac New Zealand. (n.d.-a). *FSANZ*. Available from: https://coeliac.org.nz/fsanz-codex/

Coeliac New Zealand. (n.d.-b). *How to read food labels*. Coeliac New Zealand. Available from: https://coeliac.org.nz/how-to-read-food-labels/

Coeliac New Zealand. (n.d.-c). *Welcome to Coeliac New Zealand*. Coeliac New Zealand. Available from: https://coeliac.org.nz/

Coeliac New Zealand. (n.d.-d). *Your Coeliac Journey - Where to Begin?* Coeliac New Zealand. Available from: https://coeliac.org.nz/your-coeliac-journey-where-to-begin/

Coeliac New Zealand. (2019). *Coeliac disease*. Available from: https://coeliac.org.nz/wp-content/uploads/2019/07/Coeliac-Disease.pdf

Cohen, I. S., Day, A. S., & Shaoul, R. (2019). Gluten in celiac disease more or less? *Rambam Maimonides Medical Journal*, *10*(1), 1–6. https://doi.org/10.5041/RMMJ.10360

Cook, B., Oxner, R., Chapman, B., Whitehead, M., & Burt, M. (2004). A thirty-year (1970–1999) study of coeliac disease in the Canterbury region of New Zealand. *The New Zealand Medical Journal*, *117*(1189), 8.

Dana, Z. Y., lena, B., Vered, R., Haim, S., & Efrat, B. (2020). Factors associated with non adherence to a gluten free diet in adult with celiac disease: A survey assessed by BIAGI score. *Clinics and Research in Hepatology and Gastroenterology*, *44*(5), 762–767. https://doi.org/10.1016/j.clinre.2019.12.014

Delvecchio, M., Bizzoco, F., Lapolla, R., Gentile, A., Carrozza, C., Barone, M., Simonetti, S., Giordano, P., Dargenio, V. N., Cristofori, F., & Francavilla, R. (2021). Iodine absorption in celiac children: A longitudinal pilot study. *Nutrients*, *13*(3), 1–10. https://doi.org/10.3390/nu13030808

Eastman, C. J., Ma, G., & Li, M. (2019). Optimal Assessment and Quantification of Iodine Nutrition in Pregnancy and Lactation: Laboratory and Clinical Methods, Controversies and Future Directions. *Nutrients*, *11*(2378), 1–18. https://doi.org/10.3390/nu11102378

Edmonds, J. C., McLean, R. M., Williams, S. M., & Skeaff, S. A. (2016). Urinary iodine concentration of New Zealand adults improves with mandatory fortification of bread with iodised salt but not to predicted levels. *European Journal of Nutrition*, *55*(3), 1201–1212. https://doi.org/10.1007/s00394-015-0933-y

Edmonds, S. (2016). *Coeliac suffers hard hit by high gluten-free prices*. Stuff. Available from: https://www.stuff.co.nz/business/money/83102687/coeliac-suffers-hard-hit-by -high-glutenfree-prices

Food Standards Australia New Zealand. (2006). *Final Assessment Report: Proposal P295. Consideration of mandatory fortification with folic acid*. Available from: http://www.foodstandards.govt.nz/code/proposals/documents/FAR_P295_Folic_Acid_Fortification_ Attachs_1_6.pdf

Food Standards Australia New Zealand. (2015). *Australia New Zealand Food Standards Code: Standard 2.1.1. Cereal and cereal products.* Canberra, Australia: Australian Government. Available from: https://www.legislation.gov.au/Details/F2015L00420/Download

Food Standards Australia New Zealand. (2016). *Australia New Zealand Food Standards Code: Standard 1.2.7. Nutrition, health and related claims*. Canberra, Australia: Australian Government. Available from: from: http://www.foodstandards.gov.au/code/Documents/1.2. 7 Nutrition and health claims v157.pdf

Food Standards Australia New Zealand. (2022). *Allergen labelling*. Available from: https://www.foodstandards.gov.au/consumer/labelling/Pages/allergenlabelling.aspx

Freeman, H. J. (2018). Gastroenterology and Hepatology From Bed to Bench. Role of biopsy in diagnosis and treatment of adult celiac disease. *Gastroenterol Hepatol Bed Bench*, *11*(3), 191–196.

Galli, G., Lahner, E., Conti, L., Esposito, G., Sacchi, M. C., & Annibale, B. (2018). Risk factors associated with osteoporosis in a cohort of prospectively diagnosed adult coeliac patients. *United European Gastroenterology Journal*, *6*(8), 1161–1168. https://doi.org/10.1177/2050640618784340

Gładyś, K., Dardzińska, J., Guzek, M., Adrych, K., Kochan, Z., & Małgorzewicz, S. (2021). Expanded role of a dietitian in monitoring a gluten-free diet in patients with celiac disease: Implications for clinical practice. *Nutrients*, *13*(6). https://doi.org/10.3390/nu13061859

Gładyś, K., Dardzińska, J., Guzek, M., Adrych, K., & Małgorzewicz, S. (2020). Celiac dietary adherence test and standardized dietician evaluation in assessment of adherence to a gluten-free diet in patients with celiac disease. *Nutrients*, *12*(8), 1–10. https://doi.org/10.3390/nu12082300

Gutowski, E. D., Weiten, D., Green, K. H., Rigaux, L. N., Bernstein, C. N., Graff, L. A., Walker, J. R., Duerksen, D. R., & Silvester, J. A. (2020). Can individuals with celiac disease identify gluten-free foods correctly? *Clinical Nutrition*

ESPEN, *36*, 82–90. https://doi.org/10.1016/j.clnesp.2020.01.012

Halmos, E. P., Deng, M., Knowles, S. R., Sainsbury, K., Mullan, B., & Tye-Din, J. A. (2018). Food knowledge and psychological state predict adherence to a gluten-free diet in a survey of 5310 Australians and New Zealanders with coeliac disease. *Alimentary Pharmacology and Therapeutics*, *February*, 78–86. https://doi.org/10.1111/apt.14791

Hardy, M. Y., & Tye-Din, J. A. (2016). Coeliac disease: a unique model for investigating broken tolerance in autoimmunity. *Clinical & Translational Immunology*, *5*(11), e112. https://doi.org/10.1038/cti.2016.58

Horan, M. P., Chai, S. Y., Munusamy, N., Tay, K. H., Wienholt, L., Tye-Din, J. A., Varney, M., & Badrick, T. (2018). High rates of variation in HLA-DQ2/DQ8 testing for coeliac disease: results from an RCPAQAP pilot program. *Journal of Clinical Pathology*, *71*(10), 900–905. https://doi.org/10.1136/jclinpath-2018-205209

Karla, M.-T., Sustmann, A., Katharina, W., & Koletzko, S. (2021). *Online Course " Celiac Facts " for Physicians & Dietitians: Lesson 4 - Monitoring of Celiac Disease*. Available from: https://celiacfacts-onlinecourses.eu/mod/lesson/view.php?id=725

Kenrick, K. M. (2018). *The Recognition, Diagnosis, and Management of Coeliac Disease in New Zealand*. (Thesis, Doctor of Philosophy). University of Otago. Available from http://hdl.handle.net/10523/9415

King, J. A., Jeong, J., Underwood, F. E., Quan, J., Panaccione, N., Windsor, J. W., Coward, S., Debruyn, J., Ronksley, P. E., Shaheen, A. A., Quan, H., Godley, J., Veldhuyzen Van Zanten, S., Lebwohl, B., Ng, S. C., Ludvigsson, J. F., & Kaplan, G. G. (2020). Incidence of Celiac Disease Is Increasing over Time: A Systematic Review and Meta-analysis. *American Journal of Gastroenterology*, *115*(4), 507–525. https://doi.org/10.14309/ajg.0000000000000523

Lau, M. S., Mooney, P. D., Rees, M. A., White, W. L., Marks, L. J., Hadjivassiliou, M., & Sanders, D. S. (2018). Gluten free diet adherence assessment using

CDAT and BIAGI questionnaires in patients with coeliac disease. *Gut*, *67*(160–161). https://doi.org/10.1136/gutjnl-2019-bsgabstracts.438

Lebwohl, B., Sanders, D. S., & Green, P. H. R. (2018). Coeliac disease. *The Lancet*, *391*, 70–81. https://doi.org/10.1016/S0140-6736(17)31796-8

Lee, A. R., Wolf, R. L., Lebwohl, B., Ciaccio, E. J., & Green, P. H. R. (2019). Persistent economic burden of the Gluten free diet. *Nutrients*, *11*(2), 1–8. https://doi.org/10.3390/nu11020399

Leffler, D. A., Dennis, M., Edwards George, J. B., Jamma, S., Magge, S., Cook, E. F., Schuppan, D., & Kelly, C. P. (2009). A Simple Validated Gluten-Free Diet Adherence Survey for Adults With Celiac Disease. *Clinical Gastroenterology and Hepatology*, *7*(5), 530-536.e2. https://doi.org/10.1016/j.cgh.2008.12.032

Mahadev, S., Simpson, S., Lebwohl, B., Lewis, S. K., Tennyson, C. A., & Green, P. H. R. (2013). Is dietitian use associated with celiac disease outcomes? *Nutrients*, *5*(5), 1585–1594. https://doi.org/10.3390/nu5051585

Marsilio, I., Canova, C., D'odorico, A., Ghisa, M., Zingone, L., Lorenzon, G., Savarino, E. V., & Zingone, F. (2020). Quality-of-life evaluation in Coeliac patients on a gluten-free diet. *Nutrients*, *12*(10), 1–10. https://doi.org/10.3390/nu12102981

McLean, R., Edmonds, J., Williams, S., Mann, J., & Skeaff, S. (2015). Balancing sodium and potassium: Estimates of intake in a New Zealand adult population sample. *Nutrients*, *7*(11), 8930–8938. https://doi.org/10.3390/nu7115439

McLean, R. M. (2014). Measuring population sodium intake: A review of methods. *Nutrients*, *6*(11), 4651–4662. https://doi.org/10.3390/nu6114651

Mhurchu, C. N., & Gorton, D. (2007). Nutrition labels and claims in New Zealand and Australia: a review of use and understanding. *Australian and New Zealand Journal of Public Health*, *31*(2), 105–112. https://doi.org/10.1111/j.1753-6405.2007.00026.x

Ministry for Primary Industries (MPI). (2022). *Allergen labelling - Knowing what's in your food and how to label it*. Available from: https://www.mpi.govt.nz/food

-business/labelling-composition-food-drinks/allergen-declarations-warnings-and-advisory-statements-on-food-labels/

Ministry for Primary Industries and Ministry of Health. (2016). *Mandatory iodine fortification in New Zealand: supplement to the Australian Institute of Health and Welfare 2016 report - Monitoring the health impacts of mandatory folic acid and iodine fortification*. Ministry for Primary Industries Technical Paper No: 2016/32. Available from: https://www.mpi.govt.nz/dmsdocument/12786/sitemap

Moore, S. G., Donnelly, J. K., Jones, S., & Cade, J. E. (2018). Effect of educational interventions on understanding and use of nutrition labels: A systematic review. *Nutrients*, *10*(10), 1–14. https://doi.org/10.3390/nu10101432

Muhammad, H., Reeves, S., & Jeanes, Y. M. (2019). Identifying and improving adherence to the gluten-free diet in people with coeliac disease. *Proceedings of the Nutrition Society*, *78*(3), 418–425. https://doi.org/10.1017/S002966511800277X

Muhammad, Humayun, Reeves, S., Ishaq, S., Mayberry, J., & Jeanes, Y. M. (2017). Adherence to a gluten free diet is associated with receiving gluten free foods on prescription and understanding food labelling. *Nutrients*, *9*(7). https://doi.org/10.3390/nu9070705

New Zealand Institute of Economic Research (NZIER). (2021). *A critical missing ingredient: The case for increased dietetic input in tier 1 health services* (Issue April). Available from: https://www.nzier.org.nz/publications/a-critical-missing-ingredient-the-case-for-increased-dietetic-input-in-tier-1-health-services

New Zealand Institute of Plant and Food Research. (2013). *The Concise New Zealand Food Composition Tables. 10th edition.* Palmerston North: New Zealand Institute of Plant and Food Research and The New Zealand Ministry of Health. Available from: www.foodcomposition.co.nz

Paganizza, S., Zanotti, R., D'Odorico, A., Scapolo, P., & Canova, C. (2019). Is adherence to a gluten-free diet by adult patients with celiac disease influenced

by their knowledge of the gluten content of foods? *Gastroenterology Nursing*, *42*(1), 55–64. https://doi.org/10.1097/SGA.0000000000000368

Pantaleoni, S., Luchino, M., Adriani, A., Pellicano, R., Stradella, D., Ribaldone, D. G., Sapone, N., Isaia, G. C., Di Stefano, M., & Astegiano, M. (2014). Bone Mineral Density at Diagnosis of Celiac Disease and after 1 Year of Gluten-Free Diet. *The Scientific World Journal*, *2014*, 1–6. https://doi.org/10.1155/2014/173082

Rajpoot, P., Sharma, A., Harikrishnan, S., Baruah, B. J., Ahuja, V., & Makharia, G. K. (2015). Adherence to gluten-free diet and barriers to adherence in patients with celiac disease. *Indian Journal of Gastroenterology*, *34*(5), 380–386. https://doi.org/10.1007/s12664-015-0607-y

Sharp, K., Walker, H., & Coppell, K. J. (2014). Coeliac disease and the gluten-free diet in New Zealand: The New Zealand Coeliac Health Survey. *Nutrition and Dietetics*, *71*(4), 223–228. https://doi.org/10.1111/1747-0080.12105

Suarez-Lledo, V., & Alvarez-Galvez, J. (2021). Prevalence of health misinformation on social media: Systematic review. *Journal of Medical Internet Research*, *23*(1). https://doi.org/10.2196/17187

Szymczak, J., Bohdanowicz-Pawlak, A., Waszczuk, E., & Jakubowska, J. (2012). Low bone mineral density in adult patients with coeliac disease. *Endokrynologia Polska*, *63*(4), 270–276.

Villafuerte-Galvez, J., Vanga, R. R., Dennis, M., Hansen, J., Leffler, D. A., Kelly, C. P., & Mukherjee, R. (2015). Factors governing long-term adherence to a gluten-free diet in adult patients with coeliac disease. *Alimentary Pharmacology and Therapeutics*, *42*(6), 753–760. https://doi.org/10.1111/apt.13319

Vuolle, S., Laurikka, P., Repo, M., Huhtala, H., Kaukinen, K., Kurppa, K., & Kivelä, L. (2022). *Persistent symptoms are diverse and associated with health concerns and impaired quality of life in patients with paediatric coeliac disease diagnosis after transition to adulthood*. 1–7. https://doi.org/10.1136/bmjgast-2022-000914

White, L. E., Bannerman, E., & Gillett, P. M. (2016). Coeliac disease and the gluten-free diet: a review of the burdens; factors associated with adherence and impact on health-related quality of life, with specific focus on adolescence. *Journal of Human Nutrition and Dietetics*, *29*(5), 593–606. https://doi.org/10.1111/jhn.12375

Wieser, H., Ruiz-Carnicer, Á., Segura, V., Comino, I., & Sousa, C. (2021). Challenges of monitoring the gluten-free diet adherence in the management and follow-up of patients with celiac disease. *Nutrients*, *13*(7), 9–13. https://doi.org/10.3390/nu13072274

Xhakollari, V., Canavari, M., & Osman, M. (2019). Factors affecting consumers' adherence to gluten-free diet, a systematic review. *Trends in Food Science and Technology*, *85*(December 2018), 23–33. https://doi.org/10.1016/j.tifs.2018.12.005

Zhang, Y. Y., Hughes, J., & Grafenauer, S. (2020). Got mylk? The emerging role of australian plant-based milk alternatives as a cow's milk substitute. *Nutrients*, *12*(5). https://doi.org/10.3390/nu12051254

Zhu, X., Zhao, X.-H., Zhang, Q., Zhang, N., Soladoye, O. P., Aluko, R. E., Zhang, Y., & Fu, Y. (2022). How does a celiac iceberg really float? The relationship between celiac disease and gluten. *Critical Reviews in Food Science and Nutrition*, 1–29. https://doi.org/10.1080/10408398.2022.2064811

CHAPTER 8

Discussion, Conclusion and Areas for Future Research

8.1 Discussion

Coeliac disease (CD) is a lifelong autoimmune disease which results in damage to the small intestine in response to the consumption of gluten, leading to malabsorption of nutrients (Kaur et al., 2017). In addition to gastrointestinal symptoms commonly experienced by patients with CD, comorbidities are common as a result of the inflammation and nutrient deficiencies experienced (Kreutz et al., 2020). Of these comorbidities, one that has been researched extensively is low bone mineral density (BMD), observed in both newly diagnosed children and adults (Galli et al., 2018; Lerner & Matthias, 2016). Despite extensive research indicating there is a strong relationship between CD and low BMD, a study in New Zealand found no such link (Bolland et al., 2016). Low BMD is primarily thought to be caused by nutrient deficiencies as a result of malabsorption of nutrients such as calcium, vitamin D, iodine and magnesium which is commonly experienced by patients with CD (Cardo et al., 2021). In New Zealand there has been limited research on these deficiencies in the coeliac population, however deficiencies in vitamin D and iodine are common in the general population (Brough & Skeaff, 2020; Nowson et al., 2012).

In addition to the contribution of nutrient deficiencies to low BMD in patients with CD, research suggests that low BMD persists after adherence to a gluten-free diet (GFD) implicating other potential causes (Lerner & Matthias, 2016). Previous hypothesised explanations include secondary hyperparathyroidism, activity of pro-inflammatory cytokines and uncoupling of bone formation and resorption (Kotze et al., 2016). The latter of these explanations led to the hypothesis for this PhD and may be explained by the activity of RANK ligand (RANKL). RANKL stimulates differentiation of microfold cells in the small intestine, which have an important role in translocation of luminal antigens across

the gut epithelium (Williams & Owen, 2015), as well as promoting bone resorption by stimulating activation of osteoclasts (Martin & Sims, 2015). It is suggested that the ratio of OPG:RANKL may be lower in patients with CD who have a lower BMD (Fiore et al., 2006), implicating this pathway as a potential area for research in investigation of low BMD in CD patients.

The primary aim of this study was to investigate bone mineral density (BMD) in premenopausal and otherwise healthy women with coeliac disease (CD) in New Zealand. We hypothesised that BMD would be lower in this group when compared to healthy controls and hoped to investigate the underlying mechanisms behind this observed low BMD using intestinal organoids grown from biopsy tissue from individuals with and without CD. Unfortunately, although we were able to establish the methodology to investigate these mechanisms in organoids prepared from murine tissue (chapter 3), we were unable to develop this approach using human biopsy tissue as laboratory work was unable to proceed due to issues caused by SARS-CoV-2. However, we observed no differences in BMD in premenopausal women (aged 18-40 years) with CD when compared with age-matched healthy controls, disproving our hypothesis that CD participants would have lower BMD (chapter 5). Despite seeing no difference in BMD as assessed through DXA scans, a statistically significant difference was observed in findings reported from quantitative ultrasound (QUS) supporting previous research that trabecular bone may be affected in patients with CD (Pham-Short et al., 2019) and suggesting QUS may not be the most appropriate approach to assess bone in individuals with CD. In addition, in contrast to previous research (Melini & Melini, 2019), the 4-day diet diaries (4DDD) indicated no statistically significant differences in intakes of potassium, calcium, magnesium, phosphorus or zinc between the two groups.

Assessment of the *Close to the Bone* cohort also allowed for investigation of iodine status (chapter 6), and found it was significantly lower in premenopausal women with CD compared with healthy controls. In part, this may be due to lower

iodine fortification levels of GF breads compared with their gluten-containing counterparts (New Zealand Institute of Plant and Food Research, 2013). In addition, low bread intake was observed in both the CD group and in the control group suggesting that bread is not contributing as much to iodine intake as was modelled when establishing the fortification level (Food Standards Australia New Zealand, 2006). This observed low intake of GF bread in the coeliac cohort was supported by similar low intakes in the coeliac cohort of the *A Gut Feeling* study (chapter 7).

Although the *A Gut Feeling* study found a good level of adherence to a GFD among participants, this preliminary study also highlighted apparent confusion regarding understanding of food labels, with many participants reporting they would avoid foods such as glucose syrup and caramel that contain wheat despite these being recognised as being gluten-free (GF) in New Zealand (Coeliac New Zealand, n.d.). Concerns regarding advice provided about the GFD, in particular the safety of consuming oats, indicate a need for further research to investigate the quality of information available to people newly diagnosed with CD. In addition, it was evident that there are inconsistencies in healthcare practices related to the recommended referrals for DXA scans at diagnosis and follow-up.

8.2 Areas for future research

Future research is needed to continue the investigation of the possible role of M-cells in the gut-bone cross talk and further examine the RANKL/RANK/OPG pathway in CD. Utilisation of the organoid model derived from human small intestinal biopsy samples to investigate these mechanisms is warranted. Surprisingly, despite the considerable advantages of the *in vitro* organoid model for the study of intestinal function, this was one of very few studies with only two previous publications using this model to investigate CD (Dieterich et al., 2020; Freire et al., 2019; Porpora et al., 2022).

Although the findings of our assessment of bone density indicated that there was no significant difference in BMD of premenopausal women with CD when compared to age-matched healthy controls, the sample group used for this research was small and further research is warranted to investigate these results on a larger scale. Investigation of bone density at the time of diagnosis and again a year following diagnosis in a longitudinal study of newly diagnosed CD patients would allow for more appropriate assessment of BMD with removal of potential selection biases, commonly reported in research studies (Young et al., 2020), if all newly diagnosed individuals in New Zealand were invited to take part. In addition, the *Close to the Bone* study only assessed BMD in premenopausal women; assessment of BMD in other age and gender groups is needed to confirm the observed lack of relationship between CD and low BMD. Inclusion of a young cohort who are below the age of achieving peak bone mass (PBM) would be instrumental in identifying the best practice for referral as well as nutritional and lifestyle advice, enhancing the chances of them gaining PBM despite having CD. In addition, assessment of OPG and RANKL in these individuals would provide greater understanding of the RANKL/RANK/OPG pathway in CD. Recent research investigating the role gut microbiota in bone metabolism highlights another area for future research (Rettedal et al., 2021). Gut microbiota have been implicated for both the pathogenesis and ongoing inflammatory milieu observed in CD (Galipeau & Verdu, 2022; Leonard et al., 2021). Further research to investigate the gut microbiota and the association with bone metabolism in people with CD is warranted.

The findings of the *Close to the Bone* study identify potential concerns regarding iodine status in women with CD. It is unclear whether the lower results observed in these women are a result of low dietary intake of iodine, with the GFD previously found to be a poor source of iodine (Cardo et al., 2021), or whether either the absorption of iodine or requirement for iodine may be impacted by this disease. Assessment of iodine status using the gold standard 24-hour urine

collection and assessing multiple samples is required in a larger cohort including other age and gender groups to accurately establish the iodine status in those with CD would be beneficial.

The low intake of bread in both the *Close to the Bone* and the *A Gut Feeling* studies is of concern. Bread intake in coeliac participants in both studies was well below the intake modelled for setting fortification levels for iodine (Food Standards Australia New Zealand, 2006). The bread intake in the healthy control group of the *Close to the Bone* study was similarly low. This may be due to reduced bread consumption because of concerns regarding carbohydrate intake which has been identified in recent research studies (Lockyer & Spiro, 2020). However, this low bread intake could suggest that fortification levels need to be reconsidered to establish if mandatory fortification is adequate. Further research is needed to assess the intake of bread in New Zealand, with a particular focus given to those consuming a GFD, where breads were not fortified to the same extent. In addition, an up-to-date assessment of the iodine content of GF breads is needed to establish if the low levels of iodine in GF bread reported post-fortification (New Zealand Institute of Plant and Food Research, 2013) are still relevant in 2022. Future research should consider the nutritional composition of the GFD for both individuals with CD and those with non-coeliac gluten sensitivity (NCGS) where it is assumed they are strictly adhering to the GFD in the absence of the inflammatory process observed in CD. This would allow for further assessment of iodine intake, consideration of iodine requirements in individuals with CD, and whether there is poor absorption of iodine or greater utilisation/excretion in this group.

The low intake of bread reported in these studies also has implications for the newly mandated fortification of gluten-containing bread products with folic acid (Ministry for Primary Industries, 2021). Although findings from the *Close to the Bone* study indicate no significant difference in folate status between premenopausal women with CD and healthy controls, previous research indicates

that the GFD is often low in folate (Melini & Melini, 2019), suggesting that further research is needed to explore folate status in individuals with CD after the fortification policy has been fully implemented and confirm that the fortification policy is not further exacerbating health discrepancies. This research strategy would help to establish whether there is a need for targeted preconceptual nutrition counselling for those consuming a GFD.

The findings of the *A Gut Feeling* study also highlight a need for further research investigating sources of advice and where misleading or contradictory advice originates. It is unclear from the findings of the study whether this advice is merely perceived to be contradictory or whether the advice itself is incorrect. The qualitative data from this study could be used to design a follow-up study to investigate the sources of advice, and how the advice is interpreted, in more depth. Although the Biagi adherence score indicated good adherence to the GFD, further research is needed to validate the tool in the New Zealand setting. Establishing a validated New Zealand based tool to assess adherence to a GFD which includes understanding of food labelling would assist in ensuring patients are adopting best practice, whilst not excessively restricting their diet. Results using such a tool could have important public health nutrition implications, for example for developing clear and consistent guidelines for people newly diagnosed with CD.

8.3 Conclusion

The findings of this thesis support previous research in New Zealand which suggest that individuals with CD are at no greater risk of low BMD than healthy controls. However, as the cohort assessed was small and only included premenopausal women, further research is needed to confirm if these findings are applicable to the CD population of New Zealand as a whole. One major and unexpected finding of this research was the significant difference in iodine status found between CD participants and healthy controls. With the *A Gut Feeling* study highlighting similar concerns regarding dietary iodine sources in a larger cohort of

coeliac participants, further research is needed to investigate the extent of this potential problem.

8.4 References

Bolland, M. J., Grey, A., & Rowbotham, D. S. (2016). Outcomes of bone density measurements in coeliac disease. *New Zealand Medical Journal*, *129*(1429), 40–44.

Brough, L., & Skeaff, S. (2020). Iodine status in New Zealand. In P. A. C. Richards, *Iodophor: Tamed Iodine: The Status of Iodine in Tasmania*. Gatekeeper Press

Cardo, A., Churruca, I., Lasa, A., Navarro, V., Vázquez-Polo, M., Perez-Junkera, G., & Larretxi, I. (2021). Nutritional imbalances in adult celiac patients following a gluten-free diet. *Nutrients*, *13*(8), 1–18. https://doi.org/10.3390/nu13082877

Coeliac New Zealand. (n.d.). *How to read food labels*. Coeliac New Zealand. https://coeliac.org.nz/how-to-read-food-labels/

Dieterich, W., Neurath, M. F., & Zopf, Y. (2020). Intestinal ex vivo organoid culture reveals altered programmed crypt stem cells in patients with celiac disease. *Scientific Reports*, *10*(1), 1–10. https://doi.org/10.1038/s41598-020-60521-5

Fiore, C. E., Pennisi, P., Ferro, G., Ximenes, B., Privitelli, L., Mangiafico, R. A., Santoro, F., Parisi, N., & Lombardo, T. (2006). Altered Osteoprotegerin/RANKL Ratio and Low Bone Mineral Density in Celiac Patients on Long-term Treatment with Gluten-free Diet. *Horm Metab Res*, *38*(6), 417–422. https://doi.org/10.1055/s-2006-944548

Food Standards Australia New Zealand. (2006). *Final Assessment Report: Proposal P295. Consideration of mandatory fortification with folic acid*. Available from: http://www.foodstandards.govt.nz/code/proposals/documents/FAR_P295_Folic_Acid_Fortification_ Attachs_1_6.pdf

Freire, R., Ingano, L., Serena, G., Cetinbas, M., Anselmo, A., Sapone, A., Sadreyev, R. I., Fasano, A., & Senger, S. (2019). Human gut derived-organoids provide model to study gluten response and effects of microbiota-derived molecules in

celiac disease. *Scientific Reports*, *9*(1), 1–15. https://doi.org/10.1038/s41598-019-43426-w

Galipeau, H. J., & Verdu, E. F. (2022). The double-edged sword of gut bacteria in celiac disease and implications for therapeutic potential. Mucosal Immunology, 15(2), 235–243. https://doi.org/10.1038/s41385-021-00479-3

Galli, G., Lahner, E., Conti, L., Esposito, G., Sacchi, M. C., & Annibale, B. (2018). Risk factors associated with osteoporosis in a cohort of prospectively diagnosed adult coeliac patients. *United European Gastroenterology Journal*, *6*(8), 1161–1168. https://doi.org/10.1177/2050640618784340

Kaur, A., Shimoni, O., & Wallach, M. (2017). Celiac disease: from etiological factors to evolving diagnostic approaches. *Journal of Gastroenterology*, *52*(9), 1001–1012. https://doi.org/10.1007/s00535-017-1357-7

Kotze, L. M. S., Skare, T., Vinholi, A., Jurkonis, L., & Nisihara, R. (2016). Impact of a gluten-free diet on bone mineral density in celiac patients. *Revista Espanola de Enfermedades Digestivas*, *108*(2), 84–88. https://doi.org/10.17235/reed.2015.3953/2015

Kreutz, J. M., Adriaanse, M. P. M., van der Ploeg, E. M. C., & Vreugdenhil, A. C. E. (2020). Narrative review: Nutrient deficiencies in adults and children with treated and untreated celiac disease. *Nutrients*, *12*(2). https://doi.org/10.3390/nu12020500

Leonard, M. M., Valitutti, F., Karathia, H., Pujolassos, M., Kenyon, V., Fanelli, B., Troisi, J., Subramanian, P., Camhi, S., Colucci, A., Serena, G., Cucchiara, S., Trovato, C. M., Malamisura, B., Francavilla, R., Elli, L., Hasan, N. A., Zomorrodi, A. R., Colwell, R., & Fasano, A. (2021). Microbiome signatures of progression toward celiac disease onset in at-risk children in a longitudinal prospective cohort study. Proceedings of the National Academy of Sciences of the United States of America, 118(29). https://doi.org/10.1073/pnas.2020322118

Lerner, A., & Matthias, T. (2016). Gut-bone Cross Talks and Implications in Celiac Disease. *International Journal of Celiac Disease*, *4*(1), 19–23. https://doi.org/10.

12691/ijcd-4-1-4

Lockyer, S., & Spiro, A. (2020). The role of bread in the UK diet: An update. *Nutrition Bulletin*, *45*(2), 133–164. https://doi.org/10.1111/nbu.12435

Martin, T. J., & Sims, N. A. (2015). RANKL/OPG; Critical role in bone physiology. *Reviews in Endocrine and Metabolic Disorders*, *16*(2), 131–139. https://doi.org/10.1007/s11154-014-9308-6

Melini, V., & Melini, F. (2019). Gluten-Free Diet: Gaps and Needs for a Healthier Diet. *Nutrients*, *11*(1), 170. https://doi.org/10.3390/nu11010170

Ministry for Primary Industries (MPI). (2021b). *Folic acid fortification: Summary and analysis of submissions on New Zealand Food Safety Discussion Paper No: 2019/08.* Ministry for Primary Industries Paper No. 2021/03. (Issue ISSN No: 2253-394X). Available from: https://www.mpi.govt.nz/dmsdocument/45838-Folic-acid-fortification-Summary-and-analysis-of-submissions-on-New-Zealand-Food-Safety-Discussion-Paper

New Zealand Institute of Plant and Food Research. (2013). *The Concise New Zealand Food Composition Tables. 10th edition.* Palmerston North: New Zealand Institute of Plant and Food Research and The New Zealand Ministry of Health, Available from: www.foodcomposition.co.nz

Nowson, C. A., McGrath, J. J., Ebeling, P. R., Haikerwal, A., Daly, R. M., Sanders, K. M., Seibel, M. J., & Mason, R. S. (2012). Vitamin D and health in adults in Australia and New Zealand: A position statement. *Medical Journal of Australia*, *196*(11), 686–687. https://doi.org/10.5694/mja11.10301

Pham-Short, A., Donaghue, K. C., Ambler, G., Briody, J., Garnett, S., Munns, C. F., & Craig, M. E. (2019). Abnormal cortical and trabecular bone in youth with type 1 diabetes and celiac disease. *Diabetes Care*, *42*(8), 1489–1495. https://doi.org/10.2337/dc18-2376

Porpora, M., Conte, M., Lania, G., Bellomo, C., Rapacciuolo, L., Chirdo, F. G., Auricchio, R., Troncone, R., Auricchio, S., Barone, M. V., & Nanayakkara, M. (2022). Inflammation Is Present, Persistent and More Sensitive to

Proinflammatory Triggers in Celiac Disease Enterocytes. *International Journal of Molecular Sciences*, *23*(4). https://doi.org/10.3390/ijms23041973

Rettedal, E. A., Ilesanmi-Oyelere, B. L., Roy, N. C., Coad, J., & Kruger, M. C. (2021). The Gut Microbiome Is Altered in Postmenopausal Women with Osteoporosis and Osteopenia. JBMR Plus, 5(3), 0–3. https://doi.org/10.1002/jbm4.10452

Williams, I. R., & Owen, R. L. (2015). M Cells: Specialized Antigen Sampling Cells in the Follicle-Associated Epithelium. *Mucosal Immunology: Fourth Edition*, *1–2*(Figure 1), 211–229. https://doi.org/10.1016/B978-0-12-415847-4.00013-6

Young, L. M., Gauci, S., Scholey, A., White, D. J., & Pipingas, A. (2020). Self-Selection Bias: An Essential Design Consideration for Nutrition Trials in Healthy Populations. *Frontiers in Nutrition*, *7*(November), 1–5. https://doi.org/10.3389/fnut.2020.587983

APPENDIX A

Details of Organoid Preparation

Component sources

- Murine Noggin (PT2503820) and human R-Spondin-1 (120-38-20) were purchased from Peprotech.
- Corning® Matrigel® (356231) (Growth factor reduced (GFR) basement membrane matrix, phenol red-free, LDEV-free and with a protein conc. > 9 mg/mL) was supplied by BioStrategy.
- Recombinant mouse RANKL (TRANCE/RANK L/TNFSF11 (E. coli-expressed), R&D Systems) was obtained from In Vitro Technologies.
- Advanced DMEM/F-12 (12634010), GlutaMAX™ Supplement (35050061), Penicillin-Streptomycin-Neomycin (PSN) Antibiotic Mixture (15640055), N-2 Supplement (100X), Liquid (17502048), B-27® Supplement Minus Vitamin A (12587010) and Cell strainers, 70 µm pore size, white frame (734-0003) were purchased from ThermoFisher.
- N-acetyl-L-cysteine (A9165-5G), Sodium hydroxide (795429-500G), Sodium chloride (S9888-500G), Potassium chloride (P3911-500G), Potassium phosphate monobasic (P5655-100G) and Sodium phosphate dibasic (795410-500G) were purchased from Sigma Aldrich
- Anti-GP2 (Glycoprotein 2) (Mouse) monoclonal antibody was purchased from Jomar Scientific.

- Mouse anti-occludin antibody (Clone OC-3F10) and rabbit anti-ZO-1, N-terminal region and secondary antibody biotin-XX goat anti-mouse IgG and streptavidin, Alexa Fluor 546 conjugate were purchased from Invitrogen.

Media composition

Table A.1. EDTA Crypt Isolation Solution (2mM EDTA)

Component	**Volume added**
PBS	14.79 mL
0.5M Ultrapure EDTA	60 µL
HEPES	150 µL
Osmolarity was adjusted to be in range of 280-305 mOsm	

Table A.2. Medium for last organoid centrifugation

Component	**Volume added**
F12 Media	50 mL
Hepes (1M)	500 µL (10mM)

Table A.3. Complete medium composition/ mL (used at 500 µL/well)

Component	Added	Final Concentration
F-12	1 mL	
NAC (500mM)	2 µL	1mM
HEPES	10 µL	10mM
P/S (100x)	10 µL	1X
Glutamax	10 µL	2mM
N2 (100x stock)	10 µL	1X
B27 (50x stock)	20 µL	1X

Growth Factors

	1st Plating	Subsequent REN
R-spondin (5 µL =500 ng/mL)	5 µL	2.5 µL
EGF (5 µL = 50 ng/mL)	5 µL	2.5 µL
Noggin (5 µL = 100 ng/mL)	5 µL	2.5 µL

REN = 2.5µl R-spondin, 2.5µl EGF, 2.5µl Noggin plus 45µl F-12 media

NB: Complete Media/REN was made up fresh each day it was required; REN only lasts up to 4 days in the fridge

APPENDIX B

Close to the Bone Information Sheet and Consent form

MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

Nutrition Science Group
School of Food & Advanced Technology
College of Sciences

CLOSE TO THE BONE

INFORMATION SHEET

Researcher(s) Introduction

The research group is a team of nutritionists interested in bone health. The lead researcher for this project is Katie Schraders, a PhD student from the School of Food & Advanced Technology (SFAT). Other researchers include Professor Marlena Kruger, Professor Jane Coad, Dr Louise Brough and Dr Janet Weber, also from SFAT.

Project Description and Invitation

Osteoporosis is one of the leading healthcare issues worldwide. Osteoporosis is characterised by changes in bone structure resulting in increased fragility and risk of fractures in older adults. It is estimated that about 22% of women over 50 years of age will develop osteoporosis.

This study aims to investigate the links between bone health and lifestyle factors, specifically the gluten free diet consumed by people with coeliac disease and non-coeliac gluten sensitivity.

If you would be interested in participating, please contact Katie on (06) 356 9099 ext. 85541 or email CloseToTheBone@massey.ac.nz

Participant Identification and Recruitment

We are recruiting women from the lower North Island. We are advertising the study in local media and social media, tertiary education institutes and workplaces and with the assistance of GP clinics and health practitioners. We aim to recruit a total of 141 women; 47 women with coeliac disease (CD), 47 women with non-coeliac gluten sensitivity (NCGS) and a control group of 47 healthy women. These numbers are required for the results to be statistically significant.

Inclusion criteria

To become a participant, you will need to:

- Be a healthy female
  OR
- Be a female medically diagnosed with coeliac disease, who has consumed a strict gluten free diet for a year or more
  OR
- Be a female medically diagnosed with non-coeliac gluten sensitivity, who has consumed a strict gluten free diet for a year or more
  AND
- Be aged between 18-40 years
- Live in the Palmerston North and Wellington Regions

Exclusion criteria

Unfortunately, you cannot participate if you:

- Have previously/currently use medication affecting bone metabolism e.g. corticosteroids (daily for >3 months), bisphosphonates, selective oestrogen receptor modulators
- Have a medical condition affecting bone health e.g. osteogenesis imperfecta, chronic renal (kidney) disease, uncontrolled thyroid disease, inflammatory bowel disease (Crohn's or ulcerative colitis) etc.
- Are currently pregnant or lactating
- Have a known history of inflammatory conditions (other than CD and NCGS)
- Have a BMI>30
- Have not had your condition medically diagnosed according to standard criteria (for the CD and NCGS groups)

If you are unsure whether you are eligible to participate, please contact the lead researcher for further information: Katie Schraders, CloseToTheBone@massey.ac.nz

What is involved?

Healthy control group – prior to participating

If you are part of our healthy control group, we will ask you to undergo a blood test to confirm that you do not have coeliac disease. This test looks for antibodies that are present if you have untreated coeliac disease. If the test identifies anything that needs further investigation, you will receive your test results and a letter to take with you to your GP. Unfortunately, this will mean that you are not eligible to continue with the study.

Everyone – before you come

Prior to your visit to Massey University, we will send you a diet diary and request that for 3-4 days you record everything you eat and drink. Instructions will be provided.

Everyone – Visit to the HNRU

Once you have completed this we will invite you to come into the Human Nutrition Research Unit (HNRU) at Massey University, in Palmerston North. This visit needs to be in the morning between 8-10am and before you have had any breakfast as we will be starting the visit by taking a few blood samples.
Note: if you are travelling from out of town we can schedule the fasted/time dependent blood tests at your local medical laboratory so that you don't have to travel too far without breakfast.

Participants are welcome to bring whanau or a support person along to this visit.

We will also ask you to provide a sample of urine and to do a finger prick collection of a few drops of blood to test for anaemia.

After you have provided the blood and urine samples, we will provide you with breakfast. During this time, we may ask you about your diet diary.

Once you have finished breakfast we will ask you to answer some questions about your health, lifestyle, diet and physical activity as well as some questions about how much you know about osteoporosis. This is not a test so please do not study for it, we are simply interested in information

you've picked up and where you have got this from. You will not be marked on the number of correct answers!

After you have completed the paperwork, we have a new machine that measures your bone quality by ultrasound. The Quantitative Ultrasound transmits and receives sound waves; the intensity and speed of these sound waves are altered by the properties of your bones. Quantitative ultrasound measurements are pretty close to results from medically established methods for diagnosing low bone mineral density. The best part about the Quantitative Ultrasound is that it produces your results pretty much immediately, so you can keep your results after the measurement.

For the last part of your visit we will use a DXA (Dual-emission X-ray Absorptiometry) to measure your bone density, this machine uses very low dose radiation X-rays to measure the bone density of your hip, forearm and spine, and also measures your body composition (fat mass, lean mass, and bone mass of your body).

Tests involved – in more detail:

Venous blood sample:

Trained staff will take blood samples from your preferred arm by venepuncture, which is a routine procedure whereby a needle inserted into a vein in the inside of your elbow to collect a blood sample. We know that obtaining blood samples can sometimes cause mild discomfort. We will therefore ask you to sit in a comfortable chair or lie down and the samples will be taken by trained and experienced staff who will do their utmost to reduce this discomfort. The blood sample will be no more than 44mL (equivalent to about 2 ½ tablespoons in volume).

Once collected blood samples will be sent to Canterbury Health and Auckland DHB laboratories to measure markers of inflammatory status, bone metabolism, thyroid function, calcium homeostasis and coeliac disease.

Finger prick blood sample:

Trained staff will take a capillary blood sample from your finger using lancet and HEMOCUE Hb 201+ to measure whole blood Haemoglobin value. We will ask you to stay seated in a comfortable chair and the amount of blood taken for this test will only be 2-3 drops. Results from this test only take a few seconds so we will be able to tell you your haemoglobin results straight away.

Urine sample:

For this we ask that you use the private bathroom at the research unit and provide a small mid-stream urine sample in the provided collection cup. Urine samples will be collected to estimate iodine intake (by measuring both iodine and creatinine excreted).

Quantitative Ultrasound bone scan:

Our Achilles Quantitative Ultrasound machine will provide a quick safe and comfortable scan of your non-dominant heel bone using sound-waves. You will feel a warm water filled cushion hugging your heel during your scan. The procedure is quick; just a few minutes from shoe off to shoe on. We will provide you with a printout of your results, for you to keep.

DXA Dual-emission X-ray absorptiometry bone scan:

Measurement involves you lying down on a bed fully clothed in your own clothing without any metal components (e.g. zips, studs, underwire) or surgical scrubs (that we can provide) and scan your bones on the Hologic DXA machine. This machine is used to estimate bone mineral density and bone mineral content of your hip, forearm and lumbar spine (L1-L4) and total body composition.

The DXA has X-ray beams of different energies and, while no dose of radiation is harmless, this dose is very low and unlikely to cause harm. The total effective dose of radiation to which you will be exposed to is 21.1 microsieverts (μSv), which is much lower than the range normally used in medical diagnostics. To place this in perspective, the amount of radiation you are exposed to during a return flight to the United Kingdom is 100 μSv and from a dental X-ray 50 μSv.

This procedure is quick, non-invasive and does not require anaesthetic. The room is private, and the staff are experienced and certified. It should take approximately 15 minutes. Your scan results will be assessed and approved by our consultant Radiologist. If your scan shows a T score of > 2.5 standard deviations (S.D) below normal, you will be advised and a copy of the scan, the report from the radiologist and a letter provided to take to your GP to discuss if further investigation is necessary.

Time commitment:

It is expected the diet diaries will take about half an hour to complete in total. The visit and measurements in the Human Nutrition Research Unit should take about one and a half hours.

What benefits you will get from participation:

- You will have contributed to scientific understanding of the influence of Coeliac Disease and Non-Coeliac Gluten Sensitivity on bone health in pre-menopausal women in New Zealand.
- You will not be charged for any of the measurements conducted for the study
- You will be provided with your bone scan results (both from the heel scan and DXA), blood test results and a nutrient analysis of your diet from your 3-day diet diary
- You will get a summary of the study results

Funding has been sought from Massey University and other external funding bodies.

Participation in this study will not result in any costs to you. All tests and sampling for this study will be paid for by the research funding. Participants will also receive reimbursement for their time and travel in the form of a $20 petrol or supermarket voucher.

Data Management

We will keep your name and contact details private and they will be stored in a locked filing cabinet in a locked office and then archived with our other research data before being disposed of in 10 years' time. You will only ever be identified by a code number for any data analysis and research reports.

Participants results will be kept for 10 years, as bone changes are slow and the research team may wish to invite you back for a follow-up study in future.

Please also bear in mind that it is not advisable for you to have DXA scans performed more frequently than once per year. So, if you have already participated in a bone health study recently and had a DXA scan we would like your permission to obtain a copy of your DXA results from the previous measurement in order to avoid you having a second scan.

Participant's Rights

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answer any particular question;
- Withdraw from the study at any time;
- Ask any questions about the study at any time during participation;
- Provide information on the understanding that your name will not be used unless you give permission to the researcher;
- Be given access to a summary of the project findings when it is concluded.

Project contacts

There are several researchers involved in this project; however, if you have any questions, concerns or complaints about the project or any of the tests and activities planned, please contact the lead researcher in this instance.

Katie Schraders, PhD candidate/ Lead Researcher
Phone: 06 356 9099 ext. 85541
Email: CloseToTheBone@massey.ac.nz
School of Food & Advanced Technology
Massey University
Palmerston North

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application SOA 18/73. If you have any concerns about the conduct of this research, please contact Dr Lesley Batten, Chair, Massey University Human Ethics Committee: Southern A, telephone +64 63569099 x 85094, email humanethicsoutha@massey.ac.nz

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

Nutritional Sciences Group
School of Food and Advanced Technology
College of Sciences

CLOSE TO THE BONE

PARTICIPANT CONSENT FORM

I have read the Information Sheet and have had the details of the study explained to me. Any questions I had have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree to participate in this study under the conditions set out in the Information Sheet.

Declaration by Participant:

I _____ hereby consent to take part in this study.

Signature: _____ Date: _____

APPENDIX C

Comparison of nutritional composition of bread in Palmerston North 2020

Manufacturer	Type	Loaf size g	Na mg/100g	Na mg/serving	Serving g	$ per loaf	$ per serving	Fat g/100g	Fat g/serve	Sugar g/100g	Sugar g/serve	Energy kj/100g	Energy kj/ serve
Gluten-containing breads													
NW Value	White	600	380	240	63	1.19	0.13	1.6	1	3	1.9	995	627
Nature's Fresh	White	700	390	290	74	3.29	0.35	2.2	1.6	3	2.2	1060	780
TipTop Supersoft white	White	700	453	335	74	3.59	0.38	1.9	1.4	2.5	1.8	995	736
Value wheatmeal	Toast	600	370	233	63	1.19	0.12	1.9	1.2	2.4	1.5	960	605
Molenberg Original	Toast	700	360	265	74	3.69	0.39	2.5	1.9	2.9	2.1	990	730
Sunny crust	White	600	390	245	63	2.00	0.21	1.9	1.2	3.5	2.2	1000	630
Vogels original	Mixed grain	750	375	330	88	4.29	0.50	1.4	1.2	2.7	2.4	840	740
TipTop oatilicious	White	700	383	284	74	3.69	0.39	2.7	2	3.1	2.3	1030	762
Freya's Tuscan	Mixed grain	750	390	325	83	3.79	0.42	3	2.5	3	2.5	1080	900
Ploughmans	Country grain	750	380	319	84	3.89	0.44	3.2	2.6	3.1	2.6	1030	862
AVERAGE		685	387	287	74	3	0.33	2.23	1.66	2.92	2.15	998.00	737.2
Gluten-free breads													
Pams GF everyday	White	500	410	256	62	6.99	0.87	4.1	2.6	5.2	3.3	1000	625
Burgen GF Wonderful White	White	580	374	310	83	7.49	1.07	3.4	2.8	2.5	2.1	980	813
Vogels GF & DF	White	520	415	270	65	7.89	0.99	3.5	2.3	4.1	2.7	910	590
Pams Superfoods	GF super seeded	600	380	277	73	8.29	1.01	7.7	5.6	4.6	3.4	1090	800
Vogels GF & DF	Wholemeal	580	380	275	72	7.89	0.98	5.3	3.9	4.5	3.3	930	680
Vogels keto GF & DF	Original mixed seed	580	390	285	73	8.99	1.13	19.2	14	0	0	1160	850
Pastry kitchen Allergywise	White	670	572	480	84	8.59	1.08	8.6	7.2	4.4	3.7	1240	1040
AVERAGE		576	417	308	73	8	1.02	7.40	5.49	3.61	2.64	1044.29	771.1

Source: New World website, 7th June 2022

In order of popularity (sales from New World)

APPENDIX D

A Gut Feeling Information Sheet and Consent form

MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

Nutrition Science Group
School of Food & Advanced Technology
College of Sciences

A GUT FEELING: GLUTEN-FREE DIET QUESTIONNAIRE

INFORMATION SHEET

Researcher(s) Introduction

The research group is a team of nutritionists interested in gut health. The lead researcher for this project is Katie Schraders, a PhD student from the School of Food & Advanced Technology (SFAT). Other researchers include Professor Marlena Kruger, Professor Jane Coad, Dr Louise Brough and Dr Janet Weber, also from SFAT.

Project Description and Invitation

The gluten-free diet is becomingly increasingly popular, with increases in the number of people consuming this for coeliac disease, non-coeliac gluten sensitivity and other reasons.

We are interested in the choices people make and where they find their information about the gluten-free diet.

This study is a nationwide questionnaire study which can be completed online.

If you would be interested in participating, please follow the link to the online questionnaire: https://massey.au1.qualtrics.com/jfe/form/SV_3khiijWdEFxElH7 or scan the QR code below.

If you have any questions, please contact Katie via email K.Schraders@massey.ac.nz

Participant Identification and Recruitment

We are inviting New Zealanders, over 16 years of age, who consume a gluten free diet to complete our questionnaire. We are advertising the study in local media and social media, tertiary education institutes, workplaces and with the assistance of health practitioners. We aim to recruit about 380 people.

Inclusion criteria

To become a participant, you will need to:

- Be aged 16 or over
- Be consuming a gluten free diet
- Live in New Zealand
- Have access to a computer.

If you are unsure whether you are eligible to participate, please contact the lead researcher for further information: Katie Schraders, K.Schraders@massey.ac.nz

What is involved?

Participants will be asked to access the questionnaires through the Qualtrics platform. We estimate the questionnaire should take about 15-20 minutes to complete; this may vary depending on your answers.

What benefits you will get from participation:

- You will have contributed to scientific understanding of the advice given to people consuming a gluten-free diet in New Zealand.
- You will get a summary of the study results
- You will be invited to enter the prize draw for a hamper of gluten-free goodies!

Funding has been sought from Massey University.

Data Management

We will keep your name and contact details private and they will be stored in a locked filing cabinet in a locked office and then archived with our other research data before being disposed of in 10 years' time. You will only ever be identified by a code number for any data analysis and researchreports.

Participants results will be kept for 10 years, as the research team may wish to invite you back for a follow-up study in future; you will be asked in the questionnaire if you consent to being contacted in future to further discuss your experiences related to a gluten-free diet.

Participant's Rights

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Withdraw from the study at any time;
- Ask any questions about the study at any time;
- Provide information on the understanding that your name will not be used unless you give permission to the researcher;
- Be given access to a summary of the project findings when it is concluded (an option is provided in the survey to request this be emailed to you at the conclusion of the study);
- Completion and submission of the questionnaire implies consent. You have the right to decline to answer any particular question

Project contacts

There are several researchers involved in this project; however, if you have any questions, concerns or complaints about the project or any of the tests and activities planned, please contact the lead researcher in this instance.

Katie Schraders, PhD candidate/ Lead Researcher
Phone: 06 356 9099 ext. 85541
Email: K.Schraders@massey.ac.nz
School of Food & Advanced Technology
Massey University
Palmerston North

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 20/50. If you have any concerns about the conduct of this research, please contact Dr Negar Partow, Chair, Massey University Human Ethics Committee: Southern A, telephone 04 801 5799 x 63363, email humanethicsoutha@massey.ac.nz.

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

APPENDIX E

Biochemistry Coefficient of Variations:

Biochemical markers from urine:

	Mean	SD	CV
Iodine concentration	567	11	2.0%

Biochemical markers from blood:

	Level 1			*Level 2*			*Level 3*		
	Mean	**SD**	**CV**	**Mean**	**SD**	**CV**	**Mean**	**SD**	**CV**
Anti-TPO	*8.24*	*0.47*	*5.68%*	*19.6*	*1.17*	*5.97%*			
FT4	*8.695*	*0.913*	*10.50%*	*32.515*	*2.654*	*8.16%*	*53.162*	*2.501*	*4.70%*
FT3	*3.057*	*0.253*	*8.28%*	*7.576*	*0.468*	*6.17%*	*11.846*	*0.558*	*4.71%*
TSH	*0.7624*	*0.0469*	*6.15%*	*5.6146*	*0.3218*	*5.91%*	*30.4451*	*1.4309*	*4.70%*
Vit D	*N/A*								
B12	*106.02*	*18.15*	*17.12%*	*269.1*	*26.94*	*10.01%*	*401.56*	*65.57*	*16.33%*
Folate	*7.38*	*0.41*	*5.54%*	*20.33*	*1.17*	*5.77%*	*31.2*	*1.96*	*6.27%*
Ca	*1.59*	*0.03*	*1.89%*	*2.755*	*0.04*	*1.45%*	*3.42*	*0.05*	*1.46%*
Tg	*N/A*		*4.81%*						
TgAb	*N/A*		*10.00%*						
PTH	*N/A*		*7.85%*						
CTX-I	*N/A*		*8.60%*						

N/A values were not available from the laboratory. All biochemical markers from blood were measured by Canterbury Health Laboratory, an accredited laboratory following standard practices.