Crohn's Disease and Environmental Factors in the New Zealand context

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

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2023

ABSTRACT

Background: Inflammatory bowel disease (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), are lesser-known chronic diseases of the gastrointestinal tract. The causes of IBD are unknown, although research indicates an interplay of genetic, immunological, and environmental factors. The incidence and prevalence of CD in New Zealand (NZ) are among the highest worldwide, and unlike many other Western countries, evidence suggests the incidence rate is still increasing.

Objective: The objective was to investigate the involvement of environmental factors in the aetiology, pathogenesis, and symptomatology of CD in NZ. Specifically, pathogenic bacterium *Mycobacterium avium* subspecies p*aratuberculosis* (MAP), vitamin D, diet, and urbanisation.

Methods: Patients with IBD and controls from around NZ completed a questionnaire on environmental factor exposure. Foods implicated in symptom triggering or exacerbation, the possible mechanism(s) involved, and whether vitamin D can confer protection, were investigated using an *in vitro* digestion method and *in vitro* model of the intestinal barrier. Serum vitamin D concentrations were measured and compared in patients and controls in order to explore a possible association between vitamin D and IBD. Lastly, the incidence and prevalence of IBD in the Manawatū region was determined, and the urban and rural incidence were compared.

Results: Questionnaire derived data showed significant associations between CD and exposure to rural sources of microorganisms, and a major urban birthplace (≥100,000 residents), while rainwater for drinking and cooking during childhood was protective. No associations were observed between CD and MAP exposure. Over 50% of patients implicated dietary elements in symptom onset and/or exacerbation. The *in vitro* investigation findings suggest this may result from tight junction damage. Vitamin D concentrations did not differ between patients and controls, however, were significantly lower in CD patients that reported recent disease activity. In the Manawatū region, the mean annual incidence and 2013-point prevalence of CD were 17.7 and 250.4 per 100,000, respectively, and urban residence at diagnosis was associated with a six-fold greater IBD incidence compared to rural residence.

Conclusions: The findings demonstrate that vitamin D, diet, and urbanisation are involved in CD. A greater understanding of environmental factors, especially modifiable factors, could provide opportunities for reducing CD risk, managing symptoms, or slowing disease progression.

ACKNOWLEDGMENTS

I am extremely grateful to my main supervisor, Professor Jane Coad, for her guidance and wisdom throughout this journey; the unlimited support, patience and understanding when there were bumps in the road; jubilance following every victory, big or small; and her determination to see me cross the finishing line. I would like to express my gratitude to my co-supervisor, Associate Professor Kevin Pedley, for his expertise on everything 'gut' and guidance with the laboratory work, having a consistently calm demeanour even when nothing was going to plan, and his guaranteed enthusiasm for any future cell work ideas, no matter how ambitious.

I must sincerely thank Dr James Irwin for sharing his wealth of knowledge, his constant encouragement, and always making time to answer my never-ending questions.

Many thanks to the staff in the School of Food and Advanced Technology, including the late Anne Broomfield for her assisting in taming large quantities of data. Also, to Dr Wei-Hang Chua, School of Health Sciences, for always being a friendly face and having time for a chat.

I would like to acknowledge the financial assistance from The Todd Foundation, Ken and Elizabeth Powell Bursary, New Zealand Federation for Graduate Women, and Palmerston North Medical Research Council.

I am grateful to Crohn's and Colitis New Zealand (CCNZ) for their assistance with recruitment, and a special thanks to Brian Poole QSM for readily welcoming me into the CCNZ family and supporting my research.

Thank you to all my participants, this work would not have been possible without the time you gave and the wealth of information you provided.

I would like to thank my fellow postgraduate students; especially Dr Bob Stewart for sharing his extensive knowledge of cell culture and for willingly joining the team to guide me through the formidable task of data analysis. Also, thank you to Dr Janyawat Jam Vuthijumnonk, I would not have wanted to share an office with anyone else, and to Dr Katie Schraders for letting me vent as required and for making the hours of assignment marking that little bit more bearable.

A very special thank you to my family, Dad, Miriam, Mary, Rodney, and the late Patricia, for their unwavering support and interest in my research. I am also immensely grateful to Joan and Marc, for providing a home away from home and somewhere to relax at the end of a long day. The 'medical' chat over a home-cooked meal is sorely missed.

Last, but by no means least, I would like to acknowledge and thank my wonderful husband, for putting up with countless absences, listening while I voice all my concerns and frustrations, often many times over, and never once letting me entertain the thought of not finishing.

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ABBREVIATIONS

CD	Crohn's disease
CDAI	Crohn's disease activity index
CDED	Crohn's disease exclusion diet
CDEIS	Crohn's disease endoscopic index of severity
CHO	Carbohydrate
CRP	C-reactive protein
CT	Computed tomography
DC	Dendritic cell
DSS	Dextran sulfate sodium
EN	Enteral nutrition
EPIC	European prospective investigation in cancer and nutrition
ESR	Erythrocyte sedimentation rate
FA	Fatty acid
FBS	Fetal bovine serum
FC	Faecal calprotectin
FFQ	Food frequency questionnaire
FL	Faecal lactoferrin
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides and polyols
FOS	Fructo-oligosaccharide
GI	Gastrointestinal
HBI	Harvey Bradshaw index
HC	Healthy control
IBD	Inflammatory bowel disease
IBD-AID	Inflammatory bowel disease - anti-inflammatory diet
IBDQ	Inflammatory bowel disease questionnaire
IBDU	Inflammatory bowel disease unclassified
IBS	Irritable bowel syndrome
ICD code	International Classification of Diseases code
IEC	Intestinal epithelial cell
IFN- γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin

JAM-A	Junctional adhesion molecule-A
JD	Johne's disease
KO	Knock out
LPS	Lipopolysaccharide
MAP	Mycobacterium *avium* subspecies *paratuberculosis*
M cell	Microfold cell
MDP	Muramyl dipeptide
MRI	Magnetic resonance imaging
N-3 FA	Omega-3 fatty acids
NF-κB	Nuclear factor kappa B
NOD	Nucleotide-binding oligomerisation domain
NZ	New Zealand
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PC	Paneth cell
PCDAI	Paediatric Crohn's disease activity index
PCR	Polymerase chain rection
PEN	Partial enteral nutrition
PN	Parental nutrition
PRR	Pattern recognition receptor
RCT	Randomised controlled trial
RT	Room temperature
SCD	Specific carbohydrate diet
SCFA	Short chain fatty acid
SES-CD	Simplified endoscopic score for Crohn's disease
SIBDQ	Short inflammatory bowel disease questionnaire
SVD	Semi-vegetarian diet
TEER	Trans epithelial electrical resistance
TJ	Tight junction
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
UC	Ulcerative colitis
US	Ultrasound
VDR	Vitamin D receptor

CHAPTER ONE

INTRODUCTION

Crohn's disease (CD) is an inflammatory condition of the gastrointestinal (GI) tract, that alongside ulcerative colitis (UC), are considered the inflammatory bowel diseases (IBD). Both are chronic incurable conditions that are characterised by unpredictable fluctuations between relapse and remission. New Zealand (NZ) has one of the highest reported rates of CD worldwide[1], and the incidence rate has continued to rise for both adults [2] and children[3], though recent data are only available for one of NZ's sixteen regions. The condition can present at any age, although the peak age of diagnosis is between 20 and 30 years [4]. Historically, affected individuals were of European decent, however CD now affects individuals of any race or ethnic background [5].

Chronic inflammation is the core element of CD and is thought to be caused by dysregulation of one or more components of the GI tract immune system. Altered function of the innate immune defences is evident from observations of increased intestinal permeability, a loss of tolerance due to impaired microbial antigen sensing [6], and reduced autophagy [7]. An abnormal T cell response by adaptive immune defences leads to overproduction of proinflammatory cytokines, predominantly tumour necrosis factor and interferon gamma [8], which drive intestinal inflammation and induce the symptoms of CD [9].

The most common symptoms are abdominal pain, diarrhoea, rectal bleeding, and fatigue, though CD is associated with many other symptoms, some of which affect regions outside the GI tract such as the eyes, joints, and skin [10]. A degree of overlap exists between CD and UC symptoms, however the sections of affected GI tract, complications, and disease course are quite distinct [11]. In UC, involvement is limited to the mucosal layer of the colon and extends proximally from the rectum in a continuous fashion [12], whereas involvement is typically discontinuous (skip lesions) in CD, inflammation occurs beyond the mucosal layer (transmural), and any region of the GI tract can be affected [13].

Diagnosis of CD is based on a combination of symptom history, endoscopic, histological, and radiological investigation, and biomarker testing [13]. Early intervention is important to reduce the risk of complex disease course [14], although this can be hindered by diagnosis delays owing to mild or atypical symptom presentation [11], or an under resourced healthcare system. Once diagnosis is established drug therapy usually commences with corticosteroids for the induction of remission, followed by immunosuppressants or biologicals for remission maintenance [15]. Drug therapy acts to reduce symptoms and slow disease progression, and ideally facilitate mucosal healing. Surgery is often needed in the absence of drug efficacy, and for non-responsive complications such as strictures, scarring, fistulas and abscesses [16].

Once diagnosed, upwards of 50% of patients with IBD modify their diet as a means of reducing disease symptoms or preventing relapse [17–20].

Besides physical effects, CD can have a significant impact on an individual's quality of life by reducing their ability to work, to undertake physical and social activities, and affecting their mental health [21]. The medications can also have undesirable side-effects, especially corticosteroids [22]. In addition to patient burden, CD is also associated with significant direct costs including drug therapy, hospitalisation, surgery, and specialist healthcare provision [21].

The causes of CD have not been determined, although genetic risk is evident from studies of familial clustering and of IBD [23], and incidence rate ratios of 7.8 and 2.4 have been observed among first and second degree relatives, respectively [24]. Genome-wide association studies have identified over 240 loci linked to IBD susceptibility, almost 50 specifically to CD [25]. Corresponding gene research has led to the discovery of mutations that influence innate and adaptive immunity and contribute to the immune system dysregulation, such as nucleotide-binding oligomerisation domain (NOD2), also known as CARD15, and impairment of bacterial recognition associated with mutant NOD2 [26].

Geographical disease patterns strongly suggest that CD is a disease of the developed world, and in a genetically susceptible host disease onset is triggered by environmental factors associated with industrialisation and a Western lifestyle [1]. Further support for a role of environmental factors comes from the demonstration of local disease risk acquisition in the offspring of immigrants from regions of lower risk [27]. Numerous environmental factors have been implicated in CD, though they are not well understood, and elucidating their potential involvement is complicated by the timing of exposure as immune system development and establishment of the mature microbiome may be especially impressionable during infancy and early childhood [28]. Some extensively researched environmental factors that are positively associated with risk and pathogenesis of CD include antibiotic use [29], urbanisation [30], and characteristics of a Westernised diet [31,32], while being breastfed during infancy appears to be protective [33]. Further, current cigarette smoking is protective against UC, yet increases the risk of CD [34,35]. *Helicobacter pylori*, E. *coli*, and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) [36] are just three examples of pathogenic bacteria that have been associated with CD. The latter is also the proven causative agent of Johne's disease in animals [37], a disease with many histopathological similarities to CD including intestinal wall thickening, a cobblestone appearance, ulceration and granuloma formation [38,39].

There is a growing body of evidence suggesting a role of vitamin D status in CD with earlier research reporting an inverse association between vitamin D level and both the risk and severity of disease [40]. Emerging evidence is providing a greater understanding of the many pathogenetic mechanisms involved, particularly the modulation of innate and adaptive immune responses including vitamin D stimulated production of microorganism identifying pattern recognition receptors, antimicrobial peptides, and dendritic cells which decreases T cell activation and promotes differentiation of tolerogenic regulatory T cells [41]. The expression of tight junction proteins occludin and ZO-1, and regulation of claudin-2 and claudin-4 [42] is induced by vitamin D, effects which are thought to explain observations of enhanced

intestinal epithelial barrier function. Moreover, an imbalance in microbiota populations and reduced microbiota diversity may be improved by vitamin D supplementation [43].

The nature and extent of CD symptoms, and variable efficacy of available drug therapy, presents a significant burden to patients, the healthcare system, and society. For these reasons it is essential to continue exploring how environmental factors contribute to the risk of CD, and whether exposure can be modified to improve outcomes for those with CD and ultimately reduce the risk of CD for future generations. Furthermore, ongoing investigation of epidemiological trends is necessary in order to predict the healthcare resources needed for managing CD in the future.

OBJECTIVES

1) To identify foods that patients with CD associate with the onset, exacerbation, and reduction of their GI symptoms.

2) To explore the potential of small intestinal organoids to investigate the effect of food components and environmental agents, such as MAP, on gut cell function and barrier integrity.

3) To investigate environmental factor exposure in patients with CD and controls.
   - To include environmental factors that may be pronounced in NZ including low serum vitamin D levels owing to the country's southern location, and exposure to potential sources of MAP in view of widespread farming and a high prevalence among livestock.

4) To determine the serum vitamin D concentration of patients with CD and controls.
   - To validate the blood spot method for measuring serum vitamin D.

5) To determine the incidence and prevalence of CD and UC in the Manawatū region.
   - To investigate if the risk of CD in the Manawatū region differs between rural and urban residence.

HYPOTHESES

1) Dietary elements that patients with CD associate with the onset or exacerbation, or the reduction, of their GI symptoms will have a common characteristic eg foods that contain lactose, foods that have a high fibre content, deep fried foods.

2) Small intestinal organoid barrier integrity will be compromised following exposure to food components (that patients with IBD associate with symptom onset or exacerbation).

3) CD will be positively associated with increased exposure to potential sources of MAP as a consequence of contact with ruminant farm animals, the farms on which they reside, contaminated water, hunting, or consumption of unpasteurised milk.

4) The serum vitamin D concentration of patients with CD will be significantly lower than controls.

5) The incidence and prevalence of CD in the Manawatū region will be higher than the corresponding UC rates, and the incidence of CD will be higher among populations residing in urban locations at the time of diagnosis compared to rural locations. Incidence and prevalence rates for CD and UC will match or exceed those reported by Gearry et al. [44] for 2004 and 2005, respectively.

THESIS OUTLINE

The aim of the thesis was to identify environmental factors that are involved in the aetiology, pathogenesis, and/or symptomatology of CD, and their potential mechanisms, in the NZ context. A combination of *in vitro* and *in vivo* research methods was utilised to achieve the study objectives. Some repetition is inevitable owing to chapters being prepared for publication.

The current chapter, Chapter One, provides a brief introduction about CD and demonstrates the need for continued research both within NZ and globally. The objectives, hypotheses, and thesis layout are also outlined in chapter one.

Chapter Two is a literature review that considers three aspects of CD. Firstly, the definition and diagnosis of CD, evaluation of disease activity, health effects and costs, and epidemiology. Secondly, the causes and exacerbating factors including immune system dysregulation and environmental factors. Lastly, dietary approaches to the treatment and management of CD.

Chapter Three details dietary elements that patients with IBD associate with the onset, exacerbation, and reduction of their GI symptoms, and considers characteristics of the implicated foods, additives, and cooking methods that could be responsible for the reported effects.

Chapter Four describes development of the organoid model for investigating the effect of foods that have undergone *in vitro* digestion (as described in appendix 3) on intestinal barrier integrity.

Chapter Five details identification and *in vitro* digestion of foods reported to trigger or exacerbate intestinal symptoms in CD, and the use of the Caco-2 cell model to investigate the effect of these foods. After having undergone *in vitro* digestion, on the epithelial barrier, and to determine if vitamin D protects the cells from dietary instigated damage.

Chapter Six describes the associations observed between IBD risk and exposure to environmental factors including potential exposure to Mycobacterium avium subspecies paratuberculosis, and urban or rural residence. Exposure was assessed at different stages: birth and infancy, childhood, and the ten years prior to symptom onset.

Chapter Seven investigates serum vitamin D concentration in patients with CD and controls, if vitamin D concentration is associated with recent disease activity, and factors that influence vitamin D concentration including supplementation and UV exposure. In addition, validity of the blood spot method for measuring serum vitamin D is evaluated.

Chapter Eight describes the identification of patients with a diagnosis of IBD in the Manawatū region, application of modified Lennard-Jones [45] and European Crohn's and Colitis Organisation [12,13] diagnostic criteria to confirm diagnoses between 2011 and 2015, and the presenting symptoms and phenotype of diagnoses that met the proposed criteria. Further, the 2013-point prevalence of IBD was determined.

Chapter Nine discusses and summarises the research findings, considers the strengths and weakness of the methods used, and provides recommendations for future research.

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CHAPTER TWO

REVIEW OF THE LITERATURE

1. Crohn's Disease

Crohn's disease (CD) is one of three chronic idiopathic inflammatory conditions collectively known as inflammatory bowel disease (IBD). In CD any region of the gastrointestinal tract (GIT) can be affected, whereas ulcerative colitis (UC) is confined to the large intestine. The third less common condition, inflammatory bowel disease type unclassified (IBDU), is only diagnosed when a distinction cannot be made between CD or UC and disease is confined to the large intestine [1,2].

Crohn's disease was first described in 1913 as "chronic interstitial enteritis" by Dalziel [3] but it was not until 1932 that Crohn et al. [4] comprehensively described the features of CD and it became recognised as distinct condition. In the acclaimed paper the authors noted the inflammation, bowel wall thickening, and abdominal pain that continue to be recognised as the defining characteristics of CD. Other attributes include luminal narrowing [5] that may extend to ulceration [6], strictures and fistulas [7], weight loss [8], diarrhoea, fatigue, fever, mucus and/or blood in the faeces [9]. One or more nutrient deficiencies may also present as a result of malnutrition [10], malabsorption [11], chronic diarrhoea [12], or medication side-effect.

Crohn's disease can present at any age, however peak diagnosis occurs in early adulthood (20–29 years) [13,14]. Over the last few decades it has become apparent that the average age of onset is decreasing [15] with diagnosis before 20 years of age being linked to greater disease severity [16]. A gender effect on CD risk is uncertain with some studies reporting a greater incidence of CD in males [17,18], others a greater incidence of females [19–21], and a review of 59 studies finding no clear trend towards either gender [13]. Traditionally many ethnic groups appear less prone to CD such as Chinese [22], Māori and Pacific Islanders [20,23], Indians, Koreans, and Japanese [24] which suggests that genetic factors may play a significant role in disease development. Although, as the overall incidence rates rise so too do the number of diagnoses within these and other ethnic groups previously thought to be unaffected or only marginally affected by CD [25].

1.1. DIAGNOSIS

The diagnosis of CD is based on a history of gastrointestinal symptoms, accompanied by the findings from one or more endoscopic, radiographic, pathological, and clinical examinations [9,26]. Endoscopy

is the most definitive method for diagnosing CD [27] during which time a number of small biopsy samples are taken from both affected and non-affected sites for histological examination [28]. Endoscopy has a number of significant disadvantages including its invasive nature, poor acceptance of bowel preparation by patients, and visualisation confined to mucosal surface of the colon and terminal ileum [29]. As a result of these disadvantages radiologic techniques may be used instead [30], notably ultrasound (US), magnetic resonance imaging (MRI) and computed tomography (CT), all of which differ in utility.

Ultrasound is non-invasive and does not expose the patient to radiation [31], however sonologist experience can limit the diagnostic utility of this method [29]. Magnetic resonance imaging is also radiation free, it can be used to image both the small and large intestine, and provides a high soft tissue resolution cross-sectional image enabling the detection of intestinal thickening and non-luminal manifestations [26], although it is time-consuming and expensive [32]. Computed tomography is widely available and has similar features to MRI including exposure to radiation [33]. Comparative studies of US and MRI have reported somewhat conflicting accuracy between the two methods [31–33], however a meta-analysis of thirty three studies reported US and MRI specificity of 95.6% and 92.8%, respectively, and sensitivity of 73.5% and 70.4% [34]. The specificity of CT faired greater at 95.1% but less favourably with sensitivity of 67.4% [34].

Making a diagnosis of CD is not always straightforward. Other methods that may provide secondary evidence include a combination of serum antibody testing. The most commonly tested are antibodies to yeast *Saccharomyces cerevisiae* and autoantibodies to *neutrophil nuclear lamina protein* [35] which can aid in differentiating between UC and CD that only involves the colon [36,37]. A newer technology, wireless capsule endoscopy, is a pain free alternative to traditional endoscopy but comes with several disadvantages including substantial costs, findings that may be limited by the camera angle or GIT transit time, intestinal blockage risk in the presence of a stricture [26], and the inability to acquire biopsies [38]. Scintigraphic imaging is a non-invasive method that is believed to have potential but requires further development. The method assesses blood flow and can be used to measure inflammation by determining leukocyte concentrations, and also for imaging superior intestinal regions unable to be viewed by colonoscopy [39].

1.2. DISEASE ACTIVITY

Endoscopy or radiological techniques are the most accurate methods for determining the presence of mucosal inflammation, its location [27], and thus disease activity in CD. Due to the invasive nature of endoscopy [40], the risks associated with repeated radiation exposure, the cost and time involved with these methods, and the subjective nature of classifying visual disease features [41], the preferred option is often assessment of clinical improvement or biological markers of inflammation. There are two highly sensitive biomarkers that indicate intestinal inflammation; faecal calprotectin (FC) and faecal lactoferrin (LF). Faecal calprotectin is a cytosolic protein released by stimulated or damaged neutrophils, while faecal LF is an iron-binding protein secreted by mucosal membranes and released by neutrophils during degranulation [42,43]. Faecal calprotectin has a higher sensitivity and specificity than faecal LF [44] making it

the more frequently measured marker, and it is often used to distinguish between inactive, mild, moderate, and active CD [40], as well as to predict the likelihood of clinical relapse [45]. Another commonly measured biomarker is C-reactive protein (CRP), an acute phase protein produced predominately by the liver when stimulated by interleukin-6, a cytokine produced at sites of inflammation and tissue injury [29,46]. C-reactive protein has been reported to have a good correlation with clinical, endoscopic [40], and histological markers of inflammation in CD [47] by some, yet others have reported poor correlation to endoscopically observed inflammation [48]. Other biomarkers such as erythrocyte sedimentation rate (ESR) and leukocyte count have been explored but lack the necessary specificity [42].

Disease activity can also be appraised using one of several specially developed indexes that take into account one or more combinations of endoscopic, biological, and clinical findings. Developed in the early 1970s [49], the CD activity index (CDAI) is based on eight independent variables including stool frequency, abdominal and general wellbeing, presence of CD associated complications, use of anti-diarrhoea medication, abdominal mass, haemocrit and percentage body weight decrease [49]. Although the index is widely used in trials and clinical settings, it is time-consuming as patients are required to complete a seven-day symptom diary [40], and the change in body weight variable has limited value [50]. In an attempt to eliminate some of these drawbacks a simplified version of the CDAI has been devised, the "simple index" or Harvey-Bradshaw index (HBI) [50]. The HBI excludes three of the original CDAI variables [51] and patients are only required to record their symptoms over a 24-hour period [52].

There are two well-known indexes for scoring endoscopic disease activity. The first, the CD endoscopic index of severity (CDEIS), was developed in 1989 to address the lack of endoscopic findings consideration in CD clinical trials. The index is based on four measures of ulceration [53] and is considered the gold standard for predicting endoscopic disease activity [54]. The second index, formulated fifteen years later, is the simplified endoscopic score for CD (SES-CD). Derived from the CDEIS with the aim of being easier and faster, the SES-CD considers the presence of intestinal narrowing in additional to ulceration [55].

Some disease activity indexes have also been modified or developed to cater for different CD patient groups. An index exists solely for the evaluation of post-surgical disease activity, the Rutgeerts' score [54]; some are disease location specific such as the perianal disease activity index [56] or age specific with the paediatric CDAI (PCDAI) [57]; and more recently a shortened and simplified version of the CDAI, the short CDAI [58], has been detailed. Other proposed indices are not validated, are validated but do not correlate to another suitable index or have simply failed to be adopted into routine clinical trial methodology.

Since the development of these indexes there has been a great interest in how accurately they each predict disease activity. The CDAI correlates poorly to endoscopic assessment [40,59], the CDEIS [59–62], and when compared to a number of biological markers including fibrinogen [60], FC, LF, and CRP [62], and a range of serum proteins, blood cell concentrations, and haematocrit percentage [61], the correlations are weak or absent. Similar studies investigating the predictability between both HBI [63] and CDEIS [59–62] with several biological markers fared marginally better, however considering the overall low to mediocre predictability reported the utility of such indexes appears limited. Potential explanations for a lack of agreement between

disease activity measures include the inability of endoscopy to detect transmural inflammation [61], biological marker changes brought about by pre-existing non-CD associated conditions [64], the subjective nature of clinical findings [54], and inter-observer variations in endoscopy [29] and other diagnostic instruments [26,41].

1.3. HEALTH EFFECTS AND HEALTH CARE COSTS

The physiological and psychological effects of CD can be great depending on both the severity of the condition and how well it is managed by both practitioners and the patients themselves. Difficulties can arise when coming to terms with the diagnosis or the stigma often associated with CD [65] which can lead to poor self-esteem, anxiety, or depression, especially in adolescence [66]. Family, friends and spouses of patients may also experience emotional stress in response to the difficult nature of this condition [67]. Growth failure can be an issue in paediatric cases of CD [68] and may necessitate supplemental energy and nutrients in liquid form [69]. Physical function and work productivity may be restricted [70], education or employment attendance can be affected [65], and social and emotional wellbeing often declines [71]. The ability to travel both locally and abroad may be compromised as the result of a greater need for bathroom access, the use of medications with specific storage requirements, symptom unpredictability, risk of relapse, and the expense of adequate insurance [65]. Some patients may also be dependent on a governmental disability or sickness benefit [67].

Health care costs associated with diagnosing and treating CD are significant and are rising with the increasing incidence and introduction of more effective and expensive treatments. Mean annual per patient health care costs vary between countries and are based on inpatient, outpatient, and medication costs, and often GP expenses. Estimated costs are available for Canada, Germany, USA, Europe/Israel, and England (see *Table 2.1).* Currently there are no such data for the economic costs of CD in New Zealand (NZ), but based on Australian data the closest approximation would be between $A1210 [67] and $A8119 [72]. Health care costs may be even greater in the 25% of patients that experience extraintestinal manifestations [9] such as liver disease or osteoporosis [73]. These estimates likely differ due to differences in sample size; considered cost scope, especially those associated with diagnosis; and estimates derived solely from hospital records thus skewing the data in favour of higher costs associated with hospitilisation. Further, there are undoubtedly inter-country disparities in drug therapy availability, entitlement, and costs [74], as well as government health funding and health insurance coverage.

Table 2.1 Estimated Annual Health Care Costs of Crohn's disease

Country	Year	Cost	Reference
Canada	2012	$4,232	[65]
Germany	2007	€3,767	[74]
USA	2005	$18,963	[75]
Europe/Israel	2004	€2,548	[76]
England	2000	€3,304	[77]

1.4. EPIDEMIOLOGY

Since the formal recognition of CD in the early 1930's worldwide incidence rates have continued to rise [78,79]. This increase has been observed mostly in developed countries but more recently in developing countries [80,81]. Some regions have experienced remarkable growth in the number of CD cases over relatively short time frames including an increase of 23% in Northern France between 1988 and 1999 [14], 1.9 per 100,000 in 1978-1990 to 3.4 per 100,000 in 1990-1992 in Florence [82], 0.41 to 4.68 per 100,000 in Western Hungary between 1977 and 2001 [83], a greater than three-fold increase in Hong Kong between 1989 and 2001 [22], and a prevalence increase of 46% between 1980 and 1991 in Olmsted County Minnesota [84]. The cause of these increases is not conclusively known although increased exposure to a wide array of environmental risk factors [80] and improved recognition, diagnosis, and reporting [85] have been proposed.

The highest reported CD incidence rates per 100,000 are 26.4 in the Canterbury region of NZ (2014) [86], and 20.2 for both Nova Scotia (1998-2000) [19] and Quebec in Canada [87]. The highest reported prevalence rates per 100,000 are 322 in Sicily, Italy (2002) [88] and 318.5 in Nova Scotia, Canada [19]. In NZ, the CD prevalence does not reach these levels but is still high by world standards at 155.2 in Canterbury [20]. Of note is the suggestion that reported rates, particularly from developing countries, are underestimated as a consequence of diagnostic constraints [13], study limitations [89] and misdiagnosis [18].

The mortality rate of CD patients, as evident in data obtained from 21 countries, increased by almost 100% between 1951 and 1975 and is now believed to be stable [90]. The cause and risk of mortality however remains an area of active research and several meta-analyses' have been carried out to determine whether mortality risk differs from the general population. The reported standardised mortality ratio (SMR) ranges from 1.38 to 1.52 [91–93]. Two more recent analyses identified a number of elevated cause-specific SMRs in CD patients such as 6.76 for gastrointestinal diseases (excluding CD) [93], 3.24 for infectious diseases [93], 2.82 for non-alcoholic liver disease [92], 1.44 [93] and 1.60 [92] for respiratory diseases, and 1.44 for suicide [93]. Additionally, CD is linked to an increased risk of fractures, and specifically colorectal and small bowel cancers [28,94].

2. Causes and Exacerbating Factors

2.1. DIET

2.1.1 Sugar Intake

Diet has long been thought to be involved in the aetiology of CD, a theory based on the anatomical disease site and supported by the prevalence of food intolerances in patients. Early studies suggest that elevated carbohydrate intake may trigger disease onset. In 1975, Martini [95] evaluated the intake of common foods and observed a greater intake of sweets and pastries in CD patients. A sole focus on breakfast habits led James [96] to report a correlation between the onset of CD symptoms and greater cornflake intake, and Mayberry [97] to a correlation between CD and greater consumption of added sugar at breakfast. In the following decade, the positive association between sugar intake and CD was suggested in several studies [98–104] and a potentially protective effect of greater fruit and vegetable consumption first became apparent [99,102]. Sugar intake and CD risk has been re-examined in later studies, although only a handful have identified an association [105–107]. Russel et al. [108] noted a positive association between CD risk and chocolate, cola drinks, and chewing gum, and a negative association with citrus fruits. Although in this study dietary intake evaluation was limited to ten food items and one food group (preserved food).

In some individuals the consumption of structurally different sugars, including fermentable mono-, di-, and oligosaccharides, and polyols (known as “FODMAPs”) can cause GI discomfort due to their poor absorption and subsequent colonic fermentation [109]. Research suggests that one of these FODMAPs, the monosaccharide fructose, has the potential to induce deleterious health effects, such as metabolic syndrome and bone loss, when consumed in high quantities [110]. A diet high in fructose may also precipitate gut environment disturbances that are strongly implicated in the pathogenesis of CD. Specifically, decreased expression of the tight junction (TJ) protein complexes connecting intestinal epithelial cells has been observed in mice fed a high fructose diet for three months, and also in murine colonic organoids supplemented with fructose for 24 hours [111]. Similar results were seen in mice fed a high fructose diet for two weeks, as well as impaired colonic antioxidant capacity, decreased expression of anti-inflammatory cytokines and antimicrobial peptides, reduced numbers of goblet cells and mucin secretion capacity, and altered composition of gut microbiota and microbial metabolites [112].

Work with murine models of colitis has highlighted additional fructose-induced changes that may be involved in CD. These include reduced gut barrier integrity caused by a decrease in mucus thickness (despite normal goblet cell number) and greater bacteria colonisation at the mucosal-luminal interface; and altered populations of gut microbiota, including reduced quantities of beneficial bacterial and a corresponding increase in pathogenic bacteria viability [113]. The gut microbiota changes are thought to be caused by an increase in colonic fructose levels resulting from saturation of fructose transporter GLUT5 [113]. Evidence from human studies is limited, however, a potential role of GLUT5 in CD is further supported by the demonstration of significantly decreased GLUT5 expression in ileal biopsies from CD patients compared to healthy controls [114]. An inverse relationship was also evident between GLUT5 expression and

proinflammatory cytokine expression. These findings could explain why approximately 50% [115–117] of CD patients experience a reduction in functional GI symptoms when following the low FODMAP diet.

The utility of these original studies is compromised by methodological limitations such as lengthy dietary recall durations, small sample size, and a lack of CD confirmation. Further, many inherent problems exist in the way dietary intake has been determined including evaluation of a narrow range of foods, impractical units of measures for dietary intake, and a lack of adjustment for total energy. More recent case-control studies have overcome most of these shortcomings utilising robust methodologies, notably the latter by using validated food frequency questionnaires (FFQ).

2.1.2 Diet Patterns

In a Japanese study, the five-year dietary intake was determined in 126 recently diagnosed CD patients. No negative associations were observed, however CD risk was positively associated with the consumption of sugar, sweeteners, sweets, fats and oils, and seafood [118]. Amre et al. [119] conducted a study in Canada to determine dietary habits in the 12-months prior to diagnosis in paediatric (≤20 years of age) CD patients. The FFQs were completed within one month of diagnosis and patients that made pre-diagnosis dietary changes were excluded. Data analysis of the 130 patients demonstrated a negative association between CD risk and intake of fish and nuts, and a positive association with total, monounsaturated, and saturated fat. D'Souza et al. [120] undertook a later analysis of 149 paediatric CD patients, the same cohort as Amre et al. [119] with an additional twelve month recruitment period. A positive association was observed between CD risk and "high intakes of meats, fatty foods, and desserts" in girls, and in all subjects there was a negative association between CD and a diet "characterised by vegetables, fruits, olive oil, fish, grains, and nuts" [120]. Similar findings were reported following the evaluation of dietary habits five years prior to diagnosis by Maconi et al. [121]. In these 42 recently diagnosed CD patients, meat consumption was positively associated with CD risk, while negative associations were seen with tuna and vegetable consumption.

Large-scale epidemiological studies have been utilised to explore diet and CD aetiology. Using dietary intake data collected from over 4,200 families annually from 1966-1985 in Japan, the change in diet and CD incidence was evaluated. The increase in CD incidence over this period was positively associated with total fat, animal fat, omega-6 polyunsaturated fatty acids, and milk and animal protein. The sole negative association was with vegetable protein [122]. In the United Kingdom, a sub cohort of the European Prospective Investigation in Cancer and Nutrition (EPIC) cohort has been established to investigate the role of macronutrients in the aetiology of IBD. Recruited from 1991-1998, the EPIC-IBD cohort of 401,326 have completed an FFQ at baseline and follow up (2004 or 2010). Analysis of total protein, lipid and carbohydrate intake has been carried out on participants residing in France. In this group of 30 participants with CD and 67,504 controls, a positive association was described between CD risk and animal protein intake, specifically meat or fish, but not eggs or dairy products. There was no association with vegetable protein, carbohydrates or fats [123]. To date, three studies have evaluated data gathered from the complete EPIC-IBD cohort. From >100 CD cases, no association has been seen between CD risk and total energy, total

carbohydrate, sugar, and starch [124], as well as no association with the three dietary patterns identified; high vegetable consumption, high sugar consumption, and high alcohol, animal fat, seafood, potato, and coffee consumption [125]. Lastly, in the third distinct analysis no association was observed with total fibre intake or fibre intake from cereals, fruit or vegetables [126]. These results contrast with those from a sub-cohort of the Nurses' Health Study I and II, commencing in 1984 and 1991, respectively. From >170,000 participants 269 incident CD cases were verified. Analysis of FFQ data provided every four years identified a negative association between CD risk and fibre intake, particularly fibre from fruit [127]. Andersen [126] suggests that the lack of association between CD risk and fibre in the EPIC-IBD cohort could be attributable to the 'arguably' low CD incidence number of 104, and the availability of only baseline FFQs.

When considering studies investigating fibre intake the dietary fibre definition used in the study should be taken into account. The Codex Alimentarius Commission (CAC) is a United Nations organisation that develops food standards for international food trade that are designed to protect consumer health and promote fair trade. The CAC dietary fibre definition:

> Dietary fibre means carbohydrate polymers with 10* or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories:
>
> 1. Edible carbohydrate polymers naturally occurring in the food as consumed.
> 2. Carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.
> 3. Synthetic carbohydrate polymers, which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities. ([128] p. 2).

The CAC dietary fibre definition includes types of fibre that are excluded from other definitions, for example, resistant starches, synthetic carbohydrate polymers, extracted, modified or synthesized fibres, or dietary fibre from animals [128]. The use of restrictive definitions for dietary analysis could lead to an underestimation of true intakes. The two previously mentioned studies, the EPIC-IBD cohort [126] and the Nurses' Health Study I and II [127], did not disclose how they defined dietary fibre which makes it difficult to compare the findings with other studies and also to compare the intakes to dietary guidelines.

* *The entire CAC definition includes polymers with 3-9 monomeric units. Whilst this is CACs preferred definition, this detail is optional to accommodate dissensus.*

2.1.3 Non-Food Elements

The intake of non-food elements such as ultrafine dietary particles, also known as microparticles, has been identified as a potential contender in the increasing incidence of CD [129]. While a portion of dietary microparticles are naturally occurring, namely soil and dust, it is the abundance of synthetic microparticles found in foods and pharmaceuticals, mostly as additives, that is of concern [130,131]. Ranging in size from 0.1-1.0 μm in diameter, accumulation of dietary microparticles occurs in macrophages and Peyer's patches

of human intestinal tissue [132]. Little work has been conducted in this field; however, *in vitro* work using macrophages has demonstrated minimal effect of incubation with microparticles alone, while incubation with microparticles and bacterial antigen lipopolysaccharide (LPS) together enhances the cytokine response seen in response to LPS alone. Furthermore, an inverse relationship between microparticle concentration during incubation and subsequent macrophage phagocytic capacity was observed [133]. Another mechanism has been proposed to support the notion that microparticles may induce the GIT inflammation seen in CD. This involves antigens or toxins entering the intestinal mucosa by adhering to the surface of dietary microparticles [134], although this has yet to be proven.

Two studies have trialled the efficacy of low microparticle diets in patients with CD. In the first study a significant reduction in CDAI was reported in the trial group of 9 patients compared to the control group [135]. In the second study, markers of inflammation were measured in 83 patients at the end of the four-month period and at one-year follow up, yet no reduction in disease activity was observed [136]. Similar to the first microparticle exclusion study, encouraging results were seen in a pilot study that explored a strict 6-week organic diet free from processing, additives, and contaminants such as fertiliser and pesticide. After four weeks a reduction in disease activity, as assessed by MRI or endoscopy, was seen in four of the five CD patients compared to only one of the nine control patients. Although no difference were seen in CDAI, quality of life, or inflammatory markers between the two groups [137].

Emulsifiers are another form of non-food element suspected in the aetiology of CD. An exclusion diet for CD patients is yet to be trialled, however strong positive correlation has been reported between emulsifier consumption and CD incidence based on data available during 1992-2004 for Asia, Japan, Europe, Canada and USA. This correlation was reinforced when emulsifier sales data in Japan were compared with CD incidence for 1992, 1995 and 1998 [138]. The potential effect of emulsifiers has been explored using the mucosal simulator of the human intestinal microbial ecosystem. Two commonly used emulsifiers, polysorbate 80 (P80) and carboxymethylcellulose (CMC), effectuated a change in microbial gene expression and increased the proinflammatory potential of the microbiota [139]. Earlier work in mice demonstrated an association between a 12 week intake of P80 or CMC and alterations in microbiota composition, increased gut permeability, and chronic inflammation [140]. Similarly, artificial sweeteners have been implicated in CD [141] and their potential to alter microbiota composition has been documented in several murine studies [142–144].

It is clear that further work is needed to clarify how the GIT immune system responds to the intake of non-food elements as well as those that enter the luminal mucosa. It would be of interest to establish whether microparticle uptake is greater in CD patients compared to healthy controls. Gatti [145] sought to answer this question for micro- and nanoparticles by detecting their presence in the colonic tissue of 16 patients with colon cancer or CD, and three controls. A range of particles were reported in all cancer and CD patients, but not controls, suggesting the involvement of micro- and nanoparticles in colonic disease. However, Gatti also reported a heterogeneity and altered morphology of control samples due to contamination and obligatory washing prior to fixation, potentially compromising the findings.

2.2. *IMMUNE SYSTEM DYSREGULATION*

The GIT is constantly exposed to an array of foreign substances from the external environment, many with the potential to do harm if permitted passage through the epithelial barrier. Under normal conditions homeostasis is maintained by a balance of recognition and action against pathogens, the identification and tolerance of harmless bacteria and dietary antigens [146], and maintenance of the intestinal barrier [147]. Coordinated action of the innate and adaptive immune system is needed to maintain this balance [148]. Dysregulation of one or more GIT immune system components is strongly implicated in the pathogenesis of IBD [149], namely increased intestinal permeability [150] or dysfunction of bacterial recognition [151] in genetically predisposed individuals. The GIT immune system is thought to respond to homeostatic imbalance in an over compensatory manner, bringing about the chronic inflammation observed in CD [152].

2.2.1 Intestinal Barrier

The mucosal intestinal barrier is the first line of defence against ingested pathogens [153]. Comprising a single layer of tightly connected epithelial cells covered in a dense brush border of microvilli [148] and a surface layer of mucus [154], this barrier has evolved to permit the digestion and absorption of nutrients whilst simultaneously preventing pathogen entry. Enterocytes, goblet cells, microfold cells (M cells), enteroendocrine cells, and Paneth cells (PC) form the epithelial cell layer [155], while the mucus layer is made up of mucins, defensins, and lecithin [156], and contains high concentrations of antimicrobial peptides [157].

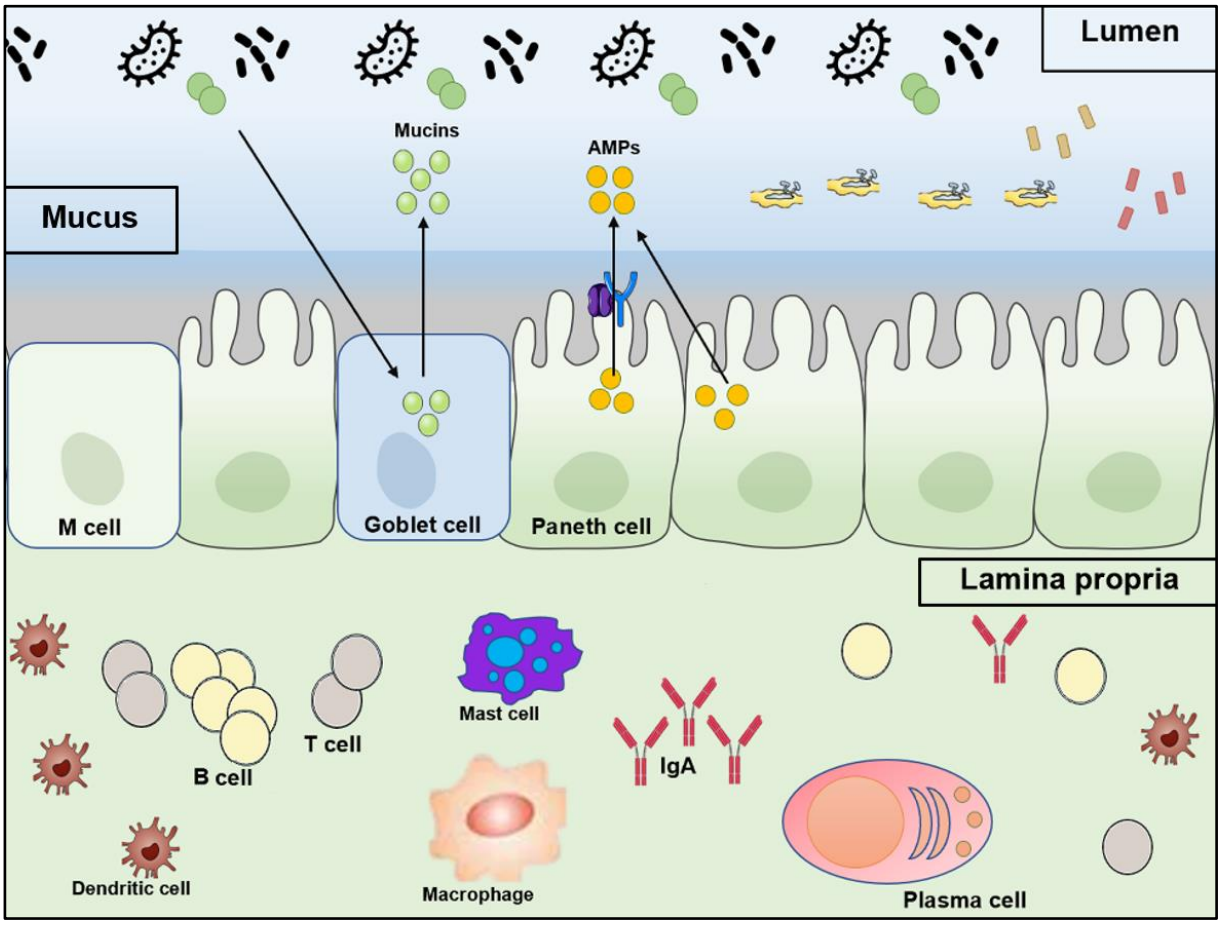

Figure 2.1 Schematic diagram of the Intestinal Barrier [158].

When barrier function is disrupted, exposure of the underlying lamina propria to luminal contents increases and the balance between immune response and suppression is altered [159]. This leads to a heightened state of inflammation and increased expression of proinflammatory cytokines, such as tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-ϒ), that may further disrupt barrier function [159]. The integrity of this barrier is dependent on the rapid and continuous cycle of cell turnover, and functioning of TJ protein complexes [160] that connect adjacent cells and selectively impede paracellular entry of macromolecules [161]. As early as 1988 Hollander [162] proposed that an increase in intestinal permeability caused by malfunctioning TJ's may play a role in the aetiology of CD [163]. Since then, increased permeability has been demonstrated in patients with CD [164,165], their spouses [166,167], and also in their first and second degree relatives [168–170]. This observation in spouses indicates that environmental elements are the likely causative agents of increased intestinal permeability, however, with the exception of some dietary substances [171], little evidence is available to explain this.

Genetic studies have identified several gene variants that may contribute to the increased intestinal permeability often seen in CD. Variants of the gene encoding nucleotide-binding oligomerisation domain (NOD2) protein were first linked to CD susceptibility in 2001 [172,173] and have subsequently been associated with increased intestinal permeability in CD patients and their first degree relatives, but not in their non-related household members [174]. Other research has shown a significantly greater incidence of increased intestinal permeability in first degree relatives of familial CD patients than those of sporadic CD patients [175]. Other genes implicated in increased intestinal permeability include variants of LAMB1, the gene that encodes a subunit of laminin, a basement membrane glycoprotein involved in cell adhesion; and CDH1, the gene that encodes E-cadherin, a transmembrane protein involved in the formation of adherens junctions which join adjacent intestinal epithelial cells (IEC) [176]. Further, increased intestinal permeability has been described in CD patients with a variant of the JAK2 gene, a protein involved in the signalling pathway of inflammatory cytokine IL-23 [177].

Tight junction irregularities have been highlighted in conjunction with CD. For instance, *in vitro* stimulation of intestinal cell line HT-29 with TNF-α and IFN-γ led to reduced expression of TJ transmembrane protein occludin [178], a manifestation also documented in colonic biopsies from IBD patients with active disease [160]. Vetrano et al. [179] explored the expression of a lesser known protein, intercellular junctional adhesion molecule-A (JAM-A), that is highly expressed at TJ's. Expression of JAM-A in intestinal biopsies was significantly down-regulated in inflamed tissue, and moderately down-regulated in non-inflamed tissue, compared to controls. Although intestinal permeability was not measured, an increase is plausible as evidenced in JAM-A silenced Caco-2 cells and in JAM-A knockout (KO) mice. Vetrano et al. [179] also reported increased susceptibility to dextran sulfate sodium (DSS) induced colitis in JAM-A KO mice, along with significantly increased production of inflammatory mediators and epithelial apoptosis following colitis induction.

2.2.2 Microorganisms

Microbiota play an essential part in development of the GIT and the immune system [180]. Essential for the breakdown of complex indigestible carbohydrates, and the production of vitamins and short chain fatty acids (SCFA), a symbiotic relationship exists between many GIT microbiota species and their host [181]. However, a loss of tolerance to these commensal flora, or unregulated microorganism entry beyond the epithelial layer to the lamina propria, may lead to chronic inflammation and IEC damage [182]. Recognition of microorganisms that pose a threat is dependent on identification of their unique pathogenic motifs [183], termed pathogen associated molecular patterns (PAMP) or microbe-associated molecular patterns [184,185], that are found on or in their cell wall [184] such as glycans, mannans, and lipopolysaccharide [186]. Pattern recognition receptors (PRR) are responsible for PAMP identification [187], namely toll-like receptors (TLR) and NOD proteins [188], and are required for distinguishing between self and non-self. This begins with the transport of small amounts of macromolecules, microorganisms and their metabolites across the small intestinal mucosa by M cells, followed by PRR presentation. Sensing of non-self by PRRs triggers signalling cascades via transcription factor nuclear factor kappa B (NF-κB) and the downstream expression of proinflammatory molecules such as TNF-α, interleukin 6 (IL-6), and interleukin 8 [189], the secretion of antimicrobial peptides by PCs [190], and activation of the adaptive immune system [188]. Threat detection also leads to the activation of additional PRRs [187], presumably in order to amplify the immune response.

2.2.3 Recognition, Processing and Autophagy of Pathogenic Bacteria

Toll-like receptors are transmembrane proteins expressed by antigen presenting cells [191], including dendritic cells (DC), macrophages, and monocytes [192], as well as small intestinal enterocytes, and PCs [193]. Variations in TLR functionality or expression, and consequently impaired bacterial clearance, have been implicated in CD. This concept is backed by findings such as significantly reduced TNF-α secretion by CD patients macrophages in response to stimulation with TLR2, TLR4, and TLR5 ligands, compared to controls [194]. In inflamed colonic biopsies from paediatric CD cases significantly increased TLR2 and TLR4 expression has been reported compared to those in remission or controls [182]. Likewise, increased TLR4 and reduced TLR3 has been documented in inflamed adult CD intestinal biopsies compared to controls [195,196]. It is unclear how increased TLR4 may pertain to CD, although gene studies have identified a significant association between CD and being a carrier of the TLR4 polymorphism Asp299Gly [197–199]. A decrease in TLR3 may confer greater CD risk based on evidence demonstrating that TLR3 induction protects against murine DSS induced colitis [200].

Nucleotide-binding oligomerisation domain protein 2 belongs to a family of intracellular bacterial receptors [187] expressed by neutrophils, IECs, macrophages, PCs and DCs [6]. The protein is specific to bacterial cell wall component peptidoglycan muramyl dipeptide (MDP) [154] and induces antibacterial activity upon recognition of the MDP constituent of Gram-negative or Gram-positive bacteria [201]. Subsequent to the identification of NOD2 as a risk gene for CD [173] the structure and function of the protein, and expression characteristics of the gene, have been researched extensively. Three polymorphisms of the NOD2 gene are strongly linked to increased CD risk; R702W, G908R, and L007fs [202]; with an odds ratio of 2.2, 2.6, and 3.8

respectively in heterozygote carriers [203]. These mutations affect the bacterial sensing leucine-rich receptor region that recognises MDP [204]. The impact on protein function is demonstrated by significantly reduced or undetectable NF-κB activation in different cell types including human embryonic kidney cells [205,206] and peripheral mononuclear cells from CD patients [207]. This loss of function has downstream implications as evidenced by Wehkamp et al's. [208] report of significantly reduced expression of PC α-defensins in CD ileal biopsies, an observation more marked in patients with a NOD2 mutation. Moreover, research involving NOD2 KO mice has shown elevated intestinal colonisation of pathogenic bacterial and an inability of intestinal crypts to kill Escherichia coli [209].

Further to the importance of NOD2 in bacterial recognition, activation of this receptor induces autophagy by recruiting bacterial autophagy pathway protein ATG16L1 [210]. Polymorphisms of NOD2 impair this process, as demonstrated by the absence of autophagy after MDP stimulation of HeLa cells transfected to replicate NOD2 polymorphism L007fs [211] and in DCs from CD patients carrying a NOD2 variant [212]. ATG16L1 gene variant T300A has also been identified as a CD risk factor [206,213,214], and is thought to impair the sensing and processing of bacteria that takes place prior to autophagy [215]. Additionally, impaired autophagy is seen with polymorphisms of another CD susceptibility gene, the immunity-related GTPase family M protein gene (IRGM) [216,217]. Specifically, single nucleotide polymorphism (SNP) rs13361189 impacts the expression patterns of IRGM which affects autophagy capacity [218].

2.2.4 Dysbiosis

Dysbiosis is an imbalance of potentially harmful and beneficial microbiota [219] that in addition to genetic determinants, is believed to be influenced by birth method, nutrition, hygiene practises, antibiotic usage, age, stress, and bacterial infection [220]. The later may be a consequence of increased intestinal permeability, impaired bacterial clearing, a loss of gut homeostasis, or simply a tenacious pathogen [221].

Some doubt surrounds the concept of dysbiosis in view of knowledge gaps around the species and concentrations that constitute a normal gut population [222]. Although a generalised characterisation of normal microbial patterns has provided a basis for making comparisons [219], significant inter-individual variation exists [223] making it difficult to identify where anticipated species diversity ends and disease associated diversity begins. Despite this, investigation of dysbiosis in CD clearly demonstrates differences in the predominance of microbial species between patients and controls [224,225] as well as between patients and non-affected relatives [226,227]. Differences in microbial composition according to the degree of disease activity are also apparent [228].

While such research has highlighted microbiota species of interest, the efficacy of treatments that aim to rectify microbiota imbalances is limited, namely prebiotics and probiotics [229–231], faecal diversion [232] and faecal transplantation [233]. Until greater advances can be made in understanding the role of microbiota species and composition in intestinal health, and the extent that microbiota can be manipulated and the lifespan of such changes, there is insufficient knowledge to maintain the notion that dysbiosis is a probable cause of CD.

2.3. MYCOBACTERIUM AVIUM PARATUBERCULOSIS

Pathogenic bacteria have been implicated in CD, yet available evidence is varied with reports of similar detection rates in CD patients and controls of some species [234], and others unable to detect the suspected species in either participant group [235]. One bacterium that cannot be overlooked and continues to intrigue investigators, as evidenced by the substantial body of research, is *Mycobacterium avium* subspecies p*aratuberculosis* (MAP). While the more than 100 species of mycobacteria are mostly innocuous [236], MAP is an exception, along with the mycobacterium responsible for Tuberculosis and Leprosy, *M. tuberculosis* and *M. leprae*, respectively [237]. The small rod shaped acid-fast gram-positive bacterium [238] is the causative agent of Johne's Disease (JD) [239], a chronic granulomatous enteritis of the intestine [240] that affects domesticated ruminants such as sheep, deer, goats, and cattle [241], dairy cattle in particular [242]. Some non-ruminants including pigs, foxes, and rabbits can also be infected [243].

Young animals are the most susceptible to MAP [244] and resistance increases with age, although a high dose can overwhelm resistance in adult animals [245]. The predominant route of infection is thought to be faecal-oral transmission, however infection can take place in-utero [245], from contaminated colostrum and milk, soil, or water [246,247]. Infection usually begins in the terminal ileum [237] and progresses gradually due to the slow growth of MAP [243]. Faecal shedding of excess MAP begins prior to the presentation of clinical symptoms, which include diarrhoea, intestinal wall thickening, and protein malabsorption with subsequent weight loss, reduced milk production, dehydration, and inevitably death [237].

Accurate estimates of JD prevalence cannot be made due to a lack of data, although the disease is known to be prevalent worldwide [240]. Containment of JD is difficult due to early faecal shedding, delayed clinical symptoms presentation of months to years following infection [240], and spread of the bacterium by a small portion of animals that remain asymptomatic throughout the disease course [248]. Herd testing and ongoing monitoring is feasible, this option is unappealing though due to the expense and requisite time commitment [249].

2.3.1 Sources, Resistance, and Detection

The survival of MAP outside the host is aided by its resistance to temperature extremes, disinfectants, antibiotics effective at treating most bacteria including *M. leprae* [242], and up to two weeks of sunlight exposure [250]. While land subjected to faecal shedding poses the greatest risk of MAP exposure, water supply can provide another source as evident from the detection of MAP DNA in drinking water reservoirs, tap water, and shower biofilm, as well as rivers and river aerosols [251,252]. A small range of foods pose a further risk of MAP exposure. The culling of asymptomatic infected cattle can result in beef products destined for human consumption containing viable MAP [253], and faecal contamination can occur in the abattoir during handling and chilling [254]. Excess MAP can also be shed into milk [255] with MAP DNA detection rates ranging from 11.8 [256] to 64% of pasteurised milk samples tested in the United Kingdom [257]. As these DNA rates account for both live and dead MAP, it could be speculated that live MAP is killed during pasteurisation. However, viable MAP detection rates of 1.8 to 10.3% [255–257] of samples suggests that some

MAP are resistant to commercial pasteurisation temperatures. This is supported by research demonstrating bovine MAP survival rates of 5 to 9% after 30 minutes at 63°C, and 3 to 5% after 15 seconds at 72°C, [258]. Viable MAP has also been detected in powdered infant formula [259].

Testing for MAP is fraught with difficulties. *In vitro* culture is the gold standard [260], however this process is expensive [261], the minimum culture period is 16 weeks [262], and sensitivity is influenced by sample location and age, clinical disease stage, and decontamination method [263]. Further, the presence of dormant MAP can lead to a drawn-out or negative culture [250]. Polymerase chain reaction (PCR) is routinely used for MAP detection following the discovery of IS900, a DNA insertion element exclusive to MAP [264]. The sensitivity and specificity of PCR is high [265], although low MAP concentration, microorganism contamination [261], difficulties lysing the tough cell wall, and buoyancy-related resistance to centrifugation all reduce the sensitivity [266]. Similarly, enzyme-linked immunosorbent assay has high specificity and is inexpensive [267], but is not suited to detection in early stages of disease when MAP concentration may be low [263].

Staining methods are seldom used as the sole method of MAP detection, instead, confirming positive samples by culture is recommended [267]. Staining is cost effective, quick, and simple [261], but the specificity and sensitivity is low [268], the number of MAP tends to be underestimated [269], and imaging can be difficult when bacterium numbers are low [270]. Further, the frequently utilised Ziehl-Neelsen stain identifies all mycobacteria species [271] and is ineffective for detection of the cell-wall deficient form (spheroplast) of MAP [272]. This form of MAP has been found in CD intestinal tissue and is believed to be dormant until the surroundings favour its return to a bacillary form [273].

2.3.2 Infection and Survival

Following ingestion MAP is transported across the small intestinal epithelium by the M cells that overlie Peyer's patches [274], then engulfed by macrophages located in the submucosa and lamina propria [241]. Pathogen destruction would typically occur during the ensuing phagosome maturation process; however, some species of mycobacteria have developed ways of interrupting this process. Early work by Armstrong and Hart [275] demonstrated that in the majority of macrophages containing intact M. *tuberculosis (*M. *tuberculosis*-phagosomes), fusion of the phagosome and lysosome does not occur. Later experiments by Clemens [276] suggest that M. *tuberculosis*-phagosomes have altered clearance of major histocompatibility complex class 1 glycoproteins and reduced expression of the lysosome-associated membrane proteins (LAMP) necessary for phagosomal and lysosome fusion. An absence LAMP-1 has also been documented in M. b*ovus*-phagosomes [277]. Phagosome acidification is another stage of maturation that mycobacteria, specifically M. *tuberculosis* and M. *avium*, can interrupt [278]. The process is not yet fully understood and may arise from one or a combination of mechanisms. One such proposed mechanism is inhibition of fusion between the vacuolar ATPase-complex and mycobacterial-phagosome [279]. Another mechanism involves protein tyrosine phosphatase, a protein secreted by M. *tuberculosis*, binding to vacuolar ATPase subunit H and halting proton transport across the membrane [280]. Together these lead to pH equilibration of the mycobacterial-phagosome at approximately pH 6.3-6.5 [279], a contrast to the expected pH 4.5 [281].

The survival of MAP is further enhanced by the bacterium's ability to reduce gene expression of proinflammatory cytokines and chemokines [282] and enhance anti-inflammatory cytokine expression, such as IL-10 and IL-6 [241]. Other proposed mechanisms that could improve MAP survival include inhibition of spontaneous or induced macrophage apoptosis [283], and inhibition of IFN-γ dependent responses including bactericidal activity and reactive oxygen intermediate production [246].

2.3.3 Crohn's Disease and MAP

Mycobacterium *avium* subspecies p*aratuberculosis* was first hypothesised to be involved in the aetiology of CD by Thomas Kennedy Dalziel in the early 1900s. In what is now widely accepted as the discovery of CD [284–288], Dalziel [3], a surgeon, described the symptoms and histological features of a chronic interstitial enteritis in several patients with suspected Tuberculosis. Although acid-fast bacilli were not observed, Dalziel refuted the Tuberculosis diagnosis and instead proposed that the observed condition and JD were one in the same. Further evidence of the possible link between CD and MAP emerged eight decades later when Chiodini and colleagues [289] isolated the bacterium from re-sectioned tissue of three CD patients. Following the isolation of MAP from CD intestinal tissue, research on CD and MAP increased exponentially. As well as a common infection site, researchers have documented many histological and clinical similarities between CD and JD including chronic transmural inflammation, ulceration, granulomata, diarrhoea, weight loss, and quiescent periods [290,291]. Advances in bacterial detection methods have led to significantly greater MAP detection in CD patients compared to healthy controls using PCR [292–294], and confocal microscopy [295]. Likewise, successful isolation of viable MAP has occurred more frequently from the intestinal tissue [292] and blood [296,297] of CD patients compared to those without CD.

Studies of the immunological response to MAP have yielded mixed results, however several observations suggest enhanced MAP exposure immunoreactivity in CD patients including an elevated antibody response [298–302], greater T cell proliferation [303–305], greater secretion of TNF-α and IL-10, and lower secretion of IFN-γ by peripheral mononuclear cells [304] and leukocytes [299] compared to UC or healthy controls. Further, significantly greater TNF-α secretion has been observed in MAP positive intestinal tissue samples from CD patients compared with samples from MAP positive UC patients, individuals with irritable bowel syndrome, and healthy controls [306].

The possible link between CD and MAP is compelling, yet counter evidence exists for almost all supportive evidence. It has been hypothesised that if CD is caused by MAP, an extended period of antimycobacterial therapy would be beneficial. A meta-analysis of the small body of research in this area found the results of uncontrolled trials and case reports to be conflicting. From an initial 29 studies identified, six randomised placebo-controlled trials were analysed and it was concluded that antimycobacterial therapy may be effective for maintaining remission in CD patients only when administered following steroid induced remission [307].

With regard to the similarities between CD and JD, a number is dissimilarities have also been documented whereby features of CD including adhesions, strictures, perforations, fibrosis, fistulae, and

obstruction have not been observed in JD [260,290,291]. As JD is predominantly found in dairy cattle, the risk of exposure should be greater to dairy farmers and associated workers such as relief milkers. Rather, an observational study in the United Kingdom reported no difference in CD prevalence between 1686 dairy farmers and the general public, and no association between CD and farm occurrence of JD [308]. Correspondingly, in a USA study of 774 veterinarians and 702 dairy and beef producers, no association was observed between CD and exposure to cattle with JD [309].

Higher levels of MAP DNA have been detected in CD patients in many studies, however others have reported a similar prevalence among CD and UC patients [297,299]. The MAP detection method may also distort the results. This is evident in Naser et al's. [297] work where MAP DNA detection rates using PCR were similar for CD and UC, 46% and 45%, respectively, whereas positive MAP culture was observed from 50% of CD patients and only 22% of UC patients. It is also argued that higher MAP detection rates are caused by increased intestinal permeability and bacterial translocation in CD patients [291], and that a portion of PCR identified MAP represents bacterium transiting the body [247].

There is little doubt MAP could be involved in the aetiology of CD, however the existing research only demonstrates association, and many confounding factors exist. While prospective studies have the potential to prove causation, the necessary duration and expense of such studies is largely impractical due to the heterogeneous nature of CD onset and disease course. In order to move forward from the current impasse, the focus needs to shift away from attempting to prove MAP is the sole cause of CD, and instead re-address what could be considered the most significant limitation of existing research, accurate MAP detection. A definitive account of environmental MAP prevalence, and the proportion of individuals (with and without CD) carrying the bacterium, would provide a solid base to further investigate alternative theories. Notably, is MAP an opportunist organism that is more likely to evade a compromised immune system [243,310], does MAP infection simply exacerbate existing CD, or could MAP be the causative agent in a sub-set of those with CD [311]?

2.4. VITAMIN D DEFICIENCY

2.4.1 Sources of Vitamin D and the Optimal Serum Concentration

Stored in adipocytes, or found in circulation, vitamin D is derived from one of two sources [312]. The leading source is exposure to ultraviolet (UV) B radiation and attendant reaction with 7-dehydrocholesterol in the skin to form cholecalciferol (vitamin D_3). Naturally occurring dietary sources of D_3 include fatty fish, eggs, red meat, and dairy products, and D_2 in mushrooms [313]. Once produced or ingested, vitamin D molecules bind to vitamin D binding protein (DBP) and are transported to the liver to be converted to calcidiol ($25(OH)D_3$), the major circulating metabolite and storage form of vitamin D, by 25-hydroxylase [314]. A second hydroxylation step by CYP27B1 takes place predominantly in the kidneys, and to a lesser extent in other tissues, to form the active metabolite calcitriol ($1,25(OH)_2D_3$) [315]. Calcitriol promotes calcium and phosphate absorption, homeostasis of plasma calcium levels, and bone mineralisation [316].

The NZ and Australian Governments currently recommend a minimum serum vitamin D concentration of ≥50 nmol/L as advised by the Working Group of the Australian and New Zealand Bone and Mineral Society, Endocrine Society of Australia and Osteoporosis Australia. This recommendation is based on concentrations below 50 nmol/L giving rise to elevated levels of parathyroid hormone and an associated increase in bone turnover [317]. Conversely, it is argued that ample reliable research demonstrates health benefits associated with higher vitamin D concentrations and that a concentration of 50-80 nmol/L is required for optimal health [318]. There is also a lack of certainty associated with determining the maximum serum vitamin D concentration. Based on supplementation studies and the occurrence of hypercalcaemia, concentrations <250 nmol/l are considered safe for adults and children. Further, it is thought that signs of toxicity are only evident once a concentration of 375nmol/L or more is attained [319].

Dietary sources providing at least 5-10 µg/day of D_2, alongside minimal sunlight exposure, is suggested for maintaining 27.5 nmol/L in adults [320]. Attaining this intake may be difficult due to the limited number of naturally occurring dietary sources and the low number of fortified foods available in NZ. These concerns are supported by estimations that dietary vitamin D intake in Australasia is 2-3µg/day [317]. While UV B exposure alone is often considered sufficient for maintaining vitamin D concentrations, many factors reduce cutaneous production such as higher latitude; lack of sun exposure due to time spent indoors, clothing, photosensitivity, sunblock use, and skin cancer concerns; lower substrate production in older people; and darker skin tone [321]. Consequently, many New Zealanders have been shown to be affected by vitamin D deficiency (<25 nmol/L) and insufficiency (25-49.9 nmol/L), 5% and 27%, respectively [322].

2.4.2 Vitamin D and Crohn's Disease

The potential link between vitamin D status and CD is thought to have originated from high incidence reports in regions of greater latitude [78,323,324] including Iceland [325], Nova Scotia Canada [19], Sweden [326], South Australia [327], and NZ [20]. Vitamin D concentrations are usually lower in regions of greater latitude due to the increased solar zenith angle and the associated reduction in UV radiation intensity, as well as reduced skin UVB exposure owing to colder temperatures [328]. In addition to geographical observations, researchers have found UV exposure to be inversely associated with CD risk [329,330], rates and duration of IBD hospitalisation [331], and risk of CD inpatient surgery [332]. Furthermore, a decreased risk of CD has been associated with being born during spring [333,334] and increased if born during winter [333].

Patients with CD frequently have lower vitamin D than healthy controls, in fact, a systematic review and meta-analysis concluded that the odds ratio of vitamin D deficiency (<50nmol/L) is 1.63 for CD patients [335]. There is no shortage of evidence demonstrating the difference in vitamin D concentrations between patients with CD and controls, however, it is argued that the reduction in vitamin D concentration takes place after CD manifests, not before. Prospective studies have attempted to clarify this debate, the largest of which is derived from the Nurses' Health Study, a series of health and disease investigations that commenced in 1976. From an initial cohort of 72,719 women, a diagnosis of CD between 1986 and 2008 was confirmed in 122 participants. Using a validated regression model to predict vitamin D status, Ananthakrishnan et al. [336] found that each 2.5

nmol/L increase in vitamin D was associated with a significant relative risk reduction of 6% for CD. Conflicting evidence has been put forward by Limketkai et al. [337] following analysis of vitamin D concentrations collected at three-time points from US military personal, 240 CD and 240 controls. When categorised as quintiles, tertiles or predefined categories (deficiency, insufficiency, and sufficiency), a significant inverse association was observed for CD and vitamin D concentrations at three months pre-diagnosis and up to 21 months post-diagnosis, while there was no association at three months to three years or three years to eight years pre-diagnosis.

Considerable work has been carried out to explore the correlation between vitamin D and GIT inflammation in patients with IBD. Significant inverse associations have been seen between vitamin D concentration and index-measured disease activity [338–347], as well as disease markers including C-reactive protein (CRP) [348–351], faecal calprotectin (FC) [352], and erythrocyte sedimentation rate [353]. Correspondingly, low vitamin D concentrations have also been associated with increased risk of hospitalisation and surgery [339,354] and increased disease duration [351,355,356]. Further, a significantly greater risk of flare-ups, hospitalisations, clinic visits, steroid use, and escalating therapy was observed in patients with severe deficiency (<37.5 nmol/L) but not moderate deficiency (37.5-75 nmol/L) or sufficiency (>75 nmol/L) [352], and a positive correlation has been reported between vitamin D concentration and quality of life score [345,357].

Vitamin D can be measured by one of several assays including RIA, enzyme immunoassay, competitive protein-binding assay, high performance liquid chromatography, and liquid chromatography tandem mass spectrometry [358]. The latter is considered the gold standard assay, though each method has both advantages and disadvantages. Inter-assay variation is a long-standing problem that can still produce significant differences in the vitamin D concentrations determined [359], accordingly the method of measurement needs to be considered when interpreting and comparing the results of studies.

2.4.3 Potential Mechanisms

Clear evidence of the regulatory involvement of vitamin D in immune function has come to light since the discovery that a number of immune cells express vitamin D receptor (VDR) including activated B and T lymphocytes, dendritic cells, and neutrophils [360–362]. Almost all tissues of the body express VDR, especially in the kidney, parathyroid gland, and small and large intestine [363]. Analysis of intestinal biopsies has demonstrated lower VDR expression in CD patients compared to controls [364,365]. The effect on vitamin D induced gene expression is not known, though VDR agonists may have the potential to mitigate any detrimental effects. One such example is BXL-62 which inhibits pro-inflammatory cytokine production by peripheral blood mononuclear cells and lamina propria mononuclear cells isolated from CD patients more effectively than $1,25(OH)_2D_3$ [366]. Genetic studies have linked VDR polymorphisms with CD including SNP rs2228570 to lower vitamin D levels [367], and SNP rs731236 to lower levels of VDR protein [368], serum vitamin D levels in CD patients [369] and an increased risk of CD among males [370,371]. Lower vitamin D concentrations have also been associated with SNP rs2282679 from the DBP gene (GC), SNP rs10741657 from the 25-hydroxylase gene (CYP2R1), and SNP rs127858787 from the enzyme 7-dehydrocholesterol reductase gene (DHCR7) [372,373].

Animal and cell line studies have also been widely utilised to investigate the relationship between vitamin D and immune system dysfunction in IBD. Of note are the murine models that replicate the GIT inflammation that characterises IBD. These models often use DSS to induce a form of colitis that exhibits increased intestinal permeability and mucosal barrier damage in a dose-dependent manner [374]. Extensive work with the DSS murine colitis model has shown that cholecalciferol treatment promotes weight regain, reduces colitis severity, reduces bacterial translocation, and attenuates increases in intestinal permeability [375]. In addition, vitamin D deficient wild type mice exhibit significantly greater GIT bactericidal activity compared to vitamin D sufficient controls [376].

Gene KO strategies have advanced the understanding of the importance of vitamin D in the development and ongoing function of the immune system. In Cyp27b1 KO mice, which lack the ability to hydroxylate calcidiol to its active form calcitriol, DSS treatment caused significantly greater weight loss compared to Cyp27b1+/- and wild type controls. Expression of VDR was also elevated in the small intestine and proximal colon of the Cyp27b1 KO mice compared to Cyp27b1+/- in response to DSS treatment [377]. Mice with VDR KO demonstrate higher mortality rates and more severe colitis, and cholecalciferol treatment reduces colitis severity and minimises of weight loss [378]. While vitamin D deficiency in IL-10 KO mice leads to significant mortality in juvenile mice, stunted growth, significantly lower body weight and significantly greater small intestinal inflammation, compared to their vitamin D sufficient or supplemented counterparts. Treatment with cholecalciferol in vitamin D deficient IL-10 KO mice reduces small intestinal inflammation and significantly ameliorates colitis symptoms [379].

Lastly, using human colon adenocarcinoma cell line, the Caco-2 cell, to model the intestinal epithelial monolayer, calcitriol treatment has been shown to increase expression of TJ proteins ZO-1 and claudin-1; and to minimise the DSS-induced decline in trans epithelial electrical resistance (TEER), monolayer disruption, and increased permeability to DSS [375]. Kong et al. [380] also observed a protective effect of calcitriol treatment against DSS-induced TEER reduction and TJ damage; as well as an increase in TJ protein expression, in this case ZO-1 and E-cadherin. Similarly, using another cancer cell line, SW480, Kong et al. [380] described increased expression of TJ proteins ZO-1, claudin-1, claudin-2, and adherens junction protein E-cadherin in response to cholecalciferol treatment.

2.4.4 Human Intervention Studies

Several intervention studies have been undertaken to establish the suitability of vitamin D as an adjunctive therapy for CD. In a prospective 12-month study, Miheller et al. [381] compared the effect of 1,000 IU/day cholecalciferol or 0.5 µg/day (20 IU/day) of alfacalcidol, a potent analogue of calcitriol, on disease activity in 36 CD patients. No significant changes were seen in the 19 patients receiving 1,000 IU/day cholecalciferol. In contrast, a significant decrease was evident in CDAI and CRP after six weeks in the 17 patients that received alfacalcidiol, although this was not sustained at 12 months. The absence of specific details, such as the compliance rate or vitamin D measurements, make it difficult to theorise the rationale behind this short-lived effect. Jorgensen et al. [382] conducted a one-month double-blind placebo randomised

controlled trial (RCT) to evaluate the effect of 1,200 IU/day cholecalciferol on maintaining remission in 94 patients with CD. Mean vitamin D rose significantly from 69 to 96 nmol/L, and fewer patients in the vitamin D group relapsed compared to the placebo group as indicated by CDAI score and biomarker levels, although the difference did not reach significance. Raftery et al. [383] reported no difference in maintenance of disease remission between 13 CD patients receiving 2000 IU/day cholecalciferol and 14 patients in the placebo group over a three month period. However, when analysed by vitamin D concentration the patients that achieved concentrations ≥75 nmol/L had significantly higher quality of life scores, significantly lower CRP, and a tendency towards a lower CDAI (*P*=0.082). Further, the mean vitamin D concentration increased from 69.2 to 91.6 nmol/L in the treatment group and decreased from 51.8 top 40.4 nmol/L in the placebo group.

Intervention studies that have used higher supplementation dosages have yielded more convincing results. In a small six-month trial, 15 CD patients were randomised to receive 1,000 or 10,000 IU/day cholecalciferol [384]. In the low dose group, vitamin D concentrations increased from 59.2 to 65 nmol/L with no significant change in HBI score, while in the high dose group vitamin D increased from 49.7 to 160.2 nmol/L with a significant improvement in HBI score. In a more recent study, a significantly lower relapse rate was seen in 12 CD patients randomised to receive 10,000 IU/day cholecalciferol for 12 months. This dosage led to a significant increase in mean vitamin D concentration after 12 months from 73.5 to 160.8 nmol/L, compared to a nominal increase of 71.3 to 82.8 nmol/L in the eight patients that received 1,000 IU/day. Interestingly, no significant changes in CRP level were observed in either treatment group [385]. Lastly, 23 vitamin D deficient (<50 nmol/L) paediatric (3-16 years) IBD patients (19 CD, 2 UC, and 2 IBDU) were given a single-oral high-dose supplement of 100,000-800,000 IU cholecalciferol depending on age. Vitamin D increased from a mean baseline of 39 nmol/L to 189 nmol/L at 1-2 months, and a significant decrease was seen in Paediatric CDAI scores, ESR, and CRP after three months. The high dosage was well tolerated and a single elevated calcium at the two-week mark had normalised on repeat measurement ten days later [386].

As an alternative to the effect of supplementation dosage on disease activity, Yang et al. [387] investigated the effect of attaining a minimum vitamin D concentration. In the 24 week pilot study, 18 CD patients received cholecalciferol incrementally up to 5,000 IU/day or until concentrations reached 100 nmol/L. At the start of the study CDAI scores indicated all patients had active disease, and by study completion disease activity had reduced significantly with 67% of patients attaining remission. Quality of life scores also improved significantly. Markers of inflammation including CRP, ESR, TNF-α, and cytokines IL-17 and IL-10 were measured at baseline and 24 weeks, though no significant changes were observed. Mean baseline vitamin D concentration increased from 40 to 112.5 nmol/L, with 14 of the 18 patients receiving the full dosage of 5,000 IU/day throughout the study, yet >100 nmol/L was only reached by half of the patients. Similarly, in a pilot study, supplementation ranged from 1,000-10,000 IU/day for 12 weeks in order to reach a vitamin D concentration of 100-125 nmol/L. A significant reduction was seen in the HBI scores of all four patients with active CD, but not in the concentrations of FC, CRP, or albumin. Vitamin D increased from a mean baseline of 49 to 105 nmol/L with three patients reaching the target concentration.

One of the three patients required 10,000 IU/day cholecalciferol, a substantial dosage even taking into account a low baseline of 27 nmol/L and mild-moderate disease activity [347].

Another point to consider when investigating supplementation dosage is absorption capacity. It is known that absorption of vitamin D is reduced by inflammation, fat malabsorption, and a reduced small intestinal surface area, yet this does not account for all instances of low vitamin D in patients with CD compared to controls. Farraye et al. [388] investigated this further by testing ergocalciferol bioavailability in 10 healthy controls and 37 patients with CD and no clinical disease activity as indicated by normal ESR and CRP levels. Vitamin D concentrations were measured at baseline and 12 hours after oral supplementation of 50,000 IU vitamin D_2. When compared to controls, bioavailability was 80% in patients with no history of resection surgery, 64-73% in patients with bowel resection history, and 50% in patients with an ileostomy. These findings illustrate the significant impact bowel surgery can have on absorption capacity and suggest that absorption may be comprised even in the absence of demonstrable inflammation. In patients that show little or no response to oral vitamin D supplementation, irrespective of surgical history, intramuscular injection may be an effective alternative.

The outcomes of these intervention studies support the notion that vitamin D could be useful in managing disease activity in CD, though some aspects do have an impact on the strength of any conclusions drawn. Notably, the generally small sample size, and the indices and biomarkers selected to evaluate changes in disease activity. For example, HBI and endoscopic disease activity are poorly correlated, and therefore should not be used to indicate remission. Additionally, there is debate around the FC thresholds that indicate mucosal healing and differentiate between active and inactive disease [389]. Despite these shortcomings, the findings demonstrate that 1,000-1,200 IU/day of vitamin D_3, and the reported increase in concentrations to 65-96 nmol/L, is not adequate to significantly reduce disease activity [381,382,384,385]. Instead, a higher dose of 10,000 IU/day may be required as seen by an improved disease activity score after six months [384], and reduced relapse rate after 12 months [385]. In these two studies the mean vitamin D concentration at study completion was 160 nmol/l, and in Martin et al.'s [386] paediatric study the mean concentration increased to 189 nmol/l and a significant decrease was seen in disease activity. Attaining a minimum concentration of $\geqslant$75 nmol/L was also beneficial as evidenced by improved quality of life scores [383,387] and reduced disease activity score [347].

While the minimum vitamin D concentration for optimal health remains uncertain, a robust review found that concentrations of 90-100 nmol are the best for various health outcomes including bone mineral density and risk of colorectal cancer [390]. Among the reviewed studies, significant changes were seen with endpoint concentrations of 75-189 nmol/L but not 65-96 nmol/L. This ineffectual range may in fact be closer to 65-83 nmol/L, as in Jorgensen et al.'s [382] one month study, supplementation of 1,200 IU/day was associated with a nonsignificant reduction in relapse. A longer study may have led to significant changes. Taking everything into consideration, the findings suggest that vitamin D concentrations of $\geqslant$90 nmol/L are required to see improvements in IBD disease activity. Regrettably, the available data is insufficient to infer the associated duration required to evoke improvements.

It is difficult to discount the wealth of evidence that suggests that low serum vitamin D concentrations play a role in the risk of CD, disease progression and severity, and potentially modulation of disease activity. Before further advances can be made in this field, it seems that a greater understanding of the immune modulating properties of vitamin D and influencing factors is needed. As this will take some time, and given the association between low vitamin D and increased risk of low bone mineral density [391], it seems prudent to monitor vitamin D concentrations in patients with IBD and to recommend supplementation to those considered to be deficient or to have insufficient concentrations.

2.5. OTHER ENVIRONMENTAL FACTORS

Despite significant advances in genetics research, and a marked increase in the identification of risk loci for CD, many of which are involved in immune pathways, the risk of disease attributed to these loci is considered to be around 13% for CD [392]. On account of this, the role of environmental factors in the aetiology of CD may be greater than initially predicted.

Tobacco smoking is one of the most established risk factors for CD. Analysis of data from 229,111 participants in the Nurses' Health Study has demonstrated a hazard ratio of 1.35 among former smokers, and 1.90 among current smokers, compared to never smokers [393]. In patients with CD, smoking has been shown to influence disease activity and progression with active smokers having a significantly greater risk of flare-ups, disease activity following surgery, and need for surgery compared with non-smokers, as well as greater risk of needing a second surgery compared with former smokers [394]. Active smokers are also reported to have a lower response rate to infliximab, a biological immune-modulating agent with high efficacy in treating CD [395]. This is marked in a study evaluating infliximab response in 100 CD patients with symptom improvement seen in 74% of non-smokers and 22% of smokers, and a prolonged response seen in 59% of non-smokers and 6% of smokers [396]. Cigarette smoking has an immunosuppressive effect on both the adaptive and innate immune responses [397]. Although the exact mechanisms responsible for an increased risk of CD are not known, cigarette smoking may alter cytokine production, mucosal blood flow, antioxidant capacity, gut permeability, and gut motility [398]. Alterations to the gut microbiome are also implicated. This was illustrated in a study examining the effects of chronic cigarette smoke exposure (24 weeks) on murine GIT bacterial diversity [399]. The outcomes included changes to microbial composition and activity in the colon, altered inflammatory gene expression in the ileum, and increased mucin expression in the ileum and colon.

Imbalances in intestinal microbiota populations, including reduced diversity, have been well documented in CD [400–402]. Consequently, the use of antibiotics has been identified as a potential CD risk factor due to the ability of this medication to induce changes in microbiota during treatment, and to influence repopulation following treatment cessation [403]. Indeed, in a Canadian case-control study of 1,025 participants with adult-onset CD and 22,346 controls, prescription of antibiotics in the two to five years prior to diagnosis was associated with a 1.3 fold increase in being diagnosed with CD [404]. The same research

group evaluated antibiotic use in 27 cases of paediatric onset CD. Of the 360 controls, 139 (39%) had one or more instances of antibiotic use before the age of one, compared to 18 (67%) of CD patients [405]. This suggests a stronger association between antibiotic use and paediatric CD risk, an observation supported by Ungaro et al's [406] meta-analysis on antibiotic exposure and CD risk among children and adults with overall odds ratios of 2.75 and 1.57, respectively. A few theories have been put forward to explain this observation. Firstly, antibiotic therapy expedites CD pathogenesis or imitates CD triggers in predisposed individuals [404]. Secondly, the infection necessitating antibiotic therapy increases the risk of CD [406]. Thirdly, the establishment of microbiota takes place largely by the age of three years [407], and exposure to antibiotics during this time disrupts the development of immune tolerance [408]. Finally, a history of antibiotic exposure during childhood is indicative of pre-existing immune impairment and is not associated with risk of CD [409].

Of the many environmental factors that have been proposed, several are linked by the hygiene hypothesis. Originating from observations of hay fever and eczema prevalence during infancy, childhood, and early adulthood, the hygiene hypothesis proposes that prenatal or early childhood infection may protect against allergic disease, while infection in older childhood may confer additional protection [410]. Exposure to a greater number and diversity of microorganisms is understood to facilitate development of immune tolerance, preventing unwarranted proinflammatory responses [411]. The hypothesis is often cited as the mechanism that may explain a lower CD risk associated with rural residence [412], larger number of siblings [413], and exposure to animals [414]. The importance of early life exposures is also evident from research on breastfeeding with two reviews concluding a protective effect of breastfeeding against CD risk [415,416]. The breastfeeding effect was also found to be dose-responsive with the highest protection associated with breastfeeding for a minimum of 12 months [416]. It is unclear how breastfeeding imparts protection against CD, although like other immune-mediated diseases, the medley of immunological molecules found in breastmilk are thought to influence the gut microbiome by providing a source of healthy gut microbiota, fostering microbiota diversity, and inducing immune tolerance [417,418].

Appendectomy may have an effect on the risk of CD. In a large cohort study incorporating data from studies in Sweden and Denmark, the hospital files of 709,353 patients that had undergone appendectomy between 1964-2004 were checked for later CD diagnosis. No association was detected among patients aged below ten years, while the standardised incidence ratio of developing CD was 1.5 overall, and 8.7 in the six months post appendectomy. The increased risk was negligible after five to ten years [419]. Similar results were observed following examination of hospital data from the Southern England Oxford Record Linkage Study, although the greatest risk was only evident in the year following appendectomy [420]. It has been suggested that the elevated risk may in part be explained by undiagnosed CD, as ileal CD may present with similar symptoms to appendicitis. This theory is supported by analysis of data from the aforementioned Danish study whereby appendectomy in patients with non-appendiceal disease was associated with 14-fold increased risk of hospitalisation due to CD in the following year [421]. In contrast, some studies did not observe any association with appendectomy [422–424], a case-control study in

Israel noted that appendectomy occurred more frequently in CD but was not a risk factor [422], while Radford-Smith et al [425] reported a negative association between appendectomy and later CD diagnosis. Many other environmental factors are associated with increased CD risk, though their interpretation is complicated. For example, conflicting results have been reported for obesity [426] and for air pollutant exposure [427], research on the oral contraceptive pill contains risk factors and variables not accounted for [428], and experimental research on stress is limited to animal models [429].

2.5.1 Epigenetics

Epigenetic modifications are potentially heritable alterations to gene structure that are not caused by DNA sequence changes [430]. The main epigenetic modifications are DNA methylation, expression of non-coding RNA, and histone modification, which can all be triggered by environmental factors such as diet [431]. The most studied epigenetic mechanism is DNA methylation which involves the covalent addition of a methyl group to cytosine [432]. This can bring about changes in transcriptional activity and the extent of gene expression, and if DNA methylation involves a gene associated with IBD the risk of developing IBD may be affected [430]. Non-coding RNA, such as microRNA (miRNA), are non-coding single-stranded molecules that can mediate gene expression [431]. Research suggests that processes strongly implicated in IBD, including T-cell differentiation, the TH17 signalling pathway, and autophagy, are modified as a result of transcriptional and post-transcriptional regulation of gene expression by miRNAs [433]. Regulation of the intestinal epithelial barrier can also be influence by miRNA [434]. Further, the expression of certain miRNAs differs between CD and UC, a discovery that could facilitate IBD diagnosis [430]. Histone modifications involve the posttranslational modification of histone proteins. This can alter gene transcription by changing transcription factor access to the DNA. Less is known about histone modification and IBD, although researchers have effected changes in the inhibitory function of regulatory T cells, apoptosis, and proinflammatory factors in mice with chemically induced colitis [434]. Research on epigenetics and IBD is still a new area that we know little about. It is anticipated that in the future a greater understanding of epigenetics will not only improve our understanding of the pathogenesis of IBD but also aid in the diagnosis of IBD and the prediction of both disease course and response to treatment [432].

3. Dietary Treatment and Management of Crohn's Disease

Currently there is no cure for CD, not even surgery [9], therefore the goal of treatment is the induction and maintenance of remission, followed by prevention of disease progression by achieving full mucosal healing [435]. As the aetiology of CD is unknown, treatment can only be targeted at the symptoms [436]. A common first step is suppression of the immune system in order to reduce inflammation [12] and its associated symptoms. Once the inflammation has subsided the future flare-up risk can be minimised through the use of medication including corticosteroids, antibiotics, aminosalicylates, and immune-modulating agents [12]. Biological immune-modulating agents (biologicals), such as TNF-α blockers, tend to be the most effective form of treatment, especially in moderate to severe cases of CD [395], however other drug therapy often takes precedence in view of the high cost of biologicals [437]. The future feasibility of widespread biological use is improving as a result of patent expirations and the development of functionally similar biotherapeutic products known as biosimilars [438]. When patients experience complications or fail to respond to drug therapy, surgery may be required [439]. Even with a reduction in the risk of surgery overtime, a meta-analysis has reported a 38.7% risk of surgery in the ten years after CD diagnosis [440].

3.1. ENTERAL NUTRITION

The risk of malnutrition in patients with CD is well documented. This is largely due to one or a combination of factors including decreased nutrient intake in response to pain or nausea; inflammation, bacterial overgrowth, or previous surgery induced malabsorption; increased nutrient loss from diarrhoea or vomiting; or greater nutrient requirements as a consequence of persistent inflammation [441]. There is a lack of up to date evidence of the prevalence of malnutrition in CD, however values ranging from 20-85% have been reported [442]. Malnourished patients, children in particular, may be placed on a liquid diet for approximately 6-8 weeks [443] to restore their nutrient and caloric levels, and also to aid in the induction and maintenance of remission [444]. A liquid diet can be administered orally or via nasogastric tube, known as enteral nutrition (EN) [442]. When EN is not tolerated by the patient, or is considered not appropriate, such as cases of severe intestinal damage or disease activity [445,446], administration is by a central line or intravenously, known as parenteral nutrition (PN) [447]. The use of PN comes with the risk of severe complications including catheter induced damage to veins or surrounding tissue, sepsis, decreased liver function, under or over provision of nutrients such as NaCl [448], and lymph duct damage [442]. By contrast, the potential side effects when commencing EN include vomiting, diarrhoea, nausea, headaches, and abdominal cramps [441,449] which can be minimised by a gradual introduction. Compliance when taking EN may also be problematic due to differences in palatability [450], low motivation [451], and nasogastric tube intolerance [452].

The four types of EN are elemental, semi-elemental, polymeric, and specialised nutrition, which differ in macronutrient structure and content. The macronutrients in elemental nutrition are hydrolysed to

their most simple and easily digestible form, semi-elemental nutrition contains partially hydrolysed macronutrients, the macronutrients in polymeric nutrition are unaltered, and specialised nutrition contains selected nutrients or biologically active substances [447]. Although not as effective as corticosteroids [453–456], exclusive EN treatment has been shown to bring about clinical remission in active CD, induce histological and endoscopic bowel healing, improve weight gain, and reduce levels of inflammatory markers and pro-inflammatory cytokines [457,458]. These improvements are more evident in children with active CD than in adults [450], an observation that reflects higher rates of compliance in children [449]. It is not fully understood how exclusive EN elicits favourable effects beyond restoring nutrient and caloric deficits, though there are a few theories. One is that the simple nature of these diets reduces digestion demands and enhances absorption in the duodenum and jejunum, thus facilitating bowel rest in the region most afflicted in CD [459], however other researchers consider bowel rest to be of little importance [448]. Griffiths et al. [453] suggests the improvements may be the result of low residue content and a subsequent decrease in the intestinal bacterial load [460]. Another theory is that exclusive EN reduces the production of proinflammatory cytokines by altering the gut microbiota [461,462]. It is also thought that replacing whole protein in the diet with free amino acids lessens the dietary antigen load, resulting in fewer immune response triggers [463].

3.2. ELIMINATION DIETS

An elimination or exclusion diet can be undertaken to explore reports of symptom triggering or exacerbation following the consumption of certain foods or dietary components, a belief held by 46-81% of CD patients [464–472]. The patients usual diet is replaced with EEN [449] until clinical remission is achieved, then foods are gradually re-introduced [11], ideally a single item per day [465]. When re-introduction of a food is followed by symptom onset the food is again removed from the diet and re-tested at a later time. In the absence of symptom onset the tested food is considered safe [451].

Many researchers have used self-administered questionnaires to identify foods or food groups that CD patients consider detrimental to their GIT symptoms (see *Table 2.2).* From the studies reviewed the most commonly reported detrimental food groups are fruits and vegetables, dairy products, spices and spicy foods, nuts and seeds, fried and fatty foods, and alcohol and carbonated beverages. Identification of individual foods is somewhat more difficult, however in more than one study corn [470,473–477], milk [468,474,478,479], raw vegetables [469,472], and lettuce [464,473] have been indicated. A smaller number of studies have also evaluated foods or food groups that CD patients consider to be beneficial. Such foods reported include rice, potatoes, yoghurt, white fish, and banana [470,473,475,480,481]. When considering the number of patients that report one or more foods to be beneficial, and the variety of foods reported, both are markedly lower than those associated with detrimental foods. This could be owing to a modest number of foods able to lessen CD associated GIT symptoms, or the consumption of beneficial foods in quantities below the threshold necessary to perceive an effect.

Nolan-Clark et al. [482] delved into the reported link between dairy product consumption and GIT symptom onset or exacerbation. Analysis of self-completed questionnaires from 165 CD patients found that more than half (55-71%) of patients reported no difference in symptoms in response to consumption of commonplace dairy products including milk, butter, ice cream, yoghurt, and cheese. The authors went on to suggest that a higher fat content, rather than dairy content, may be the symptom trigger. Earlier observations between dairy product consumption and GIT symptoms have been attributed to a greater prevalence of lactose intolerance or malabsorption in CD, however a recent meta-analysis concluded that in patients with CD lactose maldigestion is predominantly determined by ethnicity and not by disease [483]. Therefore, it is plausible that the true prevalence of dairy intolerance in CD has been overstated and a portion of patients unquestionably avoid dairy products following unfounded advice, due to symptoms experienced during periods of active disease, or owing to a different physiological mechanism that induces GIT symptoms.

Although a subset of CD patients appears to benefit from eliminating certain foods or food groups from their diet, only a small number of clinical trials have investigated elimination diets in this patient group. In a very early trial, 60 foods were gradually reintroduced to 31 patients following induced remission. Prolonged remission of 6-32 months without drug therapy was seen in 21 patients, and all 29 patients that completed the reintroduction stage were able to identify symptom triggering foods including wheat in 69% of patients, dairy 48%, yeast 31%, and corn 24% [476]. Almost a decade later, Riordan and Hunter [477] evaluated elimination diet efficacy in a RCT of 40 patients compared to 38 controls receiving tapered steroid treatment and healthy eating advice. Relapse rates were higher in the control group during the 12-week trial and at two years, 66% and 79% respectively, compared to 30% and 62% in the elimination diet group. The most frequently identified symptom triggering foods were corn 18%, wheat 15%, milk 15%, and yeast 15%. In the sole trial that meets the requisite of a true elimination diet by including a positive retest, eight of the 36 patients experienced rapid relapse and 20 were able to identify symptom triggering foods. Of the 17 patients willing to undergo re-challenge, 10 experienced further symptoms, while four of the five patients that underwent a further re-challenge experienced symptom recurrence [484]. Lastly, Slonim et al. [485] explored the effect of a predetermined elimination diet in a case-study of five paediatric patients with moderate-severe CD. Dairy products, grains, and products containing the food-additive carrageenan were excluded from the diet, and a range of supplements believed to be beneficial in IBD were simultaneously administered to promote weight gain. All patients went into remission within two months, significantly decreased ESR and CRP levels were observed at one-year follow-up, and the mean length of remission was 56 months.

Poor compliance is often observed in patients undertaking elimination diets due to their time consuming and restrictive nature [486]. As an alternative some researchers have set out to develop ‘safe’ diets that allow patients in remission to reintroduce several foods at once. One such regime is the LOFFLEX diet, a regime that is low in fibre, fat reduced, and is free of wheat, dairy, nuts, seeds, and citrus fruit. In the study of this diet CD patients were required to follow an enteral diet to induce remission, followed by one of two protocols: the LOFFLEX diet for a minimum of two weeks then gradual food reintroduction, or an

elimination diet and gradual food reintroduction. Among compliant patient's, little difference was seen between relapse rates shortly after food reintroduction and the probability of remission at two years; 12.5% and 59.4% of patients respectively in the elimination group, and 13.6% and 55.6% of patients in the LOFFLEX group. Detrimental foods were identified by 81% of patients with the most frequently reported foods comprising wheat (50%), milk and dairy (44%), coffee and tea (39%), oats (33%), rye (31%), and yeast (28%). Further, a positive trend was observed between detrimental food identification and remission at 12 months [465].

In a shorter trial, Sigall-Boneh et al. [487] evaluated induction of remission by the Crohn's Disease Exclusive Diet (CDED); a whole food diet that minimises or excludes foods linked to adverse GIT changes such as increased intestinal permeability. Forty-seven paediatric and young adult CD patients followed either the CDED exclusively, or in combination with 50% partial EN (PEN). After six weeks exclusive CDED led to a greater reduction in disease activity, higher remission rate, and decreased remission rate. In a subsequent study of longer duration, exclusive CDED was again associated with a greater remission rate than treatment with combined CDED and PEN [488]. This suggests that dietary trigger exclusion is a crucial element in inducing remission.

The outcomes of questionnaire-based studies and clinical trials strongly supports the notion that certain foods or food groups can trigger, exacerbate, or reduce CD symptoms. The ability of the patient to identify these foods however may be influenced by diet variety, nutrient interactions, ability to detect a diet related change, and whether change is related to a single food or dietary pattern [489]. Inherent methodology problems such as poor dietary recall, low subject numbers, variations in drug therapy, and crude result interpretation, must also be considered when drawing any overall conclusions. There was agreement between the findings with many foods reported to trigger or exacerbate symptoms falling into one of four categories: highly fibrous, highly processed, spicy, and nuts and seeds. A common factor was less apparent among the foods most frequently reported to reduce CD symptoms which include banana, chicken, rice, fish, and yoghurt. Although, these findings may have been affected by several questionnaire-based studies only evaluating 16 or fewer foods/food groups and thus a significant number of foods were unaccounted for. Nevertheless, where patients are suitably motivated to adhere to the protocol and are prepared for setbacks during the initial food identification phase, undertaking an elimination diet and adjusting one's diet accordingly may be suitable as a long-term adjunctive therapy if nutrient or energy intake is not compromised.

Table 2.2 Foods and Food Groups associated with Crohn's Disease Gastrointestinal Tract Symptoms

Participants	Study Design and Method	Results
England, 1984 [476] CD 31	Clinical trial - elimination diet Remission induction, medication withdrawal, gradual food reintroduction (60 foods)	Early relapse = 6% Identification of symptom triggering foods = 88% Elimination diet only and remission maintained 6-32 months (mean 15.2 months) = 67% Problem foods = wheat 69%, dairy 48%, yeast 31%, corn 24%, potato 17%, banana 17%
USA, 1988 [473] CD 71, HC 27	Cohort - questionnaire Self-completed, food tolerance (32 foods)	Most common problem foods CD (ileostomy) = corn 79%, nuts 69%, cabbage/broccoli 68%, chilli beans 65%, fizzy drinks 57%, raw fruit 57%, lettuce 52%, spices 50% CD (no ileostomy) = onions 60%, chilli beans 55%, spices 55%, beer 52%, nuts 51% HC = none Tolerated foods CD = rice, potatoes, lamb, cooked fruit, white fish, chicken/turkey, white bread HC = potatoes, lamb, cooked fruit, chicken/turkey, white bread, beef, pork, pickles
England, 1993 [484] CD 28	Clinical trial - elimination and re-challenge diet Remission induction, food reintroduction, problem food re-challenge, problem food double-blind challenge	No sensitivities identified = 8/28 patients Negative re-challenge = 7/20 Positive re-challenge = 10/17 (milk 4/10, peanuts 2/10, citrus fruits 1/10, wheat, eggs, beans, aspirin, alcohol, chicken, plums, beetroot 1/10 (each)) Negative double-blind challenge = 2/10 Positive double-blind challenge = 4/10 (milk, peanuts, wheat, aspirin 1/10 (each))

England, 1993 [477] CD 40 (diet), CD 38 (cortico-steroids)	RCT, 12 weeks - elimination diet Remission induction, medication withdrawal, Diet **or** Oral corticosteroids	Most common food intolerances = corn 18%, wheat 15%, milk 15%, yeast 15%, egg 10%, potato 10% Relapse during the trial = Diet group 30%, corticosteroid group 66% Relapse rates (2 years) = Diet group 62%, corticosteroid group 79% *Diet = tapered 'placebo', single food reintroduction daily, exclusion of symptom provoking foods* *Oral corticosteroids = tapered prednisolone (40mg/day), healthy eating advice given*
Denmark, 1997 [468] CD 53, UC 69, HC 70	Cohort - questionnaire Self-completed, food intolerance (5 food groups)	Reported intolerance = CD 66%, UC 64%, HC 14% Food groups associated with intolerance IBD = vegetables 40%, other 38%, fruit 28%, milk 27%, meat 25%, bread 23% HC = vegetables 7%, other 1%, fruit 1%, milk 0%, meat 6%, bread 1%
England, 1998 [465] CD 28 (elimination), CD 48 (LOFFLEX)	Clinical trial – elimination diet **or** LOFFLEX diet Remission induction, elimination diet, gradual food reintroduction **or** Remission induction, LOFFLEX diet >2 weeks, then gradual food reintroduction	Relapse upon food reintroduction = Elimination 10.7%, LOFFLEX 12.5% Cumulative probability of maintaining remission at 2 years = Elimination 59.4%, LOFFLEX 55.6% 1-year post-treatment = 81% patients identified food intolerance/s Most frequently reported intolerances = wheat 50%, milk/dairy 44%, coffee/tea 39%, oats 33%, rye 31%, yeast 28% *LOFFLEX diet = low in fibre, fat reduced* *LOFFLEX exclusions = Pork, fish (battered, crumbed, tinned in oil/tomato), animal derived dairy, eggs, chocolate, wheat, rye, barley, corn, oats, yeast, corn/vegetable oil, pulses, onions, tomato, sweetcorn, citrus, apple, banana, dried fruit, tea, coffee, alcohol, soft drinks, gravy mixes, salad dressings etc, nuts and seeds*

Canada, 1998 [474] CD 76, UC 49	Cohort - questionnaire Self/parent-completed, paediatric dietary practises (16 foods/food groups)	Dietary modification since diagnosis = CD 90%, UC 71% Dietary modification reported to be beneficial = CD 68.6%, UC 80% Commonly avoided foods CD = corn/corn products 71.1%, nuts/seeds 48.7%, milk 30.3%, bran 25.0% UC = milk 47%, corn/corn products 38.9%, nuts/seeds 32.7%, other dairy 32.7%
Canada, 2000 [478] CD 33, UC 27	Cohort – questionnaire Self-completed (122 foods)	Foods reported to have a positive or no effect = bananas, carrots, potatoes, rice, roast chicken Foods reported to have a negative effect = whole milk, chocolate milk, bran cereal, diet soft drinks, beer, decaffeinated coffee, Mexican, liverwurst, artificial sweetener
USA, 2007 [467] CD 1,220	Cohort - questionnaire Self-completed (14 foods)	Foods reported to worsen symptoms = 60% Foods reported to worsen symptoms = Oily (66%), spicy (62.2%), nuts (57%), dairy (51.7%), citrus fruits (49.2%), vegetables (48.7%) Foods reported to improve symptoms = low fibre foods (32.8%), breads (31.4%), white meats (27.4%)
USA, 2009 [485] CD 5 (juvenile)	Uncontrolled prospective case study, ≥12 months - elimination diet	Remission reached and medication ceased = 100% within 2 months Mean length of remission = 56.4 months Follow-up (1 year) = Significantly decreased ESR and CRP Extended remission (4.5-7 years) = CD 3 *Excluded foods = dairy, corn, whole grains, carrageenan* *Supplements = fish peptides and amino acids, bovine colostrum, herbal extract boswellia serrata, curcumin, multivitamin/mineral, probiotic lactobacillus*

NZ, 2009 [475] CD 499	Cohort - questionnaire Self-completed (44 vegetables)	Foods most associated with adverse effects = maize, mushrooms, potato chips, leeks Foods most associated with beneficial effects = pumpkin, yams, boiled potatoes, kumara
NZ, 2010 [470] CD 446	Cohort - questionnaire Self-completed (257 foods)	Foods most associated with detrimental effects = Indian meat, fish or vegetable curry (69.6 - 67.4%), Thai fish or meat curry (63.6%, 63.2%), red bull 62.5%, corn 60.5%, Indian dahl 59.7%, beer 59.1% Foods most associated with beneficial effects = fresh white fish 24.3%, yoghurt 19.1%, gluten-free products 19.1%, salmon 18.9%, porridge 17.9%, banana 17.4%
NZ, 2011 [482] CD 165	Cohort - questionnaire Self-completed (7 dairy product categories)	Dairy products most associated with detrimental effects = cream 43.6%, ice cream 37.6%, cheese 34.5%, standard cow's milk 30.9%, custard 19.4%, yoghurt 18.8% Foods most associated with beneficial effects = yoghurt 14.5%, reduced-fat cow's milk 6%, custard 4.8%, cheese 3%, sheep's milk 3%, goat's milk 2.4%
USA, 2012 [480] CD 1,526, UC 803	Cohort – FFQ questionnaire Self-completed (16 foods/food groups)	Foods most associated with detrimental effects = non-leafy vegetables 18%, spicy foods 13%, fruit 12%, nuts 10%, leafy vegetables 9%, fried foods 8% Foods most associated with beneficial effects = yoghurt 9%, rice 5%
France, 2013 [472] CD 177, UC 67	Cohort - questionnaire Self-completed, dietary beliefs and behaviour (9 food groups)	Belief that food can play a role in triggering relapse = 57.8% Foods perceived to be a relapse risk factor = spicy 80.7%, high fat 48.8%, raw vegetables 47.5%, carbonated drinks 45.1%, raw fruits 43.9%, fibrous 40.6% Diet during relapse = none 25.4%, low residue 51.6%, dairy free 13.9%, fasting/tailored 11.5%, gluten free 1.6%

Canada, 2014 [464] IBD 319	Cohort - questionnaire Self-completed, food avoidance (12 foods/food groups)	Most commonly avoided foods = alcohol 31%, popcorn 30%, legumes 30%, nuts and seeds 27%, deep-fried/higher fat food 25%, processed deli meat 25% Most commonly avoided foods during active disease = salad or raw vegetables 46%, deep-fried/higher fat food 42%, alcohol 42%, popcorn 38%, nuts and seeds 35%, raw fruit 29%
IBD 256, HC 300	Researcher-completed, dietary intake	Significantly reduced intake, IBD = red meat, saltwater fish/shellfish, eggs, dried beans, nuts, pasta, fruit, tomatoes, lettuce, fried potatoes, soft drinks, fruit juices Significantly increased intake, IBD = sausages/bacon, freshwater fish, milk, cottage cheese, spinach, other potatoes, diet soft drinks, water Significantly reduced intake, active disease = nuts, cereal, fruit, lettuce, spinach Significantly increased intake, active disease = red meat, soft drinks
Israel, 2014 [487] CD 7 (CDED), CD 40 (PEN+CDED)	Clinical trial, 6 weeks - elimination diet Crohn's Disease Exclusive Diet (CDED) **or** CDED + Partial Enteral Nutrition (PEN)	CDED = remission 6/7 patients, decrease in disease activity (PCDAI or HBI) 1/7, relapse 0/7 CDED+PEN = remission 27/40 patients, decreased disease activity (PCDAI or HBI) 3/40, relapse 10/40 CDED = ≤20g fibre/day, set portions of wholegrain bread, nuts, fruits, legumes and vegetables. Exclusion of gluten, dairy products, gluten-free baked goods and breads, animal fat, processed meats, products containing emulsifiers, canned goods, packaged products with a due date.
England, 2016 [466] CD 90, UC 66	Cohort - questionnaire Self-completed, dietary beliefs and behaviour	Belief that food affects their symptoms a lot or severely = 42%, Belief that diet is important/extremely important in controlling symptoms = 51% Most commonly [1]avoided (of 127 reported) and [2]problem foods (of 110 reported) CD[1] = spicy, dairy, fatty, meat, nuts, vegetables CD[2] = spicy, fatty, dairy, vegetables, chocolate, onion UC[1] = dairy, spicy, fatty, alcohol, beans, corn UC[2] = spicy, dairy, fruit, vegetables, green vegetables, onion

England, 2016 [469] CD 156, UC 205	Cohort - questionnaire Self-completed, dietary beliefs and behaviour (8 food groups)	Belief that diet initiates IBD = IBD 48% Modified their diet since diagnosis = 56% Belief that food has a role in triggering relapse = CD 67%, UC 53% Belief that dietary habits are more important than medicines in the control of IBD = IBD 28% Food restrictions imposed to prevent relapse = CD 77%, UC 53% Food restrictions imposed = spicy food 45%, fatty food 32%, fruits and vegetables 24%, alcohol 22%, carbonated beverages 16%, milk 15%, Foods associated with improving symptoms = high fibre 5%, low fibre 2%, starch-rich 1% Foods associated with worsening symptoms = spicy 41%, fatty 29%, alcohol 21%, raw vegetables and fruits 19%, milk and milk products 16%, carbonated beverages 12%
Israel, 2017 [488] CD 17, CD 4	Observational - retrospective CDED **or** CDED + PEN, 12 weeks	CDED = remission 3/4 patients CDED + PEN = remission 10/17 patients, Clinical response (HBI improvement or remission) = 19/21 patients Remission = 13/21 patients Inflammatory marker (CRP, ESR, FC) reduction = 17/21 Inflammatory marker level normalisation = 9/21 patients
Italy, 2017 [481] CD 54, UC 49	Cohort - questionnaire Self-completed (146 foods)	Significant inverse association with disease activity = potatoes and legumes Positive association with disease activity = meat (ns)
Netherlands, 2018 [490] CD 67, UC 32	Cohort - questionnaire Paediatric patient and caregiver completed (160 foods)	Food avoidance = 53% Foods avoided because of worsening abdominal symptoms = spicy food 46%, high fat foods 30%, dairy products 30%, cereal products 11%, onion/leek 11%, bell pepper 9%

England, 2018 [491] Active CD 5 (paediatric)	Pilot trial - elimination diet CD = 4 or 8 weeks CD-TREAT (personalised food-based diet, similar to exclusive EN)	CD, 4 weeks CD-TREAT (n=5) = clinical response 3/5 patients, clinical remission 2/5, symptom exacerbation 1/5 Significant reduction in disease activity and FC CD, 8 weeks CD-TREAT (n=4) = clinical response 4/4 patients, clinical remission 3/4 Significant reduction in disease activity or FC
Netherlands, 2018 [471] CD 146, UC 148	Cohort – questionnaire Self-completed, dietary beliefs and behaviour	Belief that diet causes IBD = 12.9% Belief that food has a role in causing relapse = 33.3% Belief that dietary change can end relapse faster = 40.5% Belief that symptoms are controlled due to dietary change = 27.4% (always), 4.8% (during relapse only), 30.1% (during remission only), 19.5% (never) Importance of dietary change compared to medicines = 12.3% (more important), 46.9% (equally important), 24.3% (less important), 13.4% (minor role), 3.1% (not important) Omitted food/s to prevent relapse = 76.5% Increased intake of food/s due to beneficial effect = 56.7% Foods associated with worsening symptoms = Spicy 74.7%, strongly seasoned 69.8%, carbonated drinks 56.4%, milk/dairy products 51.6%, energy drinks 49.3%, deep-fried 46.7% Foods associated with improving symptoms = High-fibre bread 56.2%, tea 46.7%, leafy vegetables 43.8%, fatty fish 42.0%, poultry 38.9%, exotic fruits 38.3%

ABBREVIATIONS: CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; HC, healthy control; RCT, randomised controlled trial; FFQ, food frequency questionnaire; PCDAI, paediatric Crohn's disease activity index; HBI, Harvey Bradshaw index

3.3. FOOD ANTIGENS

Hypersensitivity to dietary antigens may directly provoke inflammation by triggering luminal immune defences [492]. Measurement of food-specific serum immunoglobulin G (IgG) antibodies is a simple procedure that may indicate the presence of hypersensitivity to one or more foods. During the 1990's the measurement of yeast antibody levels in CD patients was an active area of interest following earlier observations of elevated yeast antibodies in this population group and not in UC patients or healthy controls. These observations have been replicated in several studies [493–495], along with significantly increased lymphocyte proliferation in response to yeast exposure compared to healthy controls [496]. However, it appears that only one study has investigated the effect of dietary yeast exclusion. In this cross-over RCT, 19 CD patients consumed a diet low in yeast and sugar for two periods of four weeks while taking either yeast or placebo capsules. Disease activity (CDAI) when receiving the placebo was significantly lower than at baseline measurements and when receiving yeast [495].

Several researchers have used serum dietary antigen testing to explore food hypersensitivity in IBD and have demonstrated significantly higher food triggered IgG responses in CD patients compared to those with UC [497,498] and healthy controls [499–501]. In earlier work by van den Bogaerde [502,503] the effect of dietary antigens in CD was explored using a combination of *in vitro* and *in vivo* methods. In the first study, peripheral blood lymphocytes from 31 CD patients and 22 controls were incubated with six different antigens. Lymphocytes from patients demonstrated a significantly elevated level of proliferation, and proliferation was elicited by a greater number of antigens tested [502]. In the second study of 10 CD patients and 10 controls, rectal blood flux was significantly greater in CD compared to controls, as was the number of testing sites that demonstrated increased blood flux [503]. Further, no differences were observed following skin antigen testing which demonstrates the difficulty associated with verifying food hypersensitivity.

More recently, researchers have used serum antigen testing to investigate tailored elimination diets and disease activity in CD. In a pilot study by Rajendran and Kumar [504], 29 patients followed a four week exclusion diet based on IgG reactivity to 14 antigens. A significant reduction was observed in disease activity (modified CDAI), stool frequency, and ESR. In another small pilot study, eight patients in remission followed a three-phase protocol: 10-day exclusion diet, three-day provocation diet including the highest IgG response causing foods, and a further three-day provocation diet with the addition of commercial additives. Provocation, irrespective of additive content, was associated with a significant increase in white blood cell count, CRP, CDAI, HBI, and patient reported symptoms [505]. Similar outcomes were observed in a retrospective study of patient's hospital records with disease activity (CDAI and SES-CD) increasing significantly when patients reintroduced their usual diet following exclusive EN induced remission. Furthermore, the relapse rate at 12 weeks was 25% in this patient group compared to 12.5% in those that reintroduced a diet excluding foods that triggered a strong IgG response [498].

Two studies deviated slightly from such a direct provocation approach, and instead used a sham diet to evaluate elimination diet efficacy. In the first study, 23 CD patients followed a six-week IgG based

exclusion diet, and six-week sham diet whereby foods prohibited in the true diet were exchanged with similar foods, such as broccoli replaced with cauliflower. A significant improvement was seen in stool frequency and general well-being score during the true diet compared to the sham diet [506]. In the second study, 76 CD patients were randomised to an exclusion diet prohibiting the four foods with the highest IgG response, or a sham diet prohibiting the four foods with the lowest IgG response. The exclusion diet was associated with improvement in patient symptoms and a greater tendency towards reduction in disease activity score (CDAI), 41% compared to 16% [507].

From the reviewed studies (see *Table 2.3*), the dietary antigens that most frequently trigger a response in CD patients were yeast [500–502,505], egg [498,499,504,505], wheat [504–506], and soy [497–499]; and the least responded to antigens were rice, chicken and potato [504,507]. Dairy product antigens also accounted for a large portion of antibody production, particularly cheese [504–507]. With the exception of dairy products, there was little agreement between dietary antigens that evoked the highest IgG levels and questionnaire-reported symptom triggering foods. Conversely, two of the three least responded to antigens (rice and potatoes) were in the five foods most reported by CD patients to be beneficial. It is not known why the most frequently reported symptom triggering foods and IgG evoking foods are dissimilar, although it can likely be partially explained by the number and spectrum of antigens tested. In more than half of the studies reviewed, the maximum number of antigens tested was 16 [497–499,502–504,507], and the antigens tested often overlapped, namely egg, milk, rice, wheat, soy, beef, chicken, tomato, and yeast. Dietary antigen testing is also unsuitable for the evaluation of many symptom triggering foods identified by some of the questionnaires. This is due to the questionnaires inquiring about multi-ingredient foods, such as spicy foods, foods grouped according to cooking method, such as fried foods, and food groups, such as nuts or fruit and vegetables. Aside from methodological differences, it is also plausible that elevated IgG levels are the result of impaired intestinal barrier function and an associated increase in antigen presentation, rather than food hypersensitivity.

Currently, allergy testing rates in IBD patients are low. A survey of gastroenterologist's found that 97% of United Kingdom respondents (n=363) and 82% of NZ respondents (n=51) reported only testing 0-25% of patients [508]. In addition, controversy exists around the use of dietary IgG testing as an indicator of food hypersensitivity [509], and it is argued that even elevated IgG levels are a normal response to dietary antigen presentation [510]. Despite this, convincing evidence exists to support ~~a~~ higher prevalence of food hypersensitivity in CD patients, and a portion of these patients experience symptom improvement in response to an IgG tailored elimination diet. The available studies that have investigated IgG tailored elimination diets and disease activity are mainly limited by their sample size. Future research would benefit from an increase in sample size and other forms of disease activity evaluation such as histological evaluation. Moreover, in view of the minimal health risks associated with eliminating one or more dietary components in combination with proper guidance, health care providers should consider antigen testing where a patient believes they may be hypersensitive to one or more foods.

Table 2.3 Response to Dietary Antigens in Crohn's Disease

Participants	Study Design, Method, and Assessment	Results
Scotland, 1992 [495] CD 19, HC 30	Randomised controlled trial cross-over, 4 weeks (each treatment) - exclusion diet and re-challenge Clinical assessment, disease activity (CDAI)	Yeast = significantly greater CDAI than placebo CD = significantly elevated IgG and IgA yeast antibodies compared to controls *Capsules = yeast (low-yeast diet) AND placebo* *Excluded foods = breads, malted cereals, dairy products, fruit or fruit-containing foods, alcohol, vinegar or sauces, extracts eg gravy-browning or stock cubes, vitamin B supplements, mushrooms, sugar, preserves, white flour*
Japan, 1999 [497] CD 26, UC 32, HC 116	Cohort - self-completed allergic disorder questionnaire	CD = significantly higher incidence of food allergy, drug allergy, atopic dermatitis (compared to controls)
	Cohort - serum IgG testing (5 antigens tested)	CD = significantly higher IgG to soybean (compared to UC or HC) *Antigens tested = egg white, milk, rice, wheat, soybean*
England, 2001 [502] CD 31, HC 22	Cohort - peripheral blood lymphocytes and antigen incubation (6 antigens tested)	Peripheral blood lymphocytes proliferation = CD 74%, HC 23% ≥1 Antigen response = CD 74%, HC 23% ≥4 Antigen responses = CD 52%, HC 9% Antigen response CD = peanut 52%, cabbage 52%, cereal 45%, milk 42%, citrus 29%, brewer's yeast 29%, baker's yeast 19% HC = brewer's yeast 14%, cabbage 9%, cereal 9%, milk 5%, peanut 5%, baker's yeast 5%

England, 2002 [503] CD 10, HC 10	Cohort - mucosal and cell proliferation antigen response (6 antigens tested) Skin testing and rectal mucosa injection	Antigen testing sites, immediate blood flux increase = CD 40%, HC 10% Antigen testing sites, increased blood flux (3.5 hours) = CD 40%, HC 8% Peripheral blood lymphocytes proliferation = CD 70%, HC 30%
Germany, 2010 [506]	Pilot - serum IgG testing (271 antigens tested)	Antigen response = CD 66%, HC 43%
CD 79, HC 20 CD 23	Randomised double-blind cross-over, 6 weeks, exclusion and sham diet (serum IgG based) Diet and symptom diary	Exclusion diet = reduction in daily diarrhoea frequency and abdominal pain, and improvement in general well-being score (ns change)
England, 2011 [501] CD 28, UC 25, HC 24	Cohort - questionnaire and Serum IgG testing (92 antigens tested) Self-completed food intolerance questionnaire (92 foods)	Problem foods (mean number of foods) = CD 3, UC 4, HC 0 Problem foods CD = peanut 29%, cashew 25%, lentils/broccoli 19%, hazelnut/brazil nuts 19%, chilli 19% UC = chilli 44%, wheat 40%, milk 36%, kidney/haricot beans 24%, coffee/onions 20%, oranges 20% HC = peanut 13%, cashew 13%, hazelnut/brazil nuts 13%, chilli 8%, wheat 8%, milk 8% Highest antigen response CD = yeast 82.1%, wheat 42.9%, chilli 37.9%, kiwifruit 35.7%, corn 28.6%, peanut 28.6% UC = corn 24%, millet 20%, oat 20% HC = yeast 54.2%, wheat 12.5%, chilli 8.3%, kiwifruit 8.3%
CD 12	Mucosal blood flux	Antigens associated with significant increase in rectal mucosal blood flux (3.5 hours) = yeast, milk *Antigens tested = yeast, wheat, milk, egg, kiwifruit*

England, 2011 [504] CD 29	Pilot, 4 weeks - exclusion diet (serum IgG based - 14 antigens tested) Exclusion of 4 foods with highest antigen response Self-completed symptom questionnaire, clinical assessment, and disease activity (modified CDAI)	Highest antigen response = egg white and yolk, cheddar cheese, beef, pork, wheat Lowest antigen response = rice, chicken, yeast, potato, soy, tomato Exclusion diet = significant reduction in disease activity (mCDAI), ESR, and patient reported symptoms. No change in CRP or albumin *Antigens tested = egg white, egg yolk, potato, tomato, cheddar cheese, rice, beef, lamb, pork, soya, peanuts, wheat, yeast, and chicken*
Istanbul, 2012 [505] CD 8	Pilot - exclusion and provocation diet (serum IgG based - 266 antigens tested) 10-day exclusion diet, 3-day provocative diet (highest IgG foods), further 3-day provocation diet (highest IgG foods + additives) Symptom diary, clinical assessment	Provocation = significant increase in WBC count, CRP, CDAI, HBI, and patient reported symptoms Highest food-antigen responses = wheat 5/8 patients, yeast 5/8, cow's milk and dairy products 3/8, eggs 3/8 Highest additive-antigen response = agar 7/8, guar gum 7/8
China, 2014 [499] CD 79, UC 33, HC 266	Retrospective cohort - serum IgG testing (14 antigens tested) *Antigens tested = Rice, egg, mushroom, milk, pork, chicken, beef, crab, codfish, corn, soybean, tomato, shrimp, wheat*	Antigen response = CD 75.9%, UC 63.6%, HC 33.1% Highest antigen response CD = egg 73.3%, rice 56.7%, corn 56.7%, tomato 46.7%, soybean 43.3% UC = egg 81.0%, rice 14.3%, corn 14.3%, tomato 9.5%, milk 9.5% HC = egg 69.3%, milk 14.8%, crab 14.8%, codfish 5.7%, shrimp 5.7%

Japan, 2014 [500] CD 98, UC 50, HC 52	Cohort - serum IgG testing (88 antigens tested)	Antigen response (mean number of foods) = CD 12.7, UC 2.3, HC 0.9 Highest antigen response CD = corn 67%, yeast 53%, cane sugar 52%, cabbage 48% UC = egg yolk 12%, cheddar cheese 12%, alfalfa 12%, grape 10% HC = spring bean 13%, sole 12%, kidney bean 10%, cabbage 6%
England, 2016 [507] CD 39 (true diet) CD 37 (sham diet)	Randomised double-blind, 4 weeks - exclusion **or** provocation diet (serum IgG based - 16 antigens tested) Exclusion diet (4 highest IgG4 foods excluded) **or** Sham diet (4 lowest IgG4 foods excluded) Self-completed symptom questionnaire (SIBDQ), clinical assessment, and disease activity (CDAI, HBI)	Antigen response, highest = beef, pork, egg, milk Lowest antigen response = rice, chicken, tomato, potato CDAI improvement >100 (remission <80, severe disease activity >400) = True diet 41%, sham diet 16% Exclusion diet = significant improvement in patient symptoms and all disease activity scores Exclusion and sham diet = no change in FC or CRP *Antigens tested = Milk (a-lactoglobulin, b-lactoglobulin, casein), peanuts, soya, shrimp, whole egg, tomato, pork, beef, cod fish, potato, wheat, yeast, cheddar cheese, chicken, lamb, rice*
Norway, 2017 [479] CD 16	Cohort – questionnaire (self-completed problem food, 32 foods)	Most common problem foods = cow's milk 13/16 patients, apple 11/16, wheat 11/16, ice cream 10/16, pasta 10/16, pear 10/16
CD 12	Pilot - serum IgE testing, 0-2 weeks: habitual diet, 3-4 weeks: exclusion diet Symptom diary, clinical assessment	Elevated antigen response = 2 patients (hazelnut; and wheat, soybean, and cod) Exclusion diet = significant symptom improvement *Habitual = wheat and dairy inclusion, Exclusion = based on IBS exclusion diet and FODMAP diet*

China, 2017 [498] CD 182, UC 103, HC 78	Retrospective - serum IgG testing (14 antigens tested) Hospital records, 2013-2015	<u>Antigen response (mean number of foods)</u> = CD 3.9, UC 1.3, HC 1.2 <u>Highest antigen response (CD)</u> = rice 65%, tomato 64%, egg 63%, corn 57%, soy 50% *<u>Antigens tested</u> = Beef, chicken, cod, maize, crab, egg, mushroom, milk, meat, rice, shrimp, soya, tomato, wheat*
CD 32 (exclusion diet) CD 32 (control)	Retrospective - Exclusion diet (serum IgG based - antigen numbers N/A) Remission induction, food reintroduction **<u>or</u>** control (patient's usual diet) Clinical assessment, disease activity	<u>Reintroduction diet (control)</u> = significant increase in disease activity (CDAI and simple endoscopic score) <u>Relapse at follow-up (12 weeks)</u> = exclusion diet 12.5%, control 25%

ABBREVIATIONS: CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; HC, healthy control; CDAI, Crohn's disease activity index; HBI, Harvey Bradshaw index; SIBDQ, short inflammatory bowel disease questionnaire; WBC, white blood cell; IgG/IgA, immunoglobulin G/A; IBS, irritable bowel syndrome; FODMAP, fermentable oligosaccharides disaccharides monosaccharides and polyphenols

3.4. TARGETED DIETS

3.1.1 Carbohydrates

Targeted diets have been developed to prevent or alleviate the GIT symptoms of IBD. Some such diets are based on associations made between diet and IBD risk, and others predict that certain food properties will prevent or counteract IBD-associated GIT discomfort including inflammation or excess gas production (see *Table 2.4*). Initial research stemmed from the causal link made between disease and refined carbohydrate consumption. In Heaton, Thornton, and Emmett's [511] study, dietary advice was given to 32 CD patients on reducing refined carbohydrate intake, while 32 control CD patients were given no advice. Over the 52-month follow-up period the diet group required significantly fewer and shorter hospital admissions, and fewer resection operations. Conversely, a similar study conducted in the following decade found no difference in relapse rate, disease activity, hospitalisation, or surgery between the 352 patients allocated to a diet high in either refined or unrefined carbohydrates [512].

The specific carbohydrate diet (SCD) was originally devised in the 1920's to treat coeliac disease and is relatively novel in the treatment of CD with mostly small trials involving paediatric patients. The diet excludes di- and poly-saccharides in order to prevent dysbiosis or excess bacteria populations, and resultant intestinal inflammation [513]. Dietary exclusion also extends to dairy products, grains, potatoes, soy and preservatives, and additives [514]. Positive results have been observed including remission induction rates of 66% [514], 77% [515], and 100% [516], significant improvement or normalisation of disease markers [515–517], and significant improvement in disease activity [518]. However, concerns have been raised around the difficulties in maintaining such a limited diet and possible weight loss or nutrient deficiencies [515].

A targeted diet with similarities to the SCD involves the restriction of fermentable oligo-, di-, and mono-saccharides, and polyphenols (FODMAPs) that are present in the diet as fructose, fructans, lactose, polyols, and galacto-oligosaccharides [109]. Some individuals appear to be susceptible to FODMAP induced GIT symptoms including one or a combination of bloating, abdominal pain, flatulence, and diarrhoea [115]. Two mechanisms are believed to be responsible; osmotic activity leading to luminal water uptake, and increased colonic fermentation and gas production due to incomplete small intestinal monosaccharide absorption [519]. As FODMAP associated symptoms are considered to be functional rather than of inflammatory origin, limited research has been carried out on the efficacy of a low FODMAP diet in CD patients. In Gearry et al's. [115] study, 52 CD patients with functional GIT symptoms were interviewed a minimum of three months after commencing a low FODMAP diet. Symptom reduction was reported by 56% of patients; and flatulence, overall symptoms, abdominal pain, diarrhoea, and bloating all improved significantly. Prince et al. [117] conducted a similar study involving 39 CD patients with follow-up six or more weeks after dietary consultation. Between baseline and follow-up, symptom relief satisfaction increased from 16% to 78%, and symptoms reported as either moderate or severe decreased significantly, as did reporting of seven of the eleven analysed symptoms. A smaller study of 49 IBD patients by Maagaard et al. [116] produced more modest results with 42% of patients reporting full diet effectiveness, 47% partial effectiveness, and 11% no effect. Unlike the other studies, Maagaard et al's. study permitted the reintroduction of small quantities of high

FODMAP containing foods if tolerated, and follow-up time varied greatly from 5-32 months. Despite these methodology differences, and an estimated adherence of only one third of patients, 24% of patients reported symptom resolution when following the diet. Although the low FODMAP diet does not target symptoms directly attributable to IBD, it may have merit for the 25-46% of patients that a meta-analysis report as experiencing functional GIT symptoms when in remission [520].

3.1.2 Fibre

Dietary fibre has long been known to have a therapeutic or preventative role in a number of chronic GIT diseases [521]. The insoluble fraction increases faecal transit and adds bulk, while the soluble fraction undergoes bacterial fermentation in the colon and produces SCFA's which play an essential role in maintaining colonic homeostasis [522]. Researchers have suggested that lower concentrations of SCFA's seen in IBD patients may play a role in disease development [523], which has led to investigations of the effect of increased insoluble fibre on GIT symptoms. The majority of evidence demonstrates that UC patients may benefit from this approach, but not those with CD [524]. This likely reflects disease location as UC is confined to the colon, whereas the colon is affected in less than one third of CD patients [525]. Irrespective of disease activity history or status, patients with CD have historically been found to have a lower intake of fibre compared to healthy controls [526] despite a lack of evidence suggesting IBD patients should reduce dietary fibre intake [527]. The exception is the recovery period following surgery, during periods of heightened disease activity, or in CD patients with strictures or fistulas [528].

3.1.3 Omega-3 Fatty Acids

The use of dietary omega-3 fatty acids (n-3 FA) as anti-inflammatory therapy in an array of chronic conditions is well documented [529]. The mode of action is dependent on increasing the n-3 FA concentration in cell membranes, which in turn alters gene expression and reduces inflammatory eicosanoid production [530]. Maintenance of remission is often used as a measure of n-3 FA supplementation efficacy in IBD studies. In a 12-month RCT by Belluzzi et al. [531], specially coated capsules (2.7g/day) were used to facilitate small intestinal absorption via delayed breakdown. In the treatment group, 59% of CD patients remained in remission until study completion compared to only 26% in the placebo group. Other researchers have used a similar RCT design with mixed results. The Epanova Program in Crohn's Study (EPIC-1 and EPIC-2) reported no effect from 4g of daily n-3 FA supplementation in 738 patients [532]. Lorenz-Meyer et al. [533] also reported no effect of 5g daily n-3 FA or placebo in 135 CD patients. However, in a paediatric trial 50mg/kg/day n-3 FA resulted in a significant difference between the time until relapse and the relapse rate [534]. Lastly, n-3 plasma phospholipid FA profile, baseline mean of 0.6%, was used in a short four-month pilot study to evaluate a combined n-3 FA (3g/day), prebiotic and antioxidant supplement in 20 patients. At study completion disease activity was significantly lower in CD patients with an increase in plasma phospholipid EPA to >2%, compared to those <2% [535].

3.1.4 Anti-Inflammatory

The Mediterranean diet (MD) comprises a high intake of fresh fruit and vegetables, nuts and grains, legumes, and olive oil, medium intake of dairy products, fish and poultry, and low intake of red meat and processed foods. Many health benefits have been attributed to the MD including a lower risk of cardiovascular diseases, diabetes, and neurodegenerative diseases, a reduced incidence of cancer mortality, and greater longevity [536]. These wide-ranging benefits have led researchers to investigate the anti-inflammatory potential of the MD in CD patients with active disease. A NZ based pilot study reported a promising but non-significant reduction in markers of inflammation seen in six CD patients that followed a Mediterranean inspired diet for six weeks [537]. The researchers also reported changes suggestive of microbiota profile normalisation. In an Italian study, a six-month intervention produced significant improvements including a decrease in disease activity and inflammatory marker levels [538]. The Diet to INducE Remission in Crohn's Disease (DINE-CD) study compared the effectiveness of the SCD and MD to reduce inflammation and induce symptomatic remission [539]. At both 6 and 12 weeks, no significant differences were observed in the rates of remission or inflammatory marker response. Although this disproved the authors' hypothesis, the findings could increase patient willingness to trial a dietary approach for disease management due to a broader range of MD approved foods compared to other diets suggested to CD patients. The potential importance of diet adherence has also been explored. In a Greek study of 86 CD patients, adherence to the MD was negatively correlated with inflammatory markers CRP and HBI, and positively correlated with quality of life (IBDQ) [540]. Similarly, adherence to the MD was negatively associated with FC level in an Italian study of 125 paediatric IBD patients [541]. In contrast, diet effectiveness in the DINE-CD study was similar between participants reporting adherence all the time and those reporting adherence some of the time or those without adherence data [539].

A small number of studies have developed targeted diets that incorporate foods or food components believed to have anti-inflammatory properties. Trial of the IBD-anti-inflammatory (IBD-AID) diet, which comprises select carbohydrates and fats, a high intake of pre- and pro-biotics, and reduced insoluble fibre intake, was associated with symptom improvement in 24 of the 27 IBD patients [542]. Greater success was seen in IBD patients following an autoimmune protocol diet that excludes foods, additives, or medications that could evoke inflammation, dysbiosis, or food intolerance. Remission was achieved by week 6 and maintained at week 11 in 73% of the 15 IBD patients, and disease activity scores improved significantly [543].

Presently, the utility of targeted diets in the management of CD symptoms is limited. Diets based on carbohydrate restriction have been demonstrated to be the most efficacious with symptom reduction seen in close to 50% of patients in response to the low FODMAP diet, and over 50% of patients with the SCD. However, such strict diets are difficult to maintain over an extended period of time and may even be counterproductive given the elevated risk of malnutrition and nutrient deficiencies seen with IBD. The anti-

inflammatory effects of omega-3 fatty acids in relation to the reduction of disease activity has been investigated by several researchers. While the dosages provided were ≥1 g/day, which meets or exceeds the recommendations by several scientific bodies [529], and the duration of most studies was 12 months and thus adequate to determine an effect, the results were mixed and the improvements were largely not significant or not sustained. Lesser investigated targeted diets have also yielded mixed results. In a small study (n=20), greater remission duration was seen in patients following a semi-vegetarian diet compared to those maintaining an omnivorous diet [544]. Benjamin et al. [545] explored the effect of supplementary prebiotic fructo-oligosaccharides, though no significant differences were observed between the prebiotic or placebo on markers of disease activity or rates of remission induction. Finally, in a pilot study the provision of curcumin for 90 days led to improvement of disease markers, although there was no placebo group for comparison [546].

Table 2.4 Response to Targeted Diets in Crohn's Disease

Participants	Study design and method	Assessment	Results
England, 1979 [512] CD controls 32 CD diet 32	Case-control, mean duration = 52 months Fibre rich, unrefined carbohydrate	Medical record review	Diet group = significantly fewer and shorter hospital admissions, fewer resection operations
England, 1987 [513] CD 162 (unrefined) CD 190 (refined)	Randomised controlled trial, 2 years Low unrefined CHO **or** low refined CHO	Clinical assessment	No differences observed
USA, 2014 [517] CD 7 (paediatric)	Retrospective (mean = 14.6 months) SCD	Medical record review, clinical assessment	3 months SCD = complete symptom resolution in 100% patients Normalisation of clinical markers = albumin 5/5 patients, CRP 5/5, anaemia 4/4, sedimentation rate 2/4
USA , 2014 [519] CD 9 (paediatric)	Prospective pilot study, 1 year SCD	Clinical assessment, disease activity (PCDAI or HBI)	12 weeks = significant improvement 8/9 patients, small intestinal ulcer resolution 4/4 patients 52 weeks = significant improvement 2/7 patients, no change 4/7, decline 1/7
USA, 2015 [515] CD 36, UC 9	Cohort, mean duration 35.4 months SCD	Self-completed questionnaire	Complete symptom resolution = 66% patients Effectiveness rating at sustaining remission = 92% (range 53-100%)

USA, 2016 [516] SCD = CD 20, UC 6 Control = CD 7, UC 3	Retrospective, mean duration 9.6 months SCD **or** Control (medication therapy)	Medical record review, clinical assessment	Remission Achieved = CD 7/9, UC 2/4, CD control 3/4, UC control 0/3 Maintained = CD 10/11, UC 2/2, CD control 1/3, UC control 1/1 Normalisation of elevated CRP = CD 10/14, CD control 3/6 No improvement = CD 2/20, UC 2/6, UC control 1/3 CD = Medication removal 2/20, difficulty maintaining diet 4/20
USA, 2016 [518] Strict SCD = CD 3 Simple SCD = CD 8	Retrospective, mean duration 11.8 months (strict SCD), 7.7 months (simple SCD)	Clinical assessment	Strict and Simple SCD (mean 11.9 months post SCD start) = significant improvement in albumin and ESR levels
NZ, 2013 [538] CD 8	Pilot study, 6 weeks Mediterranean inspired anti-inflammatory diet	Clinical assessment	Diet = reduced CRP (ns), reduced peripheral blood lymphocyte DNA damage (ns), improvement in microbiota abundance (closer to that of a healthy group)
USA, 2021 [540] SCD = CD 99 MD = CD 92	Prospective, 12 weeks Diet to INducE Remission in Crohn's Disease (DINE-CD) SCD or Mediterranean diet (MD)	Clinical assessment, disease activity (CDAI), 2-item patient-reported outcome (PRO2)	Symptomatic remission (%) = SCD 46.5, MD 43.5 (week 6), SCD 42.4, MD 40.2 (week 12) FC response (%) = SCD 34.8, MD 30.8 (week 6), SCD 26.1, MD 7.7 (week 12) CRP response (%) = SCD 5.4, MD 3.6 (week 6), SCD 10.8, MD 7.1 (week 12) CDAI <150 (%) = SCD 48.5, MD 47.8 (week 6), SCD 40.4, MD 46.7 (week 12) PRO2 remission (%) = SCD 34.3, MD 27.2 (week 6), SCD 33.3, MD 31.5 (week 12)

Italy, 2021 [539] CD 58	Prospective, 6 months MD	Clinical assessment, quality of life (IBDQ)	Disease activity (baseline vs 6 months) = active disease 17.0% vs 0%, mild disease 15.1% vs 3.8%, moderate disease 1.9% vs 0% Elevated CRP (baseline vs 6 months) = 44.9% vs 26.5% FC >250 (baseline vs 6 months) = 45.0% vs 27.5% Adherence to MD = significant IBDQ increase (6 months)
Australia, 2009 [115] CD 52, UC 20	Retrospective, ≥3 months Low FODMAP	Telephone interview of symptoms	Overall symptom improvement = CD 56%, UC 55% Significantly improved symptoms (CD & UC) = wind, overall symptoms, abdominal pain, diarrhoea, and bloating Least improved symptom = CD reflux 12%, UC constipation 0%
Denmark, 2016 [116] CD 12, UC 32, IBDU 5	Retrospective, 6-8 weeks Low FODMAP	Symptom questionnaire	Median follow-up time = 17 months FODMAP effectiveness (IBD); full 42%, partial 47%, no-effect 11% Symptom resolution (IBD) = 24%
UK, 2016 [117] CD 39, UC 38, IBDU 11	Retrospective, ≥3 weeks, mean follow-up 2.6 months Low FODMAP	Medical record review, symptom questionnaire	Satisfactory symptom relief = 16% baseline, 78% follow-up FODMAP = Significant reduction in symptoms (all IBD groups)
Italy, 1996 [532] CD 39 (omega-3), CD 39 (placebo)	Randomised double-blind placebo-controlled, 1 year 2.7g omega-3 daily (1.8g EPA, 0.9g DHA)	Clinical assessment and disease activity (CDAI)	Relapse during the study = fish oil 28%, control 69% Remission at follow-up (1 year) = fish oil 59%, control 26%

Germany, 1996 [534] CD 70 (omega-3), CD 65 (placebo), CD 69 (LCD diet)	Randomised double-blind placebo-controlled, 1 year Omega-3 daily (3.3g EPA, 1.8g DHA) **or** 5g placebo **or** LCD (<84g CHO/day)	Disease activity (CDAI)	Relapse = omega-3 58.5%, placebo 61.8%, LCD 54.9% Remission maintenance = omega-3 26.2%, placebo 25.5%, LCD 33.3% (no significant differences between treatments) Patient-reported diet adherence (for 1 year) = 15.9%
Japan, 2000 [548] CD 20	Controlled trial, >1 month Omega-3 fatty acid rich	Clinical assessment and disease activity (IOIBD score)	Diet = significant CRP decrease, no change in disease activity *Omega-3 fatty acid quantity not stated*
Italy, 2005 [535] CD 18 (group 1), CD 20 (group 2) (paediatric)	Randomised double-blind placebo-controlled, 1 year Group 1 - 50mg/kg/day omega-3 (400mg/g EPA, 200mg/g DHA) **or** Group 2 - placebo (olive oil)	Disease activity (PCDAI)	Relapse at 1 year = omega-3 61%, placebo 95%
Canada, Europe, Israel, USA, 2008 [533] EPIC-1/EPIC-2 CD 183/187 (omega-3) CD 180/188 (placebo)	Randomised double-blind placebo-controlled, ≤58 weeks 4g omega-3 fatty acid daily **or** placebo	Disease activity (CDAI)	EPIC-1 relapse rate at 1 year = omega-3 31.6%, placebo 35.7% EPIC-2 relapse rate at 1 year = omega-3 47.8%, placebo 48.8% *EPIC = Epanova Program in Crohn's Study*

USA, 2011 [536] CD 20	Pilot study, 4 months Omega-3, prebiotic and antioxidant supplement IBD nutrition formula (16 oz/day)	Clinical assessment, disease activity (CDAI), quality of life (IBDQ)	Increased plasma phospholipid EPA to >2% (at 4 months) n=10 = significantly higher IBDQ = significantly lower CDAI Plasma phospholipid EPA remaining <2% (at 4 months) n=10 = no change in IBDQ or CDAI *IBD nutrition formula (daily) = 2.18g EPA, 0.92g DHA, FOS and gum arabic, antioxidants vitamins and minerals*
USA, 2013 [543] IBD 27 CD 8, UC 3	Retrospective, ≥4 weeks IBD-AID (nutritionist instructed anti-inflammatory diet) Lean meats, select CHOs, high pre and probiotic intake, specific FA ratio	Diet and symptom diary Medical record review	Self-reported ambivalent/negative diet response = CD 1, UC 2 Self-reported good/very good response = IBD 24 Improved symptoms and disease activity (HBI) = CD 8, UC 3
USA, 2017 [544] CD 9, UC 6	Pilot study Autoimmune protocol (AIP) (excludes inflammation, dysbiosis, or food intolerance triggers) 6 weeks exclusion diet, 5 weeks maintenance (no food group reintroduction)	Clinical assessment and disease activity (HBI, partial mayo score)	Remission achieved (week 6) and maintained (week 11) = CD 6/9, UC 5/6 Disease activity = significant improvement from weeks 0 to 6, sustained at week 11 Clinical markers = CRP no change, FC improvement (ns) *Eliminated foods = grains, legumes, nightshades, dairy, eggs, coffee, alcohol, nuts and seeds, refined/processed sugars, oils, food additives*

Japan, 2010 [545] CD 16 (SVD) CD 6 (omnivorous)	Prospective clinical trial, 2 years Induced remission, SVD recommended to in-patients	Clinical assessment	Remission maintained (1 year) = SVD 16/16 patients, omnivorous 4/6 Remission maintained (2 years) = SVD 10/11, omnivorous 1/4
UK, 2011 [546] CD 54 (FOS) CD 49 (placebo)	Randomised double-blind placebo-controlled trial, 4 weeks Prebiotic: Fructo-oligosaccharide (FOS) (15g/day)	Clinical assessment, disease activity (CDAI), IBDQ, microbiological analysis	Remission achieved = 11% FOS, 20% placebo FOS group = significantly lower IBDQ score, significantly greater incidence and GIT symptom severity, significant increase in dendritic cell IL-10 expression and reduction in IL-6 production CDAI, CRP and Microbiota (bifidobacteria and *F prausnitzii*) = ns differences between groups
USA, 2005 [547] CD 5, UC 5	Pilot study, 90 days Curcumin supplement, 0-30 days: CD 1080 mg/d, UC 1100 mg/d 31-90 days: CD 1440 mg/d, UC 1650 mg/d		Improved CDAI, ESR and CRP = CD 80%, UC 80%

ABBREVIATIONS: CD, Crohn's disease; UC, ulcerative colitis; IBDU, inflammatory bowel disease unclassified; IBD, inflammatory bowel disease; HC, healthy control; SCD, specific carbohydrate diet; LCD, low carbohydrate diet; IBD-AID, IBD-anti-inflammatory diet; SVD, semi-vegetarian diet; FOS, fructo-oligosaccharide; FODMAP, fermentable oligosaccharides disaccharides monosaccharides and polyphenols; CHO, carbohydrate; FA, fatty acid; CDAI, Crohn's disease activity index; PCDAI, paediatric CDAI; HBI, Harvey Bradshaw index; IBDQ, inflammatory bowel disease questionnaire; IOIBD, International Organization for the Study of IBD

4. Conclusion

The incidence of CD in NZ is high by international standards and is continuing to rise. The condition can have a severe impact on a patients' quality of life, and the health care costs can be significant. Genetic advances have expanded our understanding of some mechanisms that likely contribute to the immune dysregulation seen in CD, however this is only one part of the causation puzzle. Continued research is necessary not only to build our knowledge of the environmental risk factors implicated in the risk of CD and whether or not they are modifiable, but also the complex interplay that culminates in disease onset and may continue to influence disease activity and progression. At the same time, where current evidence implicates a modifiable environmental factor, and the risk to patients is low such as diet change or vitamin D supplementation, high quality interventional studies should be conducted that will build on existing work.

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CHAPTER THREE

INFLAMMATORY BOWEL DISEASE: ARE SYMPTOMS AND DIET LINKED?

The first study reported is an investigation of dietary elements (foods, additives, and cooking methods) that New Zealand patients with inflammatory bowel disease (IBD) associate with the onset, exacerbation, or reduction of their symptoms. Patients with IBD often restrict or avoid the consumption of foods associated with symptom onset or exacerbation. This study determined the proportion of patients that may well restrict or avoid the consumption of certain foods or food groups, a strategy that can cause or contribute to inadequate nutrient intake. The most frequently reported dietary elements were also determined and compared to identify any similarities that may provide insight into the component(s) responsible for the reported effects and warrant further investigation.

PAPER PUBLISHED IN "NUTRIENTS"
Morton H, Pedley KC, Stewart RJC, Coad J (2020).
Inflammatory Bowel Disease: Are Symptoms and Diet Linked?
Nutrients 12(10):2975.

ABSTRACT

New Zealand (NZ) has one of the world's highest incidence rates of inflammatory bowel disease (IBD), a group of chronic inflammatory conditions that affect the gastrointestinal tract. Patients with IBD often believe certain foods influence their disease symptoms and consequently may alter their diet considerably. The objective of this study was to determine foods, additives, and cooking methods (dietary elements) that NZ IBD patients identify in the onset, exacerbation, or reduction of their symptoms. A total of 233 participants completed a self-administered questionnaire concerning symptom behaviour in association with 142 dietary elements. Symptom onset and symptom exacerbation were associated with dietary elements by 55% (128) and 70% (164) of all IBD participants, respectively. Fruit and vegetables were most frequently identified, with dairy products, gluten-containing bread, and foods with a high fat content also considered deleterious. Of all IBD participants, 35% (82) associated symptom reduction with dietary elements. The identified foods were typically low in fibre, saturated fatty acids, and easily digestible. No statistically significant differences were seen between the type or number of dietary elements and disease subtype or recent disease activity. The association between diet and symptoms in patients with IBD and the mechanism(s) involved warrant further research and may lead to the development of IBD specific dietary guidelines.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), collectively termed inflammatory bowel disease (IBD), are chronic inflammatory conditions that affect the gastrointestinal (GI) tract [1]. In UC only the colon is affected, whereas CD can affect any region of the GI tract. The prevalence in North America, Europe, and Oceania is estimated to exceed 0.3%, while newly industrialised countries with low prevalence rates are seeing a rapid increase in incidence [2,3]. The cause of IBD is currently unknown, although evidence suggests that genetic susceptibility, immune system dysregulation, and environmental factors are all involved [4]. Symptoms of IBD typically include diarrhoea and abdominal pain [5], and patients with CD are at greater risk of nutrient deficiency owing to increased requirements, inadequate dietary intake or composition, and malabsorption due to chronic inflammation and resection history [6]. Management of IBD involves the use of anti-inflammatory drugs, corticosteroids, and biologics to induce and maintain remission, and surgery for non-responsive patients and complications associated with disease progression [7].

Diet has long been implicated in the aetiology of IBD, a theory reinforced by the reduction of inflammation in response to enteral nutrition (EN) as well as its high efficacy for remission induction in patients with CD [8]. Early observational studies investigated the possible involvement of select dietary components including intake of sweets and pastries [9] and breakfast habits [10,11], yet many of these studies are fraught with methodological problems and many findings are un-replicated [12]. Large-scale epidemiological studies are better suited to investigating dietary patterns and the findings may provide aetiological clues; however, they are difficult to translate into practical dietary guidelines that patients with IBD frequently request.

More than half of patients with IBD believe their symptoms are induced or exacerbated by specific foods [13]. Commonly identified foods include fruit and vegetables, dairy products, spicy foods, processed foods, nuts and seeds, alcohol, and foods with a high fat content [14–17]. Promising results evidenced by prolonged remission have been observed in response to traditional exclusion and re-challenge trials [18–20]; however, there is uncertainty around the underlying mechanism(s) responsible. Investigation of GI symptoms in patients with IBD has demonstrated the coexistence of functional GI disorders (FGID), such as irritable bowel syndrome (IBS) or functional constipation [21]. An exclusion diet effective for reducing functional GI symptoms in patients with IBS is the FODMAP diet that limits fermentable oligosaccharides, disaccharides, monosaccharides, and polyols. Recently, patients with IBD reporting functional GI symptoms have also been shown to benefit from this diet [22–24]. Many other forms of exclusion diet have been trialled during the last half century such as the specific carbohydrate diet [25], CDED [26], IBD anti-inflammatory diet [27], and low residue diet [28]. Currently the evidence available is insufficient to classify dietary interventions of this nature as beneficial in the induction or maintenance of remission in IBD [29].

It is evident that dietary modification could have a role in the management of IBD symptoms. One difficulty in advancing this concept is a lack of knowledge around the scope of dietary elements implicated and the physiological processes involved. A question that needs to be answered is whether a common factor or pattern exists among dietary elements excluded by patients with IBD, as well as those they favour. In the present observational study, a self-administered retrospective questionnaire was used to investigate the extent that NZ patients with IBD associate disease symptoms with their diet. The objective of this study was to determine foods, additives, and cooking methods (dietary elements) identified in the onset, exacerbation, or reduction of IBD disease symptoms.

MATERIALS AND METHODS

Participants

Patients diagnosed with IBD were invited to complete a self-administered retrospective questionnaire on associations between foods and IBD symptoms. Advertisements were placed in gastroenterology clinics and at community IBD support organisations located throughout NZ. The inclusion criteria were 16 years or older; diagnosis of CD, UC, or IBD-unclassified (IBDU); and predominantly living in NZ for the previous two years. Screening of potential participants was undertaken by phone or in person by the primary investigator. Individuals were asked to confirm the form of IBD they have been diagnosed with, that the diagnosis had been made by a gastroenterologist or similarly qualified medical professional, and how the diagnosis was made (e.g., one or more of endoscopic, surgical, radiological, and biochemical investigation). A minimum of two years living in NZ was considered an adequate time period for immigrants to be exposed to the food items listed in the questionnaire, particularly seasonal fruit and vegetables.

Questionnaire

A review of the literature was undertaken to identify foods implicated in the onset, exacerbation and reduction of IBD symptoms. Additional foods were identified from the adult national nutrition survey to ensure inclusion of foods commonly consumed in NZ [30]. The questionnaire contained 142 dietary elements comprising 118 foods, 13 beverages, 7 additives, and 4 cooking methods (Supplementary Material *Figure 3.1*). The dietary elements were listed in columns and arranged into 15 groups: fruit, vegetables, nuts/seeds/dried fruit, grains, bread, cereals, miscellaneous, dairy products, meat, sauces, spreads, sweets/snacks/beverages, additives, and cooking methods. The complete list of dietary elements was provided in triplicate, one for each IBD symptom association; onset, worsening (exacerbation), and reduction. Participants indicated an association between a food element and their IBD symptoms and could provide additional information in open-ended question fields. Questionnaires were provided in hardcopy or via a secure online survey platform (surveymonkey.com).

Ethics

The protocol for this study was approved by the Massey University Human Ethics Committee: Southern A, Palmerston North, New Zealand (MUHEC Reference 13/58). All subjects were verbally screened and understood that by completing the questionnaire they were implicitly consenting to inclusion in the study.

Statistical Analysis

Statistical analysis was performed using JASP (version 0.11) [31]. Statistical differences between continuous demographic variables were assessed by 1-way ANOVA. Associations between disease subtype and categorical demographic variables including gender, family* history of IBD, and reported disease activity in the last 12 months were assessed by Chi-square test. Chi-square tests were also used to identify associations between dietary elements, food groups, and fruit and vegetable FODMAP content (Monash University FODMAP diet mobile application, version 3.0.3) with reported symptom effect. Associations between disease subtype (CD and UC) were also performed. T-tests were used to compare differences between participants who reported active disease in the previous 12 months versus those who reported inactive disease. Significance was set at $p<0.05$.

Comparison with disease subtype IBDU was not performed due to an insufficient number of participants with this diagnosis. When comparing FODMAP content with reported symptom effect, lentils were excluded from FODMAP analyses as the FODMAP content is considered medium.

** Family = immediate family: parent, child, sibling; and extended family: grandparent, aunt, uncle, cousin, nephew, niece.*

RESULTS

Baseline Participant Characteristics

Two hundred and fifty-six individuals registered interest in the study. Two were excluded due to not meeting the inclusion criteria. One participant withdrew during the study citing difficulties completing the questionnaire. A total of 20 participants did not complete the questionnaire and were unable to be contacted further. Of the 233 participants, 71% were female. Disease subtype comprised CD 63%, UC 32%, and IBDU 5%. The mean age of participants was 40 years with no significant differences between the three groups *(p*=0.105). The mean reported age at diagnosis was 29 years and differed significantly between participants with CD and participants with IBDU, 28 and 38 years, respectively *(p*=0.015). Mean disease duration was similar in the three groups, 11.6 years for participants with CD, 9.9 for participants with UC, and 10.7 years for participants with IBDU *(p*=0.510). A family history of IBD was reported by 29% of participants. The characteristics are given in *Table 3.1*.

Table 3.1 Characteristics of 233 Participants

Characteristics		All IBD, n=233	CD, n=146	UC, n=75	IBDU, n=12	p value
Gender, n (%)	Female	165 (71)	106 (73)	51 (68)	8 (67)	0.736
Age (years) mean ± SD		40.8 ± 14.9 n=230	40.0 ± 14.9 n=145	41.0 ± 14.5 n=73	49.5 ± 15.4	0.105
Diagnosis age (years) mean ± SD		29.7 ± 13.1 n=225	28.3 ± 12.4* n=142	31.1 ± 13.2 n=71	38.8 ± 17.4*	0.015*
Diagnosis age (years) n (%)	<20 20–40 >40	49 (22) 125 (55) 51 (23) n=225	37 (26) 76 (54) 29 (20)* n=142	10 (14) 44 (62) 17 (24) n=71	2 (16) 5 (42) 5 (42)*	0.101 0.375 0.029*
Disease duration (years) mean ± SD		11.0 ± 10.1 n=228	11.6 ± 10.1 n=143	9.9 ± 10.5 n=73	10.7 ± 6.7	0.510
Disease duration (years) n (%)	<11 11–20 >20	144 (63) 50 (22) 34 (15) n=228	83 (58) 38 (27) 22 (15) n=143	53 (73) 10 (14) 10 (14) n=73	8 (67) 2 (17) 2 (17)	0.171 0.635 0.438
Family history of IBD n (%)	Yes	65 (29) n=228	47 (33) n=144	17 (24) n=72	1 (8)	0.108
Active disease in the last 12 months, n (%)	Yes	177 (79) n=227	109 (77) n=142	60 (82) n=73	8 (67)	0.412

IBD = inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis, IBDU, inflammatory bowel disease unclassified; * statistically significant difference *P*<0.05. Statistical analyses were conducted by Chi-square test for categorical variables and by 1-way ANOVA for continuous variables.

Onset of Symptoms

An association between one or more dietary elements and symptom onset was reported by 128 (55%) participants. The mean number of dietary elements reported to initiate the onset of symptoms was 67.0 ± 5.1 for all participants, 68.9 ± 6.6 for participants with CD, and 58.4 ± 8.2 for participants with UC. The difference between CD and UC was not statistically significant (p=0.330). Seven (5.5%) participants identified a single dietary element, nine (7.0%) participants identified between two and five dietary elements, and six or more dietary elements were identified by 112 (87.5%) participants. The number of dietary elements associated with symptom onset was not significantly different between participants who reported active disease in the previous 12 months and those who reported inactive disease. Significantly more dietary elements were associated with symptom onset than symptom exacerbation (p=0.006).

The dietary elements most frequently identified in symptom onset were deep fried cooking method (73%), apple (63%), full-grain bread (62%), ice cream (61%), wheat (59%), kiwifruit (59%), corn (59%), cabbage (57%), onion (57%), and fried cooking method (57%). Supplementary Material *Table 3.2* gives the 25 most frequently identified dietary elements in symptom onset. The most frequently identified dietary elements according to group were vegetables (84%), beverages (80%), fruits (77%), dairy products (73%), cooking methods (73%), and sweets/snacks (70%).

Exacerbation of Symptoms

An association between one or more dietary elements and symptom exacerbation was reported by 164 (70%) participants. Overall, the mean number of dietary elements reported to exacerbate symptoms was 48.7 ± 4.3 for each participant. When analysed by disease subtype, there was no significant difference in the mean number of dietary elements reported to exacerbate symptoms between participants with CD and UC (43.8 ± 5.2 v 51.0 ± 7.5, p=0.429).

A single dietary element was identified by 6 (3.7%) participants, 20 (12.2%) participants identified between two and five dietary elements, and six or more dietary elements were identified by 119 (72.6%) participants. Nineteen (11.5%) participants specified one or more dietary elements in the other foods section of the questionnaire and did not identify any listed dietary elements. The number of dietary elements associated with symptom exacerbation was not significantly different between participants who reported active disease in the previous 12 months and those who reported inactive disease.

The dietary elements most frequently identified in symptom exacerbation were deep fried cooking method (60%); full-grain bread (55%); alcohol (49%); cabbage (48%); chilli sauce (48%); ice cream (47%); wheat (46%); wholemeal bread (46%); muesli (46%); and onion, chilli, and whole cow's milk (45% each). Supplementary Material *Table 3.3* gives the 25 most frequently identified dietary elements in symptom exacerbation. The most frequently identified dietary elements according to group were vegetables (80%), beverages (79%), cooking methods (70%), bread (68%), fruit (67%), and dairy products (66%).

Reduction of Symptoms

An association between one or more dietary elements and symptom reduction was reported by 82 (35%) participants. The mean number of dietary elements reported to reduce symptoms was 19.5 ± 4.5 for each participant. Participants with UC identified almost twice as many dietary elements than participants with CD, although the difference was not statistically significant (28.5 v 13.8, p=0.132). A single dietary element was identified by 10 (12.2%) participants, 28 (34.1%) participants identified between two and five dietary elements, and six or more dietary elements were identified by 34 (41.5%) participants. Ten (12.2%) participants specified one of more dietary elements in the *other foods* section of the questionnaire and did not identify any listed dietary elements. The number of dietary elements associated with symptom reduction was not significantly different between participants who reported active disease in the previous 12 months and those who reported inactive disease. Significantly fewer dietary elements were associated with symptom reduction than symptom onset (p<0.001) or symptom exacerbation (p<0.001).

The dietary elements most frequently identified in symptom reduction were banana (39%), rice (35%), white bread (33%), chicken (33%), oily fish (28%), non-oily fish (27%), eggs (26%), pumpkin (24%), yoghurt (24%), and baked cooking method (24%). Supplementary Material *Table 3.4* gives the 20 most frequently identified dietary elements in symptom reduction. The most frequently identified dietary elements according to group were fruit (53%), bread (51%), vegetables (49%), grains (47%), dairy products (46%), and meat (46%).

Other Responses

Additional information provided by participants demonstrated that the onset or exacerbation of symptoms were also associated with spicy foods (11%), foods with a high fat content (8%), takeaways or deep-fried foods (5%), raw fruit or vegetables (4%), processed foods (4%), fibrous foods (4%), foods with a high sugar content (4%), foods with a high FODMAP content (4%), and barbequed foods (1%). Other dietary elements reported in association with the reduction of symptoms were plain or bland foods (9%), soft or pureed foods (6%), boiled or mashed potato (5%), smoothies (4%), ginger beer or ale (4%), white bread or toast (4%), steamed foods (4%), yoghurt (2%), low FODMAP foods (2%), porridge (2%), and turmeric (2%).

FODMAPS

Fruits with a high FODMAP content were associated with symptom onset (p=0.004) and symptom exacerbation (p=0.009) significantly more than fruit with a low FODMAP content. Low FODMAP vegetables were associated with symptom onset (p<0.001) and symptom exacerbation (p<0.001) significantly more than high FODMAP vegetables. No difference was observed between the FODMAP content of vegetables or fruits associated with symptom reduction. There was also no difference between the FODMAP content of vegetables or fruit associated with symptom onset, exacerbation, or reduction between CD and UC.

DISCUSSION

Onset and Exacerbation of Symptoms

In the present study, 55% of participants reported an association between dietary elements and the onset of their IBD symptoms, consistent with the range of 33–68% observed in other studies [15,17,32–34]. Moreover, within the range seen in other studies (44–76.5%) [32,34–38] a greater percentage of participants (70%) reported an association between diet and symptom exacerbation.

The majority of participants identified six or more dietary elements in the onset or exacerbation of symptoms, while less than 13% of participants identified between two and five, and less than 6% identified a single item. These findings were higher than anticipated, although this is likely a reflection of the number of dietary elements evaluated and a tendency of some participants to indicate all items within a food group, notably vegetables. Surprisingly, there was no difference in the number of dietary elements identified overall or between participants with CD and UC, a finding that contradicts with observations of greater food avoidance by patients with CD [35,39,40].

Likewise, no difference was seen in the number of dietary elements identified by participants who reported active disease in the previous 12 months and those who reported inactive disease. As active disease is associated with significantly greater food avoidance [39–41], it was expected that the recall of dietary elements associated with symptoms would be heightened in participants who reported active disease and diminished in patients reporting extended remission.

Fruit and Vegetables

Patients with IBD often demonstrate an aversion for fruit and vegetables, a behaviour believed to be due to the fibre content and the effects they may experience following consumption including bloating, abdominal pain, and diarrhoea [42]. Accordingly, participants in this study frequently identified fruit and vegetables in the initiation or exacerbation of their IBD symptoms, a commonly reported finding [33,41,43] along with highly fibrous foods [14,44].

In Zallot et al.'s [15] study on dietary beliefs and behaviour, 47.5% of participants perceived raw vegetables to be a relapse risk factor. Similarly, a study on Canadian dietary habits reported avoidance of raw vegetables in 46% of participants [16]. This was not observed in the present study as the most frequently identified vegetables, including corn, cabbage, onion, chilli, broccoli, and garlic, are generally cooked before consumption. However, a small proportion of participants remarked that raw fruit and vegetables are problematic. A trend towards cruciferous [15] and brassica [39] vegetables has also been described; again, this was not supported by our findings with only two of the most identified vegetables meeting this criterion.

Research suggests more than a third of patients with IBD experience functional GI symptoms compatible with IBS [45] and that a low FODMAP diet can significantly reduce functional GI symptoms [22,23]. In fact, many of the foods frequently identified in this study have also been identified by patients with IBS as problematic including chilli, spices, onion, cabbage, alcohol, fried and fatty foods, and caffeine [46,47]. This

might indicate an association between the FODMAP content of fruit and vegetables and symptom response. This hypothesis was supported by the significant association between fruits with a high FODMAP content and both symptom onset and exacerbation; however, for vegetables the opposite was observed. It is unclear why vegetables with a low FODMAP content were associated with symptoms. This could indicate a low incidence of functional GI symptoms experienced by this cohort, or alternatively, another characteristic of vegetables could affect symptoms to a greater extent than the FODMAP content.

In line with similar studies, fruit were identified in symptom onset and exacerbation less often than vegetables. The diversity of fruit identified was also less than vegetables, making it difficult to identify a common factor. Citrus fruits have previously been singled out as being problematic [32,38,43,48], an observation not evident in this study with only one citrus fruit, orange, amongst the most frequently identified fruits. Considering each fruit individually, it is unclear how apple and orange could elicit GI irritation. Whereas the seed content of tomatoes and kiwifruit may be problematic as patients with IBD largely deem seeds to be deleterious. Additionally, kiwifruit may cause diarrhoea as a consequence of its laxative effect [49].

Dairy Products

Avoidance of dairy products is commonplace in patients with IBD. Of the nine dairy products evaluated in this study, ice cream and cream were most frequently identified by all participants. Participants with CD also identified hard cheese and fruit yoghurt in association with symptom onset, and both whole and reduced fat cow's milk were identified by participants with UC. In some participants, the association between symptoms and dairy products could possibly be attributable to lactose intolerance, a term used to describe the onset of GI symptoms in response to lactose consumption, usually due to lactase deficiency induced malabsorption [50]. A degree of overlap exists between symptoms of IBD and lactose intolerance including bloating, abdominal pain, and diarrhoea [51]; thus, participants may be unaware that these symptoms can occur independently of IBD or be unable to distinguish between symptoms.

An NZ study investigating the effects of dairy products in 165 patients with CD found that the worsening of symptoms correlated with fat content, with the most problematic dairy products being cream, cheese, ice-cream, and standard milk [52]. The findings of this study support the notion that fat content could be a key component in symptom onset or exacerbation, although participants with CD also frequently identified fruit yoghurt which usually has a low-fat content. Further, butter was not identified as often as some dairy products with significantly lower fat content, a mutual finding that somewhat undermines the fat content theory or could equally reflect that consumption quantities are usually inadequate to elicit an effect [52].

Gluten

An unexpected finding was the association between bread and the onset and exacerbation of symptoms. Of the five types of bread listed in the questionnaire, only gluten-free bread was well tolerated, a finding also seen in Triggs et al.'s [53] study of food intolerances. The coexistence of coeliac disease or non-

coeliac gluten sensitivity are possible reasons for these findings and are further supported by the frequent reporting of wheat and wheat-based cereal in this study. However, the likelihood of undiagnosed coeliac disease in a patient with IBD is low despite the relative risk of coeliac disease being almost four times higher in patients with IBD [54]. Moreover, taking into account the estimated Oceanic coeliac disease prevalence of 0.8% [55], this would suggest a prevalence of only 3.2% in the NZ IBD population. Non-coeliac gluten sensitivity, on the other hand, affects approximately 30% of patients with IBD [56]. Significant benefit to patients with IBD has also been demonstrated in response to a gluten free diet trial with 66% of the 314 patients reporting symptom improvement and reduced flare frequency and severity in 38% [57].

Other

Of interest are the strong associations that participants have with cooking methods. Deep frying was the dietary element most frequently identified in symptom onset and exacerbation, ahead of foods, and frying was also frequently identified. Both cooking methods generally involve the addition of fat as a cooking medium, and thus, they are suggestive of deleterious effects in response to the ingestion of foods with high fat content. The study was not designed to explore the perceived effects of macronutrients; however, from the other responses received, 8% of participants specified foods with a high fat content, and 5% specified takeaways or deep-fried foods. These findings are in agreement with previous and current studies where patients with IBD have identified fast food [35,48], fatty food [15,17,34,36,37], fried and fatty foods [14], deep-fried and fatty food [16,39], oily food [38], and fatty meats [58] in symptom onset or exacerbation. Furthermore, evaluation of dietary intake changes in over 4200 families in Japan over two decades found positive associations between increasing CD incidence and total fat, animal fat, and omega-6 fatty acids [59]. Whether GI irritation is evoked in response to the type or content of fat ingested remains to be elucidated.

A large percentage of participants identified alcohol, fruit juice, chilli sauce, and coffee in the onset and exacerbation of symptoms. Given that alcohol can cause GI symptoms in healthy individuals, it was unsurprising that wine and beer specifically, and alcohol in general, were frequently identified. Fruit juice was likely identified for the same reason as fruit and vegetables, and the capsaicin content could explain why chilli sauce, and perhaps spicy foods also, are frequently deemed problematic [40]. Unlike most studies, the perceived effects of coffee and tea were investigated individually, where coffee was frequently associated with symptom onset and exacerbation, and black tea was well tolerated. Generally, tea and coffee appear to be problematic as evidenced by their association with increased risk of relapse [15], negative effects on symptoms [48], significantly reduced consumption in CD patients experiencing active disease [60], and overall avoidance [16,58]. However, taking into account de Vries et al.'s [32] observation that tea is the second most symptom improving food among 294 patients with IBD, and the findings of the present study, coffee and tea should be considered separately when investigating their purported effects on IBD symptoms.

Reduction of Symptoms

The view that diet can directly influence IBD symptoms is most convincingly demonstrated by the ability of EN to ameliorate inflammation. Although not definitive, EN is thought to reduce inflammation by downregulating proinflammatory cytokine levels, facilitating mucosal healing and restoring GI barrier function, and improving nutritional status [61]. In the current study, less than half of the participants (35%) reported an association between dietary elements and symptom reduction. Dietary elements identified in previous studies as being associated with symptom improvement include bananas, chicken, rice, white bread, oily fish, white fish, yoghurt, gluten free foods, and herbal tea [14,32,38,48,53,62]. Cooking foods by baking or grilling were also frequently identified. Additionally, participants specified that they associate symptom reduction with plain or bland foods, soft or pureed foods, boiled or mashed potato, smoothies, ginger beer or ale, and steamed foods.

Reduction of inflammation and ultimately mucosal healing is the goal of IBD management. The anti-inflammatory effects of omega-3 fatty acids are well established, particularly for improving health outcomes in those affected by chronic conditions [63]. While the evidence from available studies is considered inadequate to make recommendations, omega-3 fatty acid supplementation in patients with IBD has been shown to reduce disease activity, extend remission, improve endoscopic scores, and decrease levels of inflammatory markers [64,65]. As oily and non-oily fish were frequently identified by participants, and avocado by participants with UC, the reported reduction of symptoms could be facilitated by omega-3 or monounsaturated fatty acid intake. This is further supported by a recent dietary belief and behaviour study where 2% of patients with inactive UC reported consumption of oily fish or omega-3 supplements to prevent relapse [17].

Theoretically, attributes of the dietary elements identified in symptom onset and exacerbation are deemed potentially harmful by the immune system and consequently trigger an inflammatory response. Thus, the content of suspected irritants may be lower in dietary elements identified in symptom reduction. Accordingly, their overall fibre content is low; dairy product number is limited and low in lactose; saturated fatty acid levels are minimal; wholegrains, spicy foods, alcohol, and caffeine are notably absent; gluten is avoided, and the cooking methods require little or no added fat. The extent of these dietary restrictions possibly equates to a significantly reduced antigenic load and provision of nutrients in a form that eases digestion requirements, elements also believed to be of importance to EN [61].

In agreement with Triggs et al. [53], the views regarding diet and IBD symptoms demonstrate that individual foods cannot be classified as either detrimental or beneficial for all patients with IBD. This indicates that like other aspects of IBD, including disease trajectory and response to therapy, patients respond to dietary elements in a heterogenous fashion. Whole cow's milk is a prime example with 57% of participants with UC reporting an association with symptom exacerbation, and 30% with symptom reduction. Similarly, 39% of participants with CD identified orange in symptom exacerbation, and 16% in symptom reduction. Other instances may be attributable to changes in food properties following preparation and/or cooking. For example, apple was identified in symptom onset by 66% of participants

with CD, and in symptom reduction by 18%. In the case that mechanical processing and digestibility are directly correlated to symptoms, it is plausible that the effect of raw apple could be distinct to that of peeled and stewed apple.

Dietary Modification

According to studies on dietary beliefs and behaviours, 56–90% [33–35,39,41,48] of patients modify their diet following their diagnosis of IBD. The most recent of these studies reported that foods rich in fibre, fruit and vegetables, and grains are the most modified foods [41], putting patients at risk of inadequate intake of micronutrients such as folate, potassium, magnesium, vitamins A and C, and B vitamins. Dairy product avoidance is also common, especially during periods of disease activity [16]. Without adequate alternate sources of calcium, this can be especially detrimental to patients with IBD given the increased risk of osteoporosis and osteopenia associated with corticosteroid treatment and prolonged inflammation [66].

Under the guidance of a professional, the risk of inadequate nutrient intake due to dietary modification can be mitigated by the provision of education around alternate sources of key nutrients, or supplemental nutrition where appropriate. However, patient accessibility to reputable sources of dietary information and advice can be limited, a challenge that can be hampered by medical practitioner scepticism around the utility of dietary therapy as an adjunct to drug therapy. For instance, a survey of 414 UK and NZ gastroenterologists found views of the efficacy of dietary exclusion in IBD and IBS symptom management to be mixed and the majority referring less than 25% of these patients to dietetic services [67]. Consequently, patients seek dietary information and advice from other sources including websites, online forums and books, and fellow patients [33,39] that can lead to self-imposed dietary restrictions and an increased risk of inadequate nutrient intake.

In the current study, a high number of dietary elements were identified with symptom onset and exacerbation, specifically, fruit, vegetables, and dairy products. These results suggest that self-imposed dietary restrictions could contribute to the high rates of malnutrition seen in the IBD population, a concept recently reported by Lim, Kim, and Hong [58]. Health providers may be unaware of the extent of patient views regarding diet and symptoms and should be encouraged to offer their patients nutritional counselling not only soon after diagnosis but also when attempts to suppress ongoing disease activity are ineffective. Besides ensuring patients understand and are able to implement the principles of a balanced diet, any dietary modification could be appropriately monitored, and the risk of inadequate nutrient intake reduced.

While the success of available exclusion diets is limited, we may be overlooking the potential of an antigen-response based exclusion diet. Patients with IBD have been shown to have significantly higher rates of food hypersensitivity (allergies and intolerances), and encouraging results have been observed in response to IgG or IgE-titre based exclusion diets [68–75]. Further research in this area using objective measures of disease activity is needed to determine if the intake of specific dietary antigens correlates directly with IBD symptoms.

It is irrefutable that a greater understanding of the role of diet in symptom-based disease behaviour is needed. Well-designed prospective trials are needed to investigate how the immune system of patients with IBD responds to different foods, specifically, whether foods alter intestinal permeability, gene expression, or microbiota composition. Improved evidence could lead to development of evidence-based diet recommendations, a necessary tool for the improved management of IBD. Dietary approaches could also provide a low-risk alternative for when drug therapy is responsible for severe side-affects or has lost its effectiveness.

Limitations

The study design was observational and thus causality cannot be ascertained. Although participants were asked to indicate dietary elements they associate with their symptoms, some may have been identified due to personal preference or as a result of information sought or advice received, rather than a perceived association with symptoms. Further, recall bias is an inherent and unavoidable limitation associated with retrospective dietary studies and could have influenced the study findings.

The prevalence of IBS in IBD patients is 35–44% [45], and the odds ratio of IBS in women compared with men is 1.67 [76]. Given the predominance of female participants (71%) and difficulties distinguishing between IBD and IBS symptoms [77], some participants may have mistakenly reported associations between dietary elements and IBS symptoms. As the baseline prevalence of IBS in this cohort was not determined, potential differences between participants affected and not affected by IBS were unable to be investigated.

The questionnaire did not distinguish between foods that can be consumed both raw and cooked, in some cases limiting the ability to deduce the primary symptom effect. Disease phenotype data were not collected, preventing the analysis of possible differences in effects dependent upon disease location and extent, and stricturing or penetrating disease in participants with CD. Finally, the study did not have a target ratio of patients with CD and UC, resulting in almost double the number of patients with CD. While this is consistent with recent patterns of IBD diagnosis in NZ [78,79], the smaller number of participants with UC may have resulted in this disease subtype being underrepresented.

CONCLUSION

This study showed that patients with IBD strongly believe diet can affect the duration and severity of their symptoms. The dietary elements most frequently identified with symptom onset and exacerbation are predominantly high in fibre, dairy-based or cooked by deep frying; while symptom reduction was associated with distinct dietary elements including banana, rice, white bread, and white meat. Further research is required to determine the extent of self-imposed symptom-associated dietary restriction in this patient group, and their effect on nutrient status.

Supplementary Material

APPENDIX 1: Foods or Food Combinations you associate with the **ONSET** of your IBD symptoms

Please tick (√) the food/s you associate with the **onset** of your IBD symptoms (excluding allergies/intolerances that cause discomfort **DISTINCT** from your IBD symptoms) and rate the effect of the food/s (1 = little effect 2 = moderate effect 3 = definite effect), and/or list other foods and/or food combinations below this table.

√	FRUIT	1	2	3
	Apple			
	Apricot			
	Avocado			
	Banana			
	Cherry			
	Grapes			
	Grapefruit			
	Kiwifruit			
	Mandarin			
	Nectarine			
	Orange			
	Peach			
	Pear			
	Pineapple			
	Plum			
	Strawberry			
√	VEGETABLES	1	2	3
	Asparagus			
	Beans			
	Beetroot			
	Broccoli			
	Brussels Sprouts			
	Cabbage			
	Carrot			
	Capsicum			
	Cauliflower			
	Celery			
	Chickpeas			
	Chilli			
	Corn			
	Courgette			
	Cucumber			
	Garlic			
	Kumara			
	Leek			
	Lentils			
	Lettuce			
	Mushroom			
	Onion			
	Parsnip			
	Pumpkin			
	Spinach			
	Tomato			

√	NUTS/SEEDS/ DRIED FRUIT	1	2	3
	Almond			
	Apricot			
	Brazil			
	Cashew			
	Cranberry			
	Date			
	Fig			
	Hazelnut			
	Macadamia			
	Peanut			
	Pine nut			
	Pistachio			
	Pumpkin			
	Raisin			
	Sunflower			
	Walnut			
√	GRAINS	1	2	3
	Barley			
	Oats			
	Popcorn			
	Rice			
	Wheat			
√	BREAD	1	2	3
	Brown			
	Full-grain			
	Gluten-free			
	White			
	Wholemeal			
√	CEREALS	1	2	3
	Bran based			
	Corn based			
	Rice based			
	Wheat based			
	Muesli			
√	MISC	1	2	3
	Eggs			
	Pastry			
	Tobacco			

√	DAIRY PRODUCTS	1	2	3
	Butter			
	Cheese – hard			
	Cheese – soft			
	Cream			
	Ice-cream			
	Cow's Milk – light			
	Cow's Milk – whole			
	Yoghurt – fruit			
	Yoghurt – dairy food eg caramel			
√	MEAT	1	2	3
	Beef			
	Chicken			
	Fish – non oily eg hoki, snapper			
	Fish – oily eg salmon, tuna			
	Lamb			
	Pork			
	Processed eg salami, luncheon			
	Seafood eg mussels			
	Turkey			
	Veal			
√	SAUCES	1	2	3
	Barbeque			
	Chilli			
	Chutney			
	Mayonnaise			
	Maple syrup			
	Salad dressing			
	Tomato			
√	SPREADS	1	2	3
	Honey			
	Jam			
	Margarine			
	Marmalade			
	Peanut Butter			
	Marmite or Vegemite			

√	SWEETS/SNACKS	1	2	3
	Biscuits			
	Cake			
	Chocolate			
	Crackers			
	Corn chips			
	Dips			
	Lollies			
	Muesli bars			
	Pizza			
	Potato chips			
√	BEVERAGES	1	2	3
	Alcohol – any			
	Beer			
	Coffee			
	Energy drinks			
	Fruit juice			
	Hot Chocolate			
	Milo			
	Soft drinks - any			
	Soft drinks – sugar free			
	Spirits			
	Tea – black			
	Tea – herbal			
	Wine			
√	ADDITIVES	1	2	3
	Artificial Sweetener			
	Food colouring			
	Herbs			
	Pepper			
	Salt			
	Spices			
	Sugar			
√	COOKING METHODS	1	2	3
	Baked			
	Deep Fried			
	Fried			
	Grilled			

Other Foods and/or Food Combinations:

_____

_____

Supplementary Figure 3.1 Inflammatory Bowel Disease (IBD) Questionnaire

Supplementary Table 3.2 Dietary Elements Most Frequently Identified in IBD Symptom Onset, according to disease subtype

IBD, n=165	**%**	**CD, n=146**	**%**	**UC, n=75**	**%**
Deep Fried[1]	73	Deep Fried[1]	74	Deep Fried[1]	67
Apple	63	Apple	66	Alcohol – any	64
Full-grain bread	62	Full-grain bread	66	Apple	56
Ice-cream	61	Ice-cream	63	Cabbage	56
Wheat	59	Chilli sauce	63	Wheat	56
Kiwifruit	59	Kiwifruit	61	Ice-cream	56
Corn	59	Wholemeal bread	61	Coffee	56
Cabbage	57	Chilli	59	Kiwifruit	53
Onion	57	Onion	59	Broccoli	53
Fried[1]	57	Tomato	59	Corn	53
Chilli	56	Wheat	59	White bread	53
Chilli sauce	56	Corn	58	Muesli	53
Coffee	56	Bran based cereal	58	Cow's Milk – light	53
Fruit juice	56	Fruit juice	58	Beer	53
Broccoli	55	Fried[1]	58	Fried[1]	53
Wholemeal bread	55	Cabbage	57	Full-grain bread	51
Bran based cereal	55	Brown bread	57	Cream	51
Muesli	55	Cheese – hard	57	Chilli	49
Cream	55	Cream	57	Garlic	49
Wheat based cereal	55	Broccoli	55	Onion	49
Alcohol – any	55	Wheat based cereal	55	Wheat based cereal	49
Wine	55	Yoghurt – fruit	55	Wine	49
Brown bread	54	Chocolate	55	Fruit juice	49
White bread	54	Coffee	55	Chocolate	49
Chocolate	54	Wine	55	Cow's Milk – whole	49

Cooking methods are marked with [1].

Supplementary Table 3.3 Dietary Elements Most Frequently Identified in IBD Symptom Exacerbation, according to disease subtype

IBD, n=165	**%**	**CD, n=146**	**%**	**UC, n=75**	**%**
Deep Fried[1]	60	Deep Fried[1]	57	Deep Fried[1]	67
Full-grain bread	55	Full-grain bread	51	Alcohol – any	65
Alcohol – any	49	Chilli sauce	49	Full-grain bread	63
Cabbage	48	Cabbage	45	Cabbage	57
Chilli sauce	48	Onion	44	Wholemeal bread	57
Ice-cream	47	Chilli	43	Ice-cream	57
Wheat	46	Wheat	43	Cow's Milk – whole	57
Wholemeal bread	46	Ice-cream	43	Muesli	55
Muesli	46	Alcohol – any	43	Wine - red or white	55
Chilli	45	Muesli	42	Wheat	53
Onion	45	Apple	41	Chilli	51
Cow's Milk – whole	45	Corn	41	Popcorn	51
Fried[1]	44	Peanut	41	Wheat based cereal	51
Broccoli	43	Wholemeal bread	41	Coffee	51
Cream	43	Fried[1]	41	Fried[1]	51
Coffee	43	Broccoli	40	Broccoli	49
Apple	42	Cream	40	Cream	49
Popcorn	42	Orange	39	Chilli sauce	49
Wheat based cereal	42	Tomato	39	Muesli bars	49
Corn	41	Cashew nuts	39	Pizza	49
Wine	41	Cow's Milk – whole	38	Apple	47
Brown bread	40	Fruit juice	38	Beer	47
Peanut	40	Garlic	38	Onion	47
Fruit juice	40	Kiwifruit	38	White bread	47
Garlic	40	Brown bread	38	Cow's Milk – light	47

Cooking methods are marked with [1].

Supplementary Table 3.4 Dietary Elements Most Frequently Identified in IBD Symptom Reduction, according to disease subtype

IBD, n=165	**%**	**CD, n=146**	**%**	**UC, n=75**	**%**
Banana	39	Banana	37	Chicken	50
Rice	35	White bread	33	Rice	50
White bread	33	Rice	25	Fish – non oily	46
Chicken	32	Kumara	23	Fish – oily	46
Fish – oily	28	Eggs	21	Banana	42
Fish – non oily	27	Chicken	21	Rice based cereal	42
Eggs	26	Yoghurt – fruit	19	Baked[1]	38
Pumpkin	24	Fish – oily	19	Pumpkin	33
Yoghurt – fruit	24	Marmite/Vegemite	19	Gluten-free bread	33
Baked[1]	24	Pumpkin	17	Eggs	33
Kumara	23	Oats	17	Yoghurt – fruit	33
Rice based cereal	23	Fish – non oily	17	Tea – herbal	33
Tea – herbal	22	Baked[1]	17	Avocado	29
Gluten-free bread	21	Apple	15	Lettuce	29
Grilled[1]	21	Carrot	15	Spinach	29
Oats	20	Rice based cereal	13	White bread	29
Honey	20	Tea – herbal	15	Cow's Milk – whole	29
Marmite or Vegemite	20	Grilled[1]	15	Beef	29
Avocado	18	Gluten-free bread	13	Honey	29
Carrot	18	Cauliflower	13	Grilled[1]	29

Cooking methods are marked with [1].

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CHAPTER FOUR

DEVELOPMENT OF AN *IN VITRO MODEL* TO INVESTIGATE INTERACTIONS AT THE INTESTINAL BARRIER

Investigation of the *in vitro* digestion protocol (appendix 3) offered the opportunity to robustly investigate the effects of foods on small intestinal tissue. This approach began with the establishment of the methodology for isolating intestinal crypts and culturing murine intestinal organoids ("mini-guts"), a novel cell model that closely resembles normal intestinal tissue. Organoid culture provides the opportunity to maintain intestinal epithelium *in vitro* and investigate tissue at a structural and cellular level, using live cell imaging that cannot be applied to intact intestine. Further, to characterise the interactions between murine intestinal tissue and *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of Johne's disease in ruminants which has many similarities to Crohn's disease in humans. An *in vitro* model of infection was developed to infect organoids by microinjecting macrophages containing MAP into the lumen of the organoid.

NOTE The methodology for culturing intestinal organoids was successfully established, and initial experiments involving microinjection of macrophages were conducted. However, following methodology difficulties and limitations associated with microinjection and culture medium matrix viscosity, the research was halted.

INTRODUCTION

Mycobacterium avium subspecies paratuberculosis (MAP) is the pathogen that causes Johne's disease (JD), a chronic enteritis that affects cattle, sheep, deer, and goats [1]. Weight loss and weakness, and diarrhoea in cattle, are the first visible signs of disease, followed by progression to irreversible emaciation, anaemia, and hypoproteinemia [2]. Pathological features of JD include thickening of the mucosa, enlargement of the lymph nodes, and granulomatous lesions that typically affect the ileum, caecum, and colon [3]. This combination of weight loss, diarrhoea, mucosal thickening, granulomata, and the regions affected by JD lead to the hypothesis that MAP could be the cause of Crohn's disease (CD) in susceptible individuals [4].

Based on current knowledge, the proposed mechanism of infection suggests that once consumed MAP is engulfed by intestinal macrophages and induces the production and release of proinflammatory molecules responsible for intestinal inflammation. These molecules prompt the differentiation of T cells to helper T cells, which exacerbates intestinal inflammation by further stimulating macrophage production of proinflammatory molecules [5]. An alternate theory proposed by Hermon-Taylor and El-Zaatari [6] is that MAP infection causes the intestinal wall to become chronically leaky, causing increased antigen exposure and a constant state of inflammation.

Of the evidence that supports a link between MAP and CD, the most compelling is the odds ratio of 7:1 for MAP DNA detection in tissue from patients with CD compared to controls [7]. Other supportive evidence, besides the aforementioned similarities between the clinical features and pathologies of JD and CD, is the potential for MAP contact via infected livestock, environments in which they reside, as well as contaminated water, raw meat, milk, and cheese [8]. Further, antibody reactivity to MAP antigens has been shown to be significantly higher in patients with CD compared to controls [9], and the CD-predisposing gene mutation for the nucleotide-binding oligomerisation domain protein 2 (NOD2) may increase susceptibility to mycobacterial infection [10]. Conversely, conflicting evidence highlights the absence of a positive association between MAP exposure and CD prevalence [11], detection of MAP in individuals that do not have CD, and that antibiotic therapy sufficient to eradicate MAP only produces a short-term benefit in patients with CD [12].

Attempts to expand our understanding of how MAP may be associated with CD have been hampered by a limited ability to investigate how MAP interacts with intestinal tissue. The development of techniques that allow isolation of entire intestinal crypts, and the identification of growth factors essential for stem cell differentiation, has enabled the culture of intestinal tissue and establishment of an *in vitro* model of the small intestinal epithelium. The small intestinal 'organoid' model is a three-dimensional structure that includes stem cell derived Paneth cells, goblet cells, enterocytes, and enteroendocrine cells [13]. The culture process is highly reproducible, enabling expansion of very small tissue samples [14], and the organoids can be maintained for months due to self-renewal of the stem cells [15]. This is in sharp contrast to the use of intact intestinal tissue and tissue biopsies which can be maintained *in vitro* for 1-2 hrs and are poorly accessible for monitoring and manipulation of cell function. Use of the small intestinal organoid model offers the opportunity to investigate how MAP interacts with intestinal tissue, which currently cannot be performed using other *in vivo* or *in vitro* methods. Expanding our knowledge of the interactions between MAP and

intestinal tissue has implications for understanding the mechanisms involved in the pathogenesis of JD, and may aid in resolving whether MAP is involved in other health conditions including type-1 diabetes mellitus [16,17], Blau Syndrome (an inherited inflammatory disorder) [18], and irritable bowel syndrome [19].

AIMS

Firstly, to apply a novel *in vitro* model, that we recently established in our laboratory, to investigate the effect of exposure to foods reported to trigger or exacerbate intestinal symptoms in CD on intestinal barrier integrity. Secondly, to use the same model to characterise the interactions between murine intestinal tissue and MAP thus establishing the methodology which could subsequently be applied to investigate human intestinal tissue exposed to MAP. Live cell imaging will be used to monitor the infectivity of MAP, and to directly assess whether infection by this pathogen can disrupt the ability of intestinal tissue to maintain its barrier to gut contents.

- To culture organoids from murine tissue to investigate the effect of foods, after *in vitro* digestion, on the intestinal barrier.
- To develop an *in vitro* model of infection to infect organoids with MAP.
- To identify live cell imaging parameters most suited to monitoring MAP infectivity, e.g., long-term time-lapse duration and the optimal fluorescent probes to be used.
- To identify some of the mechanisms MAP uses to infect tissue using live cell imaging, and whether MAP infection can disrupt the ability of intestinal tissue to maintain its barrier to gut contents.

METHODS

Intestinal organoids can be cultured from either male or female mice. Intestinal epithelial stem cells, from which all intestinal epithelial cells are derived both *in vivo* and in organoids *in vitro*, have higher proliferation rates in female than male mouse tissue, an occurrence independent of gender-related steroid hormones [20]. Female mice were therefore chosen as the source of intestinal tissue for organoid culture in this study. Murine small intestinal crypt isolation and culture were adapted from Sato et al.'s protocol [13].

Crypt Isolation

The small intestine was removed immediately post-euthanasia, placed in ice-cold PBS, and cut longitudinally. Villi and debris were removed by gently stroking the luminal surface with a glass coverslip, the tissue placed in fresh ice-cold PBS, and cut into 0.5cm pieces. Tissue pieces were cleaned further by gently pipetting with a 5ml pipette in ice-cold PBS, allowing the supernatant to settle briefly before removing, replacing with fresh ice-cold PBS, and repeating until the supernatant was almost clear.

The final supernatant was removed, replaced with ice-cold crypt isolation buffer (*Table 4.2*), and gently rocked on ice for 30 minutes. The supernatant was then allowed to settle briefly, removed, and

replaced with fresh ice-cold PBS. The tissue was vigorously pipetted up and down for 30 seconds, and after the tissue settled the supernatant was removed and inspected for crypts using an inverted microscope.

PBS replacement, tissue pipetting, and supernatant inspection were repeated until intact crypts were obtained. A further two fractions were produced, and the fraction containing the most intact crypts and the least debris (usually in fraction 3, 4, or 5) was filtered through a 70µm filter and spun at 300g for 5 minutes at 4°C. The supernatant was removed, the crypt pellet resuspended in 10ml of chilled medium, and re-spun at 150-200g for 2 minutes at 4°C. This final supernatant removal, pellet resuspension, and re-spin was repeated once.

Table 4.1 Crypt Isolation and Culture Materials

ITEM		SOURCE
Female C57B6 mice, aged 6-10 weeks		Gifted
Phosphate buffered saline (PBS):	- Sodium chloride	Sigma Aldrich
	- Potassium chloride	Sigma Aldrich
	- Potassium phosphate monobasic	Sigma Aldrich
	- Sodium phosphate dibasic	Sigma Aldrich
Glass coverslip		
Serological pipettes, 5ml		Sigma-Aldrich
Cell strainers, 70µm		Thermo Fisher Scientific
Ethylenediaminetetraacetic acid (EDTA)		
HEPES, 1M		Sigma-Aldrich
Round coverslips, 13mm		
Basement membrane matrix matrigel (phenol red free, growth factor reduced)		BD Biosystems
DMEM F-12		Thermo Fisher Scientific
Glutamax		Thermo Fisher Scientific
Penicillin-Streptomycin-Neomycin (PSN)		Thermo Fisher Scientific
N2 supplement		Invitrogen
B27 supplement		Invitrogen
N-acetyl-L-cysteine (NAC)		Sigma-Aldrich
Murine Recombinant Noggin		PeproTech
Recombinant Human R-spondin 1		R&D systems
Murine recombinant Epidermal Growth Factor (EGF)		Invitrogen Gibco

Table 4.2 Crypt Isolation Buffer

ITEM	QUANTITY
PBS	15ml
EDTA	60µl
HEPES	150µl
280-305mOsm, pH 7.30-7.35	

Organoid Culture

The supernatant was removed, and the crypt pellet was resuspended in 200µl of thawed Matrigel and plated as 3 or 4 small domes per round coverslip. The plate was incubated (37°C, 5% CO_2) for 5 to 10 minutes to allow the domes to solidify before 500µl of complete culture medium (*Table 4.3)* pre-warmed to 37°C was added to each well. The medium was replaced with fresh complete culture medium every 4 days post-plating, and fresh R-spondin, EGF, and Noggin, also known as REN (*Table 4.4*), was added to each well 2 days post-plating and then 2 days post each fresh medium replacement.

Table 4.3 Complete Culture Medium

ITEM	QUANTITY
DMEM F-12	1ml
Glutamax	10µl
HEPES	10µl
PSN	10µl
N2 supplement	10µl
B27 supplement	20µl
NAC	2µl
EGF	5µl
Noggin	5µl
R-spondin	5µl

Table 4.4 REN

ITEM	QUANTITY
DMEM F-12	45µl
EGF	2.5µl
Noggin	2.5µl
R-spondin	2.5µl

Microscopy

The culture medium was removed, the domes washed twice with PBS, then fixed with paraformaldehyde (4% in PBS) overnight at 4˚C. The paraformaldehyde was removed, the domes washed three times with PBS, then stored in PBS at 4°C with the plate sealed with Parafilm to avoid dehydration until required. In preparation for imaging, the domes were washed with blocking buffer (5% goat serum in PBS), then permeabilised in blocking buffer (0.5% Triton-X100 in PBS) for 60 minutes at room temperature.

Infection Model

Murine intestinal tissue organoids will initially be microinjected (borosilicate glass, 15-20µm) with murine macrophages (RAW264.7). The intention was to follow this with microinjection of macrophages that have been incubated with MAP, and to visualise this process, and the effects of MAP infection on cell function and barrier integrity, using live cell imaging and a rabbit anti-MAP polyclonal antibody.

ETHICS

The protocols for this study were approved by the Massey University Human Ethics Committee: Southern A, Palmerston North, New Zealand (MUHEC Reference 17/26 (murine intestinal tissue culture), 13/36 (human intestinal tissue donation), 13/37 (human intestinal tissue culture)).

RESULTS AND DISCUSSION

The first aim of culturing intestinal organoids was achieved (*Figure 4.1*). During this time, it became apparent that the firmness of the Matrigel may impede penetration of the supernatant from foods that underwent *in vitro* digestion, or that some supernatant particles may be too large to penetrate the Matrigel. To address this, experimentation was planned to determine the minimum incubation period necessary for the supernatant to penetrate the Matrigel and whether filtration is required, or to remove the organoids from the Matrigel prior to incubation. This work was postponed to allow progression on other study aims first.

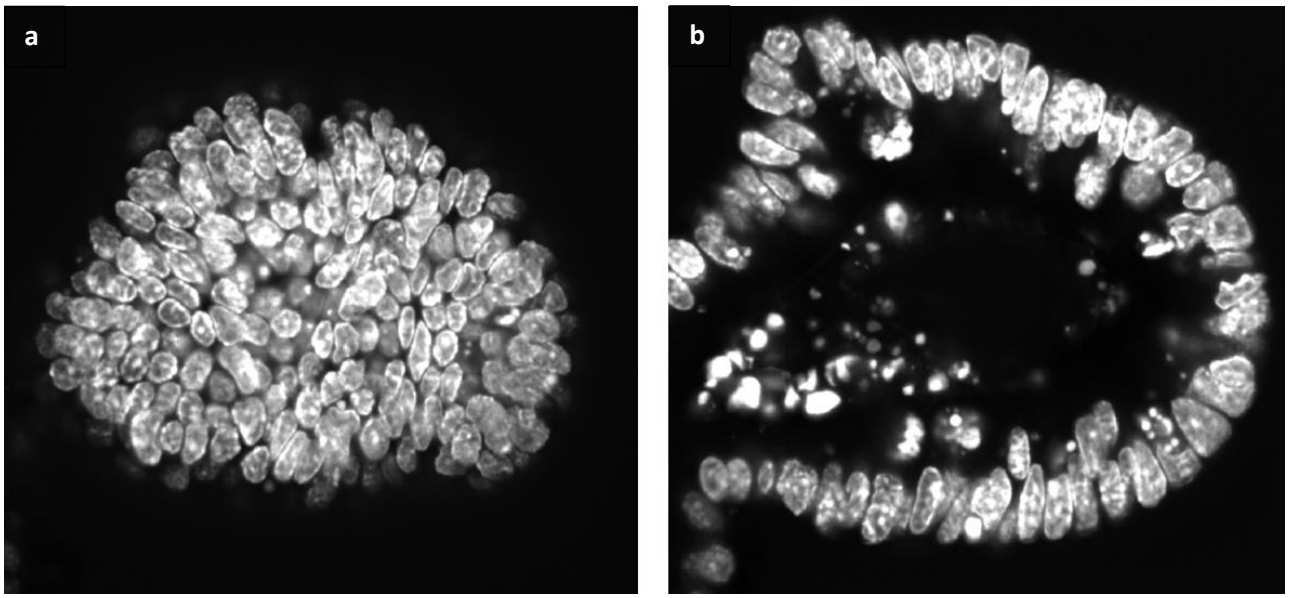

Figure 4.1 Murine Intestinal Organoid Structure

The structure of a mouse intestinal organoid with cell nuclei stained with Hoechst 33258 and viewed by confocal microscopy. The collection of z-series, in which images are serially captured while progressively focusing deeper in 5µm steps into the tissue, demonstrates the ability to visualise individual cells throughout the crypts with a resolution which cannot be achieved with conventional fluorescence microscopy. **a.** shows the top surface **b.** shows half-way into the depth of the crypt with 58 images collected in the z-plane.

The second study aim was to develop a model of infection to infect organoids with MAP by microinjecting macrophages into the lumen of the organoid (*Figure 4.2* and *Figure 4.3*). Initial experimentation included determining the smallest micropipette tip size that would allow passage of the macrophages without clumping and blockage. The most effective size was 15-20µm. Throughout microinjection experimentation several difficulties were encountered including the micropipette tip being blocked by Matrigel, organoid rupture during microinjection, and extraction of the micropipette disrupting the Matrigel dome and causing damage to the organoids. As a result, the rate of successful macrophage microinjection was approximately one in every ten attempts.

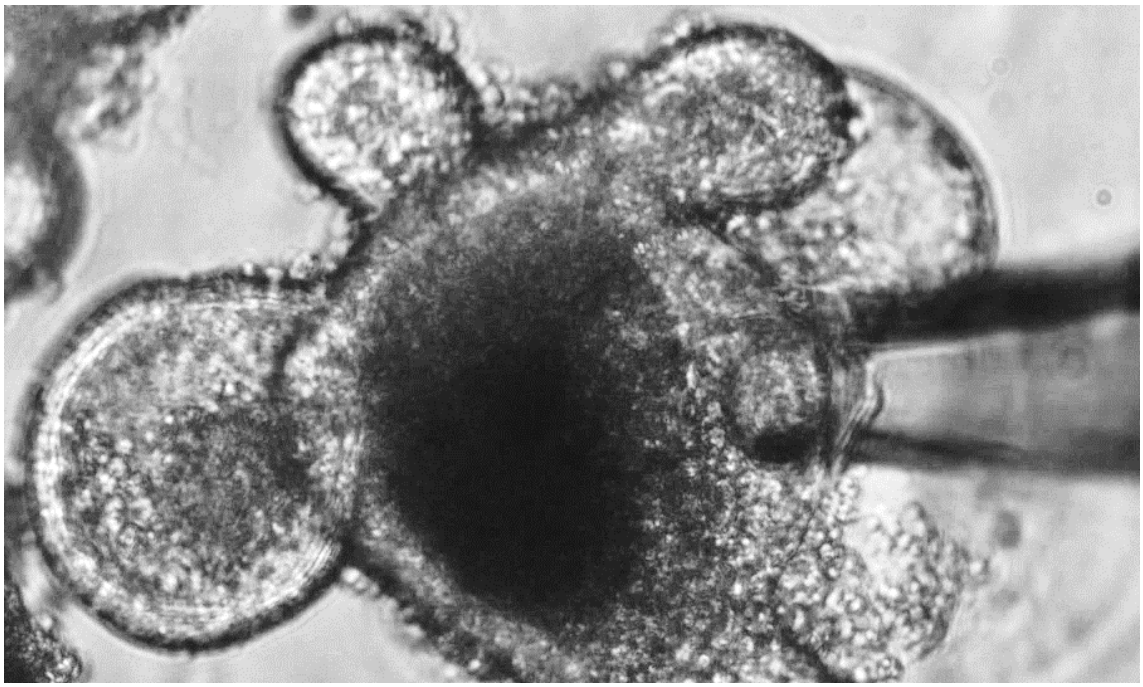

Figure 4.2 Microinjection of a Murine Organoid

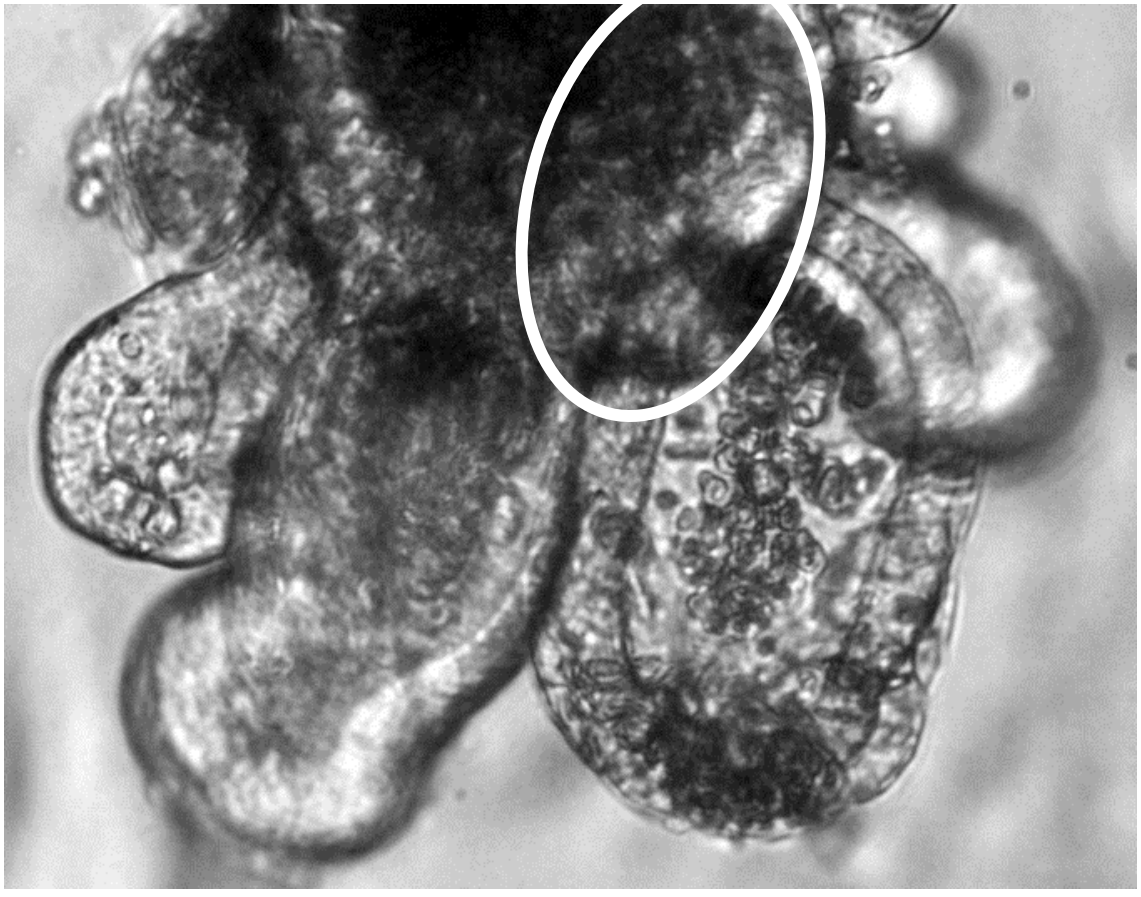

Figure 4.3 Organoid Containing Microinjected Macrophages

Organoid grown from murine small intestinal tissue, 10 days after isolation and grown under tissue culture conditions. Note cluster of macrophages (circled) injected during preliminary development of *in vitro* model of infection.

Incubation with Foods

The experimental plan was to expose organoids to foods that patients with IBD associate with symptom onset or exacerbation (identified in chapter three). The planned method is as follows.
The selected foods will be prepared following the *in vitro* digestion protocol (detailed in chapter five), and the supernatant will be frozen until required. The culture medium will be removed from mature organoids, the domes washed twice with DMEM F-12, and 500µl of supernatant pre-warmed to 37°C will be placed in each well. After 60 minutes incubation, the supernatant will be removed, the domes will be washed twice with DMEM F-12, and the domes and organoids viewed using confocal microscopy.

Infection Model – experimental plan

The planned method for the continuation of macrophage microinjection for infecting organoids with MAP is as follows.
Organoids will then be cultured from small intestinal biopsy tissue samples. The tissue will be obtained from consenting human patients over the age of 16 years, scheduled to undergo investigative intestinal biopsy or colonoscopy, or patients with confirmed CD scheduled to undergo an intestinal surgical procedure, at the Gastroenterology Department of MidCentral Health Palmerston North. Exclusion criteria; patients positive for blood-borne diseases such as HIV or Hepatitis. Once established, the organoids cultured from human intestinal tissue will be infected with macrophages containing MAP. This process, and the effects of MAP infection on cell function and barrier integrity, will be visualised using live cell imaging.

As research progressed, a change in circumstances led to re-evaluation of the study. After taking this into consideration, alongside the difficulties already encountered, the research was halted.

CONCLUSION

The methodology for isolating intestinal crypts and culturing murine intestinal organoids was established, and confocal microscopy was utilised to visualise and capture the three-dimensional organoid structure and individual epithelial cells throughout the crypts. These provide the necessary foundation for advancing organoid culture and the development of the live cell imaging protocol. Further, an *in vitro* model of organoid infection was developed that has the potential to be tailored and exploited for the investigation of infection with other bacteria.

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CHAPTER FIVE

DIET AND THE EPITHELIAL BARRIER: IMPLICATIONS FOR CROHN'S DISEASE

A compromise in epithelial barrier integrity due to paracellular tight junction disruption, and a subsequent increase in intestinal permeability and entry of luminal antigens into the lamina propria, is implicated in Crohn's disease. This study used the *in vitro* digestion protocol developed in a collaborative study (appendix 3), with an established model of intestinal epithelium, to explore the effect of foods reported to trigger or exacerbate intestinal symptoms in CD on the epithelial barrier.

An advantage of the Caco-2 cell model is the detection of changes in monolayer integrity as indicated by trans-epithelial electrical resistance, a measure of tight junction permeability. Additionally, microscopy techniques allow visualisation of the three primary proteins that comprise the paracellular tight junction complex. The culture conditions can also be modified to explore the effect of environmental agents, such as increasing vitamin D availability which is suggested to mitigate intestinal barrier damage.

As discussed in chapter four, investigation of the effects of foods on murine intestinal organoids was discontinued. The Caco-2 model was employed instead, and whether vitamin D can protect cells from dietary instigated damage was also determined.

ABSTRACT

Background: Approximately 50% of patients with Crohn's disease (CD) associate the triggering or exacerbation of their gastrointestinal symptoms with certain foods. Dietary changes have been shown to influence relapse rates, symptom severity, and inflammatory marker levels, however there is an absence of research on the potential mechanisms involved.

Aims: To identify foods reported to trigger or exacerbate intestinal symptoms in CD and to investigate the effect of a selection of these foods, after *in vitro* digestion, on the epithelial barrier using human epithelial colorectal adenocarcinoma (Caco-2) cells. Furthermore, to determine if vitamin D protects Caco-2 cells from any dietary instigated damage.

Methods: Literature published from 1970 to 2016 was comprehensively reviewed to identify foods reported by patients with CD as problematic relative to their symptoms. Five of the ten most frequently reported foods, and a saline control, underwent *in vitro* digestion, followed by digestive enzyme inactivation and collection of the supernatant. Vitamin D was added to the culture medium of half of the cell wells 24 hours prior to incubation with the supernatants. Confluent Caco-2 cells were incubated with the supernatants for 60 minutes, and monolayer permeability was measured at three time points over the following 24 hours.

Results: The ten most frequently reported foods to trigger or exacerbate CD symptoms include milk, non-milk dairy products, corn, spicy/hot food or curry, nuts or peanuts, eggs, wheat, tomato, onions, and citrus. Incubation with the supernatant of the five selected foods (whole milk, corn, curry, egg, and raw unsalted peanuts) caused a significant increase in Caco-2 cell monolayer permeability, indicative of damage to the tight junctions. Treatment with vitamin D significantly reduced the decrease in monolayer permeability in response to incubation with *in vitro* digested curry supernatant.

Conclusions: In patients with CD, certain foods may be capable of triggering or intensifying gastrointestinal symptoms by disrupting or damaging the tight junctions, while vitamin D supplementation may have a protective effect against dietary induced impairment of the epithelial barrier. Further research is needed to establish the mechanisms responsible for the association between diet and CD symptoms.

INTRODUCTION

Crohn's disease (CD), one of two forms of inflammatory bowel disease (IBD), is an incurable chronic inflammatory condition of the gastrointestinal (GI) tract. The cause of IBD is unknown, although more than 200 genomic risk loci have been identified [1]. It is thought that disease manifestation is not due to genetics alone, rather an interaction that occurs between genetic susceptibility, immune system dysregulation, and exposure to environmental risk factors [2]. A wide range of environmental risk factors have been implicated, particularly in CD, including smoking, antibiotic use, pathogenic organisms, and low vitamin D [3]. Disease onset can occur at any age although the peak age is 20 to 30 years, and a smaller peak at 50 years [4]. The condition is characterised by a relapsing and remitting course and treatment typically involves one or more

of immunosuppressive therapies, anti-inflammatory medications, and surgery [5]. The cause of these intermittent periods of heightened disease activity, or flare-ups, is not currently known.

One possibility is the consumption of certain foods or food groups. This theory is often contested by health professionals owing to the absence of robust evidence [6], yet it is difficult to dismiss given 33-67% of patients associate certain foods with symptom triggering or exacerbation [7–13] and that abdominal pain and diarrhoea are the symptoms most frequently experienced [14]. The efficacy of exclusive enteral nutrition (EEN) for inducing remission is perhaps the most supportive evidence. When taken as the sole energy source, the liquid formulation of carbohydrates, proteins and fats [15] induces remission in 20 to 84% in patients with CD and is comparable to corticosteroid therapy [16]. However, little understanding has been gained from the success of EEN due to uncertainty of the mechanisms involved [17]. Available evidence supports microbiome changes with a subsequent reduction in pro-inflammatory bacteria, decreased inflammation by upregulation of anti-inflammatory cytokines and down-regulation of inflammatory cytokines, alterations in microbial metabolites such as short chain fatty acids, and the absence of potentially detrimental dietary components [18,19]. Further, differences in the fat content (high or low) or protein form (whole or amino acid based) of EN does not affect its efficacy [16]. The partial success of research driven exclusion diets has provided some supportive evidence, usually by demonstrating improvements to markers of inflammation, relapse-rate, or disease activity index. While such findings are useful, the scope of evaluated foods is often limited and subsequent research to explore the underlying mechanisms is lacking. Studies that have drawn on patient experience have implicated individual foods and food groups, as well as cooking methods or additives, yet follow-up research is similarly absent.

An alternative for investigating the potential association between diet and the triggering or exacerbation of CD symptoms is the use of an *in vitro* model of the intestinal barrier. Comprising a monolayer of human epithelial colonic adenocarcinoma (Caco-2) cells, the Caco-2 cell model has been extensively used as an *in vitro* model for absorption studies [20]. When grown to confluence under standard culture conditions, Caco-2 cells exhibit a number of properties analogous with the human small intestinal epithelium including a brush border, tight junctions (TJ), and cell polarity [21]. Located between adjacent cells, TJs play an essential role in maintaining the intestinal barrier by selectively regulating paracellular transport of water, ions, and solutes [22]. The TJ structure comprises transmembrane proteins including claudins, tricellulin, and junctional adhesion molecules (JAM), as well as cytosolic proteins, zonula occludens (ZOs) [23]. Disruption of the TJ complex can increase intestinal permeability, a phenomenon associated with CD [24]. Changes in permeability can be determined by measuring the trans-epithelial electrical resistance (TEER) of the monolayer [25], making the Caco-2 model suitable for investigating if certain foods can potentially disrupt the intestinal barrier *in vivo*. Additionally, experimental conditions can be manipulated to simulate alternate conditions. Vitamin D is of interest due to the growing body of research linking low levels with CD, along with an expanding understanding of the key role vitamin D and its receptor, VDR, in intestinal barrier integrity, immunomodulation, and anti-inflammatory pathways [26,27]. The purpose of this study was to investigate the effect of foods reported to trigger or exacerbate symptoms in

patients with CD, after *in vitro* digestion, on the epithelial barrier using the Caco-2 cell model, and to determine if vitamin D mitigates any deleterious effects.

MATERIALS AND METHODS

Literature Search

Literature published from 1970 to 2016 on scientific databases 'Web of Knowledge' and 'Scopus' were searched to identify individual foods reported by patients with CD to trigger or exacerbate CD symptoms. The keywords used were "Crohn's", "IBD", "diet", "nutrition", and "food". Accepted methods of identification included elimination/exclusion diet and reintroduction, questionnaire, and positive serum IgG antibody test.

Materials

Enzymes, chemicals, vitamin D (cholecalciferol), and cell stains were purchased from Sigma Aldrich (St. Louis, MO, USA). Tissue cell culture medium reagents were obtained from Invitrogen (CA, USA). Human epithelial colorectal adenocarcinoma (Caco-2) cells were sourced from American Type Culture Collection (Manassas, USA), cultured in T75 flasks (Nunc, Rochester, NY), and plated on ThinCert chambers (polyethylene terephthalate (PET) capillary pore membranes) (Greiner Bio-One, Germany). Measurement of trans-epithelial electrical resistance (TEER) was made using an EVOM manual TEER meter and STX2 electrode (World Precision Instruments, Sarasota, FL, USA).

Sample Preparation

The three raw foods (unsalted peanuts, egg, and corn) were prepared in keeping with the state in which they are typically consumed. Specifically, the peanuts were de-husked; the egg was boiled for ten minutes, and the shell removed; and the corn husk was removed, the cob boiled for five minutes, and the corn kernels removed. The digestion model established by Glahn et al. [28] was modified to produce a final sample dilution of 1:5 as observed in the duodenum [29–31]. 50g samples of raw unsalted peanut, boiled egg, boiled corn, whole milk, and curry were blended for 30 to 120 seconds, then for a further 30-60 seconds with 24ml of saline until homogenised.

In Vitro Digestion

The gastric phase of digestion was simulated by adding 1M HCl in small increments to duplicate 10ml samples of each food and a saline control until pH2 was reached. Pepsin (activated in 0.1M HCl) was added to each sample to achieve a concentration of 25 mg/ml, followed by incubation on a rocking platform shaker for 60 minutes at 37^{o}C.

For the intestinal phase 1M $NaHCO_3$ was added to each sample in small increments until pH6 was reached. 500µl pancreatin and bile extract solution (activated in 0.1M $NaHCO_3$) was added to samples to

achieve concentrations of 9.3 and 55.5 mg/ml, respectively, followed by further incubation on a rocking platform shaker for 120 minutes at 37^{o}C.

At the completion of digestion, the samples were rapidly chilled and adjusted to pH2 with 1M HCl. After five minutes the samples were adjusted to pH8 with 1M NaOH to inactivate any remaining enzymes. A final adjustment of samples to pH7 with 1M HCl was made. Osmolarity was then corrected to 280-300mOsm with a 1.4M NaCl 50mmol KCl solution and saline was added to bring the sample dilution to 2:5 (sample volume 25 ml). Digested samples were then centrifuged at 1000rpm for five minutes and the supernatant collected.

Cell Culture

Human epithelial colorectal adenocarcinoma (Caco-2) cells were thawed at passage 28 and cultured in T75 flasks in 15mL of cell culture medium at 37°C with 5% CO_2 and 90% humidity. Culture medium contained Dulbecco's modified Eagle's minimal essential medium, fetal bovine serum (FBS) (10%), Penicillin-Streptomycin-Neomycin (1%), non-essential amino acids (1%), and Glutamax (1%), and was adjusted to pH 7.4. Cells were used between passage 32 and 35 and seeded in a 12 well plate at a density of 60,000 cells/cm^2 on ThinCert chambers. The medium was replaced every alternate day for 21 days post-seeding. Confluence was achieved for all cell monolayers as indicated by TEER of > 500Ωcm^2 (after subtraction of the membrane resistance) [32].

Vitamin D Treatment

Vitamin D was solubilised in ethanol, then diluted in cell culture medium to a final concentration of 75nmol/L. Vitamin D supplementation was randomly allocated to 52 of the 104 wells, and cells were treated with vitamin D supplemented medium for 24 hours, a period of time shown to induce gene expression of intestinal tight junction proteins [33,34]. Cell culture medium was replaced 24 hours prior to incubation with supernatants for vitamin D supplemented and non-supplemented wells.

Cell Incubation

The supernatants were randomly allocated to two wells in each of the nine plates and evenly distributed between vitamin D supplemented and non-supplemented wells. The apical membrane culture medium was removed and replaced with 250µl of fresh medium and 250µl of supernatant. After 60 minutes the supernatants were removed, the apical and basolateral reservoirs washed twice with culture medium, and fresh culture medium added. Duplicate TEER measurements were taken immediately prior to initial culture medium removal (baseline), immediately after supernatant removal and culture medium wash and replacement (time 0), and at six and 24 hours post supernatant removal and culture medium replacement.

Immunocytochemical Staining Protocol: Occludin and ZO-1

After cell treatment, culture medium was removed from the chambers and replaced three times with phosphate buffered saline (PBS) to remove traces of protein from the FBS. The cells were then fixed in 2% paraformaldehyde (in PBS) for 30 minutes at room temperature (RT), then washed again three times with PBS and stored at 4°C.

Prior to staining the transwells were removed from each chamber, the filters cut from each transwell, then incubated in 95% ethanol for 30 minutes at 4°C, followed by three minutes at RT in acetone (stored at -20°C). The filters were then brought to RT and permeabilised for 15 minutes with 2mls of 0.2% Triton (in PBS), then washed three times with PBS. This medium was removed and replaced with filtered (0.22µ) blocking buffer (BB) containing 2% normal goat serum in PBS. The filters were left in BB for 60 minutes and all subsequent media were prepared in BB to minimise non-specific binding of antibodies. Immediately prior to staining, the transwell filters were laid on coverslips in a 60mm dish (see *Figure 5.1*), so that 100µl Ab solution was sufficient for each treatment.

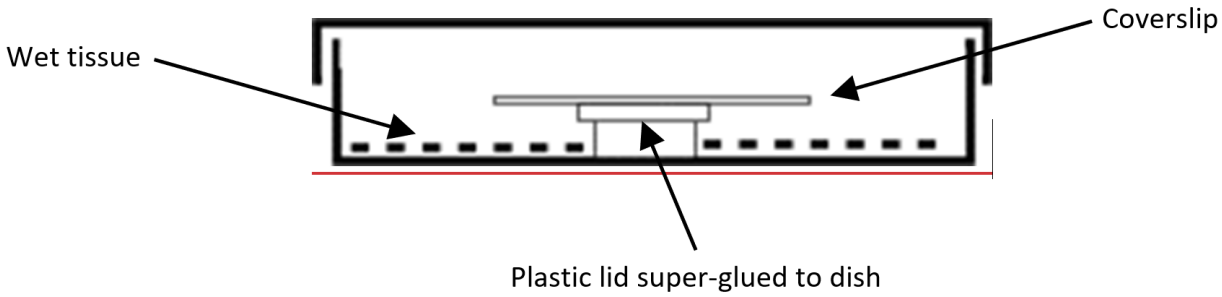

Figure 5.1 Staining Platform

Staining Order

1. Mouse anti-occludin (33-1500, Clone OC-3F10), 2.5µg/ml for 60 minutes at RT, wash 3x with BB
2. Biotin-XX goat anti-mouse (B-2763, (H+L)), for 60 minutes at RT, wash 3x with BB
3. Rabbit anti-ZO-1 (40-2300, N-terminal), 2.5µg/ml for 60 minutes at RT, wash 3x with BB
4. Alexa Fluor 488 goat anti-rabbit IgG (A-11034, (H+L), highly cross-adsorbed), 10µg/mL for 60 minutes at RT, wash 3x with BB
5. Streptavidin Alexa Fluor 546 conjugate (S11225), 10µg/mL for 60 minutes at RT, wash 3x with BB
6. Hoechst (33258), 1µM for 20 minutes
7. Filters were mounted onto microscope slides using the anti-fading agent Prolong Gold and covered with coverslips to maintain a flat surface.

All cell imaging reagents were purchased from Thermofisher Scientific New Zealand.

Microscopy

Fluorescence microscopy was performed using a Nikon TE2000 inverted microscope equipped with a Nikon C1 confocal scanhead, multi-wavelength laser (405nm, 488nm and 546nm; Coherent Scientific) and Z-series stepping motor. This enabled the three fluorescent dyes to be visualised simultaneously and distinguished in the same Caco-2 monolayers with a 60x CFI Plan Apochromat VC water immersion objective (Nikon). The EZ-C1 software enabled the three fluorescent dyes and the corresponding brightfield images to be captured and superimposed, and also Z-series confocal stacks to be acquired to provide a 3-dimesional fluorescent image. NIH ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018) was used for final image processing and Adobe Photoshop for image presentation.

Statistical Analysis

Correlations between TEER and supernatant, time and vitamin D supplementation were analysed using ANOVA test (SAS 9.4, North Carolina, USA).

RESULTS

Literature Search

The ten foods most frequently reported by patients with CD to trigger or exacerbate their symptoms include milk [7–13,35–43], non-milk dairy products [8–13,35,37–41,43], corn [11,35–38,41–45], spicy/hot food or curry [8,9,11–13,36–39,41,44], nuts or peanuts [8,10,11,13,37,38,41–43], eggs [35,37,42,43], wheat [35,41–43], tomato [9,35,37,41,43], onion [7,11,37,39,43,45], and citrus [7–9,13,37,42,43].

Effect of Supernatant on TEER

Incubation with the *in vitro* digested supernatants for 60 minutes, followed by washing and medium replacement (baseline and time 0), caused a decrease in all TEER values, although the control supernatant led to a significantly smaller decrease compared to all other supernatants *(p*<0.001).

The largest single TEER decrease was observed during the incubation period in response to curry supernatant (-35.5%) and was significantly greater than the TEER decrease of monolayers incubated with corn *(p*=0.003) and milk *(p*=0.031). Curry treated monolayers subsequently underwent an extended recovery period as indicated by an increase in TEER at time 0 to time 6 (13.4%), and time 6 to time 24 (10.6%), which were both significantly greater *(p*<0.001) than any other increases observed at either time point.

Incubation with milk or peanut caused a decrease in TEER at baseline, followed by a period of recovery at time 0 to time 6. Continued TEER recovery occurred at time 6 to time 24 for peanut treated monolayers, while a second TEER decrease was seen for milk treated monolayers. The effect of incubation with corn or egg supernatant resulted in a TEER decrease at all three time points.

Table 5.1 Effect of *In Vitro* Digested Foods on Caco-2 Monolayer Trans-epithelial Electrical Resistance (TEER)

	Change in TEER (%)			
Supernatant	**Baseline to Time 0**	**Time 0 to Time 6**	**Time 6 to Time 24**	**Total**
Control (saline)	-11.5	-0.5[b]	-3.1[b]	-15.1
Corn (fresh, boiled)	-24.8[*b]	-1.6[b]	-4.4[b]	-29.8[*b]
Curry (traditional)	-35.5[*a]	+13.4[a]	+10.6[a]	-19.4[a]
Egg (boiled)	-28.0[*]	-0.9[b]	-1.4[b]	-29.7[*b]
Milk (whole)	-26.6[*b]	+0.7[b]	-6.6[b]	-31.5[*b]
Peanut (raw unsalted)	-27.7[*]	+1.2[b]	+0.4[b]	-27.5[*]

* = significantly different from control, dissimilar letters = significantly different from one another

Effect of Time on TEER

The change in TEER for monolayers treated with curry supernatant was significantly different between each timepoint; baseline to time 0 compared to time 0 to time 6 *(p*=0.007), and time 0 to time 6 compared to time 0 to time 24 *(p*=0.016). No significant differences were observed between timepoints for any other supernatant.

Effect of Vitamin D on TEER

The mean TEER decrease of vitamin D supplemented monolayers was significantly lower than non-supplemented monolayers following incubation with curry (24.1% v 30.2%, *p*=0.029). The TEER decrease following incubation with corn, peanut and the control supernatant were also lower in vitamin D treated monolayers, although the differences were not statistically significant.

Table 5.2 Effect of *In Vitro* Digested Foods on Vitamin D Supplemented and Unsupplemented Caco-2 Monolayer Trans-epithelial Electrical Resistance (TEER)

	Change in TEER (%) over 24 hours		
Supernatant	**+ Vitamin D**	**- Vitamin D**	***p* value**
Control (saline)	-11.7	-14.3	0.249
Corn (fresh, boiled)	-25.3	-28.4	0.093
Curry (traditional)	-24.1	-30.2	**0.029***
Egg (boiled)	-28.9	-28.8	0.956
Milk (whole)	-28.4	-27.9	0.829
Peanut (raw unsalted)	-24.9	-29.6	0.105

* = significantly different TEER change between vitamin D + and Vitamin D - Caco-2 monolayers

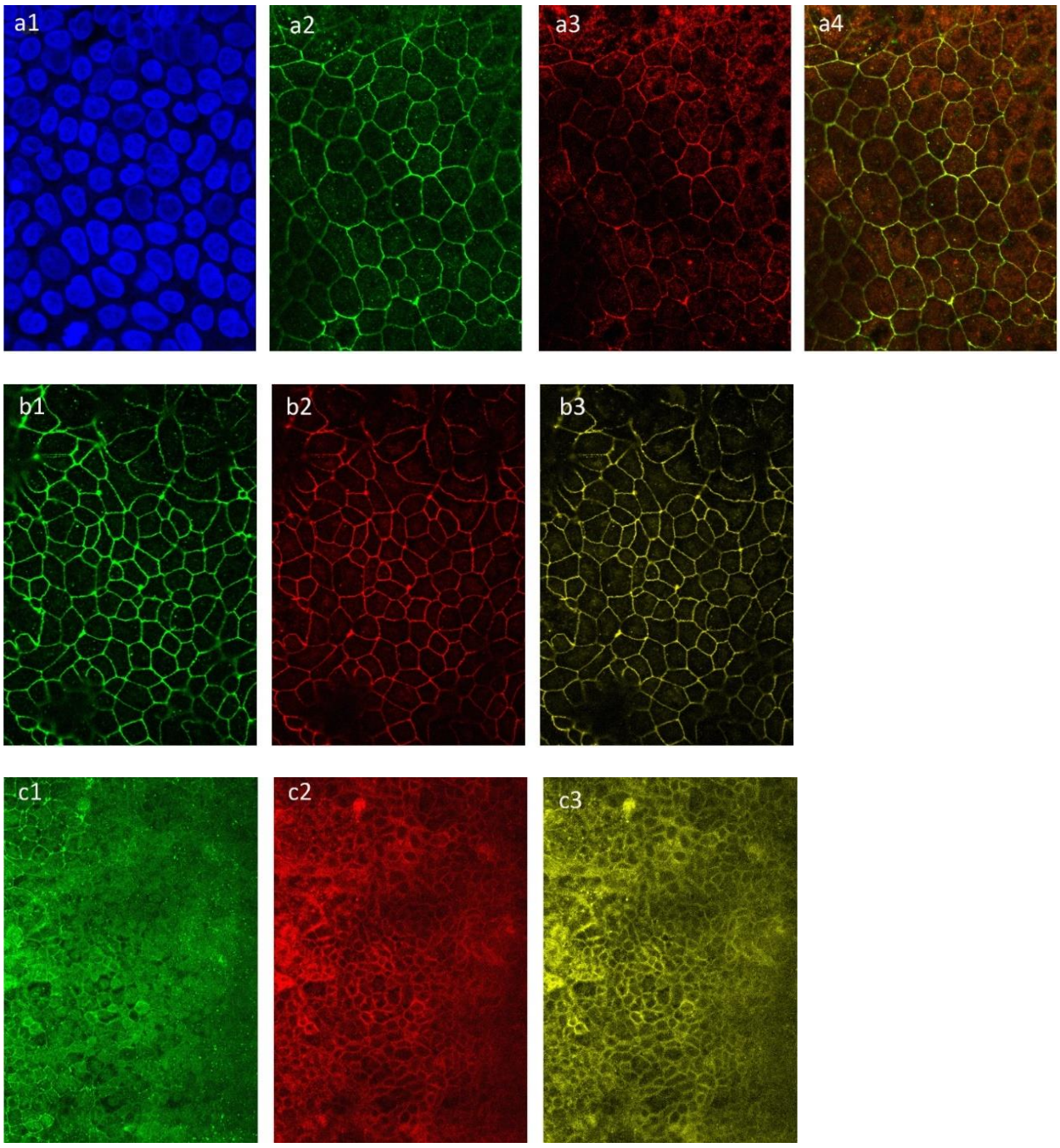

Figure 5.2 Hoechst, ZO-1, and occludin immunocytochemical staining of Caco-2 monolayers after supernatant exposure

a. Saline control supernatant exposed Caco-2 monolayers. **a1.** Cell nuclei (405nm excitation). **a2.** TJ protein ZO-1 (488nm excitation). **a3.** TJ protein occludin (546nm laser line). **a4.** 24-hours post incubation with control supernatant, colocalisation of both TJ proteins appear yellow when the green and red fluorescence staining images are overlayed.

b. Raw unsalted peanut supernatant exposed Caco-2 monolayers. **b1.** TJ protein ZO-1. **b2.** TJ protein occludin. **b3.** ZO-1 and occludin remain colocalised 24-hours post incubation with peanut supernatant.

c. Whole milk supernatant exposed Caco-2 monolayers. **c1-3.** This supernatant caused a decrease in resolution of confocal images, presumably reflecting the non-specific binding of fluorescent probes to remaining milk components covering the cell monolayers. Maintenance of colocalisation could not be demonstrated.

DISCUSSION

Incubation with the supernatant of all five *in vitro* digested foods led to a decrease in Caco-2 monolayer TEER, reflecting TJ disruption and a reduction in monolayer integrity. These effects on increasing monolayer permeability were most probably due to an increased permeability of TJs but, as shown in *figure 1b* in the peanut treated monolayer, is a more subtle effect than complete TJ breakdown. Agents which do cause such a breakdown are known to cause a re-distribution of TJ proteins, a loss of colocalization, and therefore a near complete loss of TEER. At each time point the effect of corn, egg, milk, and peanut supernatant on TEER were comparable, while the effect of curry differed significantly from two or more foods. The change in TEER induced by curry was most pronounced immediately after the incubation period (baseline to time 0), decreasing by more than one third. As existing evidence concerning CD symptoms and foods strongly implicates hot/spicy food, and associations are with various forms of curry, including vegetarian and non-vegetarian [44], particular spices may be the deleterious component. Supportive evidence comes from Jensen-Jarolim et al.'s [46] research on the effect of spices and their major components on the permeability of human ileocecal adenocarcinoma cells (HCT-8). Application of chilli pepper, cayenne pepper, and paprika each caused a significant TEER decrease. Similarly, capsicum and ginger extract have been shown to cause a significant decrease in Caco-2 TEER [47].

The greatest total decrease in TEER was seen in response to incubation with milk supernatant. While it is difficult to speculate about the components that may be responsible, milk proteins are doubtful based on the reported TEER stabilising effect of β-lactoglobulin and β-casein on Caco-2 cells cultured in serum free medium [48,49]. The fat content however, and a subsequent delay in gastric emptying has been suggested to be the underlying cause. In the study that examined the reported association between CD symptoms and dairy products, symptom worsening was most frequently attributed to dairy products with a high fat content and was not associated with the lactose content or the patients disease activity [40]. The fat content of the whole milk was also a likely cause of the poor resolution of confocal images.

Supplementation with vitamin D prior to incubation had a small protective effect against a corn, peanut or control supernatant induced TEER decrease, and significantly protected against a curry induced TEER decrease. These results are in agreement with other Caco-2 research that suggests a protective effect of vitamin D supplementation against intestinal barrier damage. Kong et al. [33] have demonstrated that a 24-hour treatment with 10^{-8} M $1,25(OH)_2D_3$, the active form of vitamin D, protects against dextran sulfate sodium (DSS) induced disruption to the monolayer and TJs compared to untreated cells. These findings were reproduced by Zhao et al. [50] when treating cells with 10^{-9}, 10^{-8}, or 10^{-7} M $1,25(OH)_2D_3$. Additionally, monolayer permeability was assessed with 10^{-8} M $1,25(OH)_2D_3$ being the most effective concentration for protecting against a DSS induced increase in permeability. In both studies, immunofluorescence staining indicated that the mechanisms responsible were an increase in TJ protein expression, specifically ZO-1, claudin-1, claudin-2, and E-cadherin. Murine models of colitis have also been used to explore the effect of interrupting the actions of vitamin D. Specifically, an absence of the vitamin D receptor is associated with

inhibition of genes involved in regulating the production and signalling of inflammatory cytokine TNF-α [51], as well as faster symptom onset, greater weight loss, and higher mortality [52–54].

Dysfunction of the epithelial barrier is a prominent feature of CD and may be evident as one or more of increased epithelial apoptosis, altered production of antimicrobial peptides, mucus [55], and TJ claudins, [56], and a decreased number of TJ strands and increased number of TJ strand breaks [57]. The ability of all five tested foods to induce a TEER decrease *in vitro* could suggest that a compromised epithelial barrier, as seen in CD, increases susceptibility to diet induced TJ disruption. On the other hand, increased intestinal permeability has been reported in CD patients with active disease [58] and has been linked to inflammatory cytokines such as interferon gamma, interleukin 6, and TNF-α [22], as well as patients with inactive disease [59] due to genetically determined subclinical inflammation [60,61] or functional abnormality [62]. In the event that intestinal permeability is already increased, dietary components may have the potential to exacerbate existing TJ disruption. In either scenario, a consequent increase in intestinal permeability could permit excess translocation of luminal antigens, pathogenic organisms, and toxins into the lamina propria, resulting in the initiation of inflammatory responses by resident and circulating immune cells [55] and a heightened risk of further TJ disruption and associated permeability changes [63].

This study has several limitations. All efforts were made to keep culture conditions consistent, however some differences in Caco-2 monolayer morphology could have been present due to variations in cell seeding density and the degree of confluence between passage [64]. Although TEER is the gold standard for measuring intestinal permeability *in vitro* [65], cell models including the Caco-2 model lack mucus secreting goblet cells and thus are unable to simulate the mucus layer and its protective functions including acting as a barrier to large particles [66]. Lastly, TEER may have been influenced by a small content of active digestive enzymes remaining in the supernatant.

CONCLUSION

Using Caco-2 cells as a model of the intestinal barrier, we have demonstrated that foods associated with symptom triggering or exacerbation in patients with CD can increase monolayer permeability by disrupting tight junction function. If certain foods or food components can effect a transient increase in TJ permeability *in vivo*, even if contingent on pre-existing epithelial barrier dysfunction, this could explain the clear association many patients have between diet and symptoms. Alternatively, IgG antibodies against food antigens have been shown to be significantly elevated in CD patients [67]. The triggering or exacerbation of gastrointestinal symptoms could be attributable to inadvertent intake of food antigens and activation of an inflammatory response equivalent to those associated with a flare-up.

Future research should assess a wider range of foods, including those associated with symptom improvement. Additionally, one of the primary cytokines associated with CD, such as TNF-α, could be utilised in conjunction with a cell model to simulate an inflammatory state and increased TJ permeability.

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CHAPTER SIX

ENVIRONMENTAL FACTORS AND INFLAMMATORY BOWEL DISEASE IN NEW ZEALAND

While Crohn's disease is associated with diet, a number of other environmental factors have been implicated. This study investigated a selection of these environmental factors with a particular focus on increased risk of exposure to *Mycobacterium avium* subspecies *paratuberculosis*, and urban and rural residence.

ABSTRACT

Background and Aim: An increasing incidence of the inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), has been associated with environmental factors. We aimed to investigate the risk of IBD in relation to environmental factors.

Methods: A population-based case-control study was carried out in New Zealand (NZ). A total of 146 participants with CD, 75 with UC, and 105 healthy controls completed a retrospective self-administered questionnaire on exposure to environmental factors.

Results: A major urban birthplace (OR 2.06, 95% CI 1.06-4.05), ≥5 days visiting a farm prior to IBD symptom onset (OR 1.82, 95% 1.01-3.33), and contact with freshwater prior to IBD symptom onset (OR 2.19, 95% CI 1.25-3.84) were significantly associated with CD. In UC, significant associations were seen with a major urban residence during childhood (OR 3.17, 95% CI 1.43-7.25), major urban birthplace (OR 3.78, 95% CI 1.73-8.49), and a small urban residence prior to IBD symptom onset (OR 4.66, 95% CI 1.06-28.3). Drinking and cooking water sourced from a rainwater tank during childhood was protective against CD (OR 0.49, 95% CI 0.24-1.00), and handling dirt or soil prior to IBD symptom onset was protective against UC (OR 0.37, 95% CI 0.15-0.87). A history of smoking was positively associated with CD (OR 2.67, 95% CI 1.19-6.47) and UC (OR 4.85, 95% CI 2.05-12.31).

Conclusion: The results support the importance of early life exposure events and future risk of IBD and are in agreement with the hypothesis that sanitation improvements arising from urbanisation increase the risk of IBD.

INTRODUCTION

The inflammatory bowel diseases (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), are chronic incurable diseases of the gastrointestinal tract that follow a relapsing and remitting course [1]. Symptoms typically include abdominal pain, diarrhoea, and rectal bleeding, although comorbidities affecting the skin, eyes, joints and the biliary tract may also occur [2]. The aetiology of IBD is unknown, though the widely held belief is that it involves the interaction of genetic predisposition, immune system dysregulation, and environmental factors [3].

Advances in genetics research have led to the identification of over 200 risk loci for IBD, many associated with immune function such as intestinal homeostasis and pathogen recognition [4]. This knowledge, in combination with an expanding comprehension of the inflammatory cascade, has facilitated the identification of some disease mechanisms and subsequently the development of targeted therapies [5]. The discovery of environmental factors involved in IBD, and more importantly the pursuit of high-quality evidence that categorically confirms an association with disease risk or onset, has proved more difficult.

Westernisation has long been suggested to play a role in IBD risk owing to higher incidence and prevalence rates seen in developed countries [6]. An increasing incidence of IBD is also being seen in developing countries where the condition was previously rare, such as China and India [7], and in Canada, children of immigrants from less developed countries have been shown to assume an IBD risk similar to non-immigrants [8], which further supports the theory that western lifestyle factors are involved and also implicates industrialisation [9]. Evidence has

demonstrated positive associations with improved sanitation [10] adoption of a western diet [11], and increased antibiotic use [12], while investigation of air pollution has yielded mixed results [13].

Of the many other environmental factors that have been proposed, the most irrefutable evidence exists for the increased risk of CD, and decreased risk of UC, associated with smoking [14]. Appendectomy is associated with a reduced risk of UC [15,16], and exposure to infectious microorganisms, such as *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is speculated to increase the risk of CD [17]. An increased risk of IBD has also been associated with caesarean delivery [18], formula feeding versus breastfeeding [19,20], oral contraceptive use [21], and low plasma vitamin D levels [22].

The highest incidence rates of IBD are reported in Western countries including Canada, Australia, and New Zealand [6,23,24]. New Zealand (NZ) has also documented a dramatic increase in the incidence of paediatric IBD during the previous two decades in the Canterbury region [25]. While such rapid changes in incidence are in part attributable to an increased awareness of IBD, and improvements in access to healthcare and diagnostic technology [9,26], Western society-specific environmental factors are thought to be predominantly responsible. As exposure to certain environmental factors is potentially modifiable, continued research of both new and existing environmental factors implicated in the risk of IBD is of immense importance. The aim of this case-control study was to investigate potential associations between exposure to environmental factors and the risk of IBD in NZ residents.

METHODS

Participants

Participants aged 16 years and above, diagnosed with IBD or healthy controls with no family history of gastrointestinal disorders, responded to advertisements (see appendix 4) placed in gastroenterology clinics and at community IBD support organisations located throughout NZ. A diagnosis of IBD included CD, UC, and IBD-unclassified (IBDU), a diagnosis reserved for colonic-IBD where definitive features of CD and UC are absent [27].

Questionnaire

Participants were asked to complete a self-administered retrospective questionnaire developed expressly for this study concerning demographic data, location of residence, and exposure to environmental factors. Questions concerning residence location and environmental factor exposure pertained to childhood (0-12 years) for all participants, as well as the ten years prior to IBD symptom onset for participants with IBD, and ten years prior to questionnaire completion for controls. Questionnaires were provided in hardcopy or via a secure online survey platform (surveymonkey.com).

Ethics

The protocol for this study was approved by the Massey University Human Ethics Committee: Southern A, Palmerston North, New Zealand (MUHEC Reference 13/58). All subjects were verbally screened and understood that by completing the questionnaire they were implicitly consenting to inclusion in the study.

Statistical Analysis

Statistical analysis was performed using JASP (version 0.16.3) [28]. Associations between categorical demographic variables including gender and family history of IBD, and between risk factors including smoking history and domicile, were assessed by Fisher's exact test with odds ratios and 95% confidence intervals. A P value of < 0.05 was considered significant.

Domicile data were coded into urban and rural categories defined by NZ's official data agency: major urban, ≥100,000 residents, large urban, 30,000–99,999 residents; medium urban, 10,000–29,999 residents; small urban, 1,000–9,999 residents; and rural, <1,000 residents [29]. As some countries consider <10,000 residents to be rural [30], additional analyses were performed whereby domiciles small urban and rural were combined, termed rural-small urban (<10,000 residents). For domicile during childhood and the ten years prior to IBD symptom onset/questionnaire completion, ≥2 differing categories were coded 'mixed'. Locations outside NZ were excluded from analysis. The number of participants with IBDU was insufficient to include in disease subtype comparisons.

RESULTS

Participant Characteristics

Four hundred and sixty-six individuals registered interest in the study. Of the 423 individuals that met the inclusion criteria, 56 declined to participate, 27 did not complete the questionnaire, two withdrew from the study, and 12 had a diagnosis of IBDU. A total of 326 participants completed the questionnaire: 146 with CD, 75 with UC, and 105 controls (*Table 6.1*). The age range of participants with IBD was 16 to 84 years, and the mean age was 40.0±14.9 years for CD and 41.0±14.5 years for UC. Control participant age ranged from 19 to 81 years with a mean of 43.3±14.7 years. No statistically significant differences were observed between IBD and control participants for gender, age, and ethnicity.

Table 6.1 Baseline Characteristics of the 326 Participants

	Control, n=105	CD, n=146	UC, n=75
GENDER *n* (%)			
Female	76 (72.4)	106 (72.6)	51 (68.0)
Male	29 (27.6)	40 (27.4)	24 (32.0)
Mean age, years	43.3 ± 14.7	40.0 ± 14.9	41.0 ± 14.5
ETHNICITY *n* (%)			
European	95 (90.5)	138 (94.5)	68 (90.7)
Māori	5 (4.8)	4 (2.7)	2 (2.7)
Indian	3 (2.9)	2 (1.4)	3 (4.0)
Asian	2 (1.9)	1 (0.7)	2 (2.7)
African		1 (0.7)	

ABBREVIATIONS: CD, Crohn's disease; UC, ulcerative colitis

Risk Factors

Table 6.2 shows the association between environmental factors and risk of CD and UC. Birth factors including birth method (natural, caesarean section), nutrition source from birth to three months (breast milk, formula, cow's milk, mixed), birth country (NZ, outside NZ), or birth island of NZ born participants (North, South) were not found to be associated with CD or UC. When compared to all other domicile categories a birthplace classified as major urban significantly increased the risk of CD (OR 2.06, 95% CI 1.06-4.05, p=.023) and UC (OR 3.78, 95% CI 1.73-8.49, p<.001). No association was seen with small urban, rural, or rural-small urban birthplace.

A major urban residence during childhood was a significant risk factor for UC (OR 3.17, 95% CI 1.43-7.25, p=0.002) but not CD. There was a trend toward small urban childhood residence to be protective against CD (OR 0.49, 95% CI 0.21-1.11, p=0.079), and a rural-small urban residence to be protective against UC (OR 0.46, 95% 0.19-1.07, p=0.059), though neither were statistically significant. Drinking and cooking water sourced from a rainwater tank during childhood significantly decreased the risk of CD (OR 0.49, 95% CI 0.24-1.00, p=0.043). Water sourced from public supply, though not significant, tended to increase UC risk (OR 2.06, 95% CI 0.93-4.80, p=0.073). No associations were observed with wellbore or supplied tank water. The type of milk consumed (pasteurised, unpasteurised, organic, none), ≥5 days visiting a farm or farms, farm animal contact (cattle, sheep, deer, goats, rabbits), dirt/soil handling (gardening, digging, mud slide, hand-washing potatoes, clay-modelling, other), freshwater contact (creeks, waterways, rivers, dams, ponds), and hunting (cattle, sheep, deer, goats, rabbits) during childhood were not associated with CD or UC.

Similar proportions of participants resided outside NZ in the ten years prior to IBD symptom onset, CD 8.8% and UC 10.0%, compared with no controls in the ten years prior to questionnaire completion. Small urban residence was associated with UC risk (OR 4.66, 95% CI 1.06-28.3, p=0.023), no association was seen between residence and CD. There was a trend toward reduced CD risk with drinking and cooking water sourced from a rainwater tank (OR 0.42, 95% CI 0.13-1.2, p=0.091), though this was not statistically significant. No associations were seen between CD or UC and the type of milk consumed in the ten years prior to IBD symptom onset, although only participants with IBD reported drinking unpasteurised milk during this period. ≥5 days visiting a farm or farms in the ten years prior to IBD symptom onset was significantly associated with risk of CD (OR 1.82, 95% 1.01-3.33, p=0.039). Neither farm animal contact or hunting were associated with CD or UC. Handling dirt or soil was significantly protective against UC in the ten years prior to IBD symptom onset (OR 0.37, 95% CI 0.15-0.87, p=0.016), but not CD (OR 0.52, 0.23-1.11, p=0.084), and contact with freshwater was found to be a significant risk factor of CD (OR 2.19, 95% CI 1.25-3.84, p=0.007), but not UC (OR 1.47, 0.71-3.11, p=0.307).

Passive smoke exposure (cigarettes or other form of tobacco) in the ten years before the onset of IBD symptoms, or being a current smoker, were not associated with CD or UC. A history of smoking was positively associated with CD (OR 2.67, 95% CI 1.19-6.47, p=0.014) and UC (OR 4.85, 95% CI 2.05-12.31, p<0.001). When gender was taken into account the association only remained significant for females with UC (OR 5.39, 95% CI 1.91-16.95, p<.001). Oral contraceptive use in the ten years prior to IBD symptom onset was not a risk factor for CD or UC. A family history of IBD was seen in a greater proportion of CD participants compared to UC, 32.6% and 22.6% respectively, though the difference was not statistically significant.

Table 6.2 Environmental Factors and Risk of IBD

				CD vs Control		UC vs Control	
Environmental Factor, *n* (%)	**CD, 146**	**UC, 75**	**Control, 105**	**OR 95% CI**	***p***	**OR 95% CI**	***p***
BIRTH							
Method of birth							
Natural (vaginal)	122 (83.6)	65 (86.7)	95 (90.5)	0.64, 0.21-1.78	ns	0.96, 0.25-4.00	ns
Caesarean section	14 (9.6)	5 (6.7)	7 (6.7)	1.56, 0.56-4.74	ns	1.04, 0.25-4.01	ns
No answer	10 (6.8)	5 (6.7)	3 (2.9)				
Nutrition source (0-3 months)							
Breast milk (solely)	79 (54.1)	37 (49.3)	65 (61.9)	0.66, 0.35-1.25	ns	0.57, 0.27-1.21	ns
Formula (solely)	18 (12.3)	13 (17.3)	10 (9.5)	1.35, 0.56-3.47	ns	2.13, 0.79-5.89	ns
Cow's milk (solely)	5 (3.4)	1 (1.3)	4 (3.8)	0.90, 0.19-4.68	ns	0.36, 0.01-3.72	ns
Mixed	21 (14.4)	10 (13.3)	10 (9.5)	1.62, 0.69-4.09	ns	1.54, 0.54-4.46	ns
No answer	23 (15.8)	14 (18.7)	16 (15.2)				
NZ birth country							
Yes	117 (80.1)	56 (74.7)	77 (73.3)	1.47, 0.77-2.77	ns	1.07, 0.52-2.25	ns
No	29 (19.9)	19 (25.3)	28 (26.7)				
Island of birth							
North	72 (61.5)	41 (73.2)	46 (59.7)	1.08, 0.57-2.02	ns	0.55, 0.24-1.21	ns
South	45 (38.5)	15 (26.8)	31 (40.3)				
Birthplace (NZ)							
Major (City) Urban	51 (43.6)	33 (58.9)	21 (27.3)	**2.06, 1.06-4.05**	**0.023**	**3.78, 1.73-8.49**	**<.001**
Large Urban	34 (29.1)	13 (23.2)	29 (37.7)	1.47, 0.76-2.83	ns	0.50, 0.21-1.15	ns
Medium Urban	6 (5.1)		12 (15.6)	0.30, 0.09-0.90	ns		ns
Small Urban (<10,000)	23 (19.7)	10 (17.9)	15 (19.5)	1.01, 0.46-2.26	ns	0.90, 0.33-2.37	ns
Rural (<1,000)	3 (2.6)						
CHILDHOOD							
NZ Country of residence							
Yes	123 (84.2)	66 (88.0)	80 (76.2)	1.40, 0.69-2.84	ns	1.92, 0.78-5.10	ns
No	23 (15.8)	9 (12.0)	21 (20.0)				
No answer			4 (3.8)				
Residence							
Major (City) Urban	34 (27.6)	28 (42.4)	15 (18.8)	1.69, 0.82-3.63	ns	**3.17, 1.43-7.25**	**0.002**
Large Urban	21 (17.1)	9 (13.6)	14 (17.5)	0.99, 0.44-2.27	ns	0.75, 0.26-2.01	ns
Medium Urban	7 (5.7)		9 (11.3)	0.49, 0.15-1.54	ns		ns
Small Urban (<10,000)	15 (12.2)	11 (16.7)	18 (22.5)	0.49, 0.21-1.11	ns	0.69, 0.27-1.70	ns
Rural (<1,000)	11 (8.9)		6 (7.5)	1.23, 0.40-4.24	ns		ns
Mixed	33 (26.8)	18 (27.3)	18 (22.5)	1.29, 0.64-2.67	ns	1.29, 0.57-2.94	ns
No answer	2 (1.6)						
Drinking + cooking water source							
Mains water supply	113 (77.4)	62 (82.7)	75 (71.4)	1.50, 0.80-2.82	ns	2.06, 0.93-4.80	ns
Tank (rainwater)	19 (13.0)	10 (13.3)	25 (23.8)	**0.49, 0.24-1.00**	**0.043**	0.50, 0.20-1.18	ns
Tank (supplied)	4 (2.7)		2 (1.9)	1.48, 0.21-16.64	ns		
Bore	7 (4.8)	2 (2.7)	3 (2.9)	1.75, 0.39-10.72	ns	0.95, 0.08-8.47	ns
No answer	3 (2.1)	1 (1.3)					

Environmental Factor	CD, 146	UC, 75	Control, 105	CD vs Control	*p*	UC vs Control	*p*
Cow's milk consumed							
Pasteurised	127 (87.0)	62 (82.7)	92 (87.6)	1.06, 0.45-2.44	ns	0.73, 0.29-1.88	ns
Un-pasteurised	13 (8.9)	9 (12.0)	10 (9.5)	0.94, 0.36-2.51	ns	1.31, 0.45-3.82	ns
Organic	1 (0.7)	1 (1.3)	1 (1.0)	0.73, 0.01-57.61	ns	1.42, 0.02-112.79	ns
None	3 (2.1)	2 (2.7)	2 (1.9)	1.10, 0.12-13.34	ns	1.43, 0.10-20.11	ns
No answer	2 (1.4)	1 (1.3)					
≥5 days on any farm/s							
Yes	67 (45.9)	38 (50.7)	47 (44.8)	1.07, 0.63-1.84	ns	1.34, 0.71-2.55	ns
No	77 (52.7)	35 (46.7)	58 (55.2)				
No answer	2 (1.4)	2 (2.7)					
Farm animal contact							
Yes	102 (69.9)	45 (60.0)	71 (67.6)	1.14, 0.64-2.02	ns	0.74, 0.38-1.45	ns
No	43 (29.5)	29 (38.7)	34 (32.4)				
No answer	1 (0.7)	1 (1.3)					
Dirt/soil handling							
Yes	130 (89.0)	65 (86.7)	95 (90.5)	0.91, 0.35-2.28	ns	0.76, 0.26-2.25	ns
No	15 (10.3)	9 (12.0)	10 (9.5)				
No answer	1 (0.7)	1 (1.3)					
Time spent in freshwater							
Yes	109 (74.7)	56 (74.7)	84 (80.0)	0.76, 0.39-1.45	ns	0.82, 0.38-1.82	ns
No	36 (24.7)	17 (22.7)	21 (20.0)				
No answer	1 (0.7)	2 (2.7)					
Hunting							
Yes	32 (21.9)	12 (16.0)	25 (23.8)	0.91, 0.48-1.73	ns	0.64, 0.27-1.45	ns
No	113 (77.4)	60 (80.0)	80 (76.2)				
No answer	1 (0.7)	3 (4.0)					
10 YEARS PRIOR TO IBD SYMPTOM ONSET							
NZ country of residence							
Yes	124 (84.9)	63 (84.0)	103 (98.1)				
No	12 (8.2)	7 (9.3)					
No answer	10 (6.8)	5 (6.7)	2 (1.9)				
Residence							
Major (City) Urban	51 (41.1)	27 (42.9)	38 (36.9)	0.88, 0.49-1.56	ns	1.22, 0.61-2.44	ns
Large Urban	26 (21.0)	8 (12.7)	17 (16.5)	1.29, 0.63-2.73	ns	0.71, 0.25-1.89	ns
Medium Urban	5 (4.0)	1 (1.6)	10 (9.7)	0.38, 0.10-1.27	ns	0.15, 0.00-1.08	ns
Small Urban (<10,000)	8 (6.5)	8 (12.7)	3 (2.9)	2.22, 0.52-13.4	ns	**4.66, 1.06-28.3**	**0.023**
Rural (<1,000)	8 (6.5)	1 (1.6)	8 (7.8)	0.79, 0.25-2.53	ns	0.19, 0.00-1.45	ns
Mixed	26 (21.0)	18 (28.6)	24 (23.3)	0.84, 0.43-1.66	ns	1.27, 0.58-2.74	ns
No answer			3 (2.9)				
Drinking + cooking water source							
Mains water supply	123 (84.2)	65 (86.7)	86 (81.9)	1.81, 0.82-4.05	ns	1.79, 0.70-5.03	ns
Tank (rainwater)	7 (4.8)	6 (8.0)	12 (11.4)	0.42, 0.13-1.20	ns	0.70, 0.20-2.13	ns
Tank (supplied)	2 (1.4)		5 (4.8)	0.30, 0.03-1.85	ns		
Bore	6 (4.1)	2 (2.7)	2 (1.9)	2.33, 0.41-24.11	ns	0.45, 0.10-20.40	ns
No answer	8 (5.5)	2 (2.7)					

Environmental Factor	CD, 146	UC, 75	Control, 105	CD vs Control	*p*	UC vs Control	*p*
Cow's milk consumed							
Pasteurised	131 (89.7)	67 (89.3)	99 (94.3)	0.61, 0.18-1.80	ns	0.58, 0.15-2.12	ns
Un-pasteurised	2 (1.4)	4 (5.3)					
Organic	2 (1.4)		1 (1.0)	1.46, 0.08-87.20	ns		
None	9 (6.2)	3 (4.0)	5 (4.8)	1.33, 0.39-5.22	ns	0.85, 0.13-4.51	ns
No answer	2 (1.4)	1 (1.3)					
≥5 days on any farm/s							
Yes	54 (37.0)	25 (33.3)	26 (24.8)	**1.82, 1.01-3.33**	**0.039**	1.53, 0.75-3.10	ns
No	89 (61.0)	49 (65.3)	78 (74.3)				
No answer	3 (2.1)	1 (1.3)	1 (1.0)				
Farm animal contact							
Yes	68 (46.6)	35 (46.7)	56 (53.3)	0.78, 0.46-1.34	ns	0.81, 0.42-1.53	ns
No	76 (52.1)	38 (50.7)	49 (46.7)				
No answer	2 (1.4)	2 (2.7)					
Dirt/soil handling							
Yes	116 (79.5)	54 (72.0)	93 (88.6)	0.52, 0.23-1.11	ns	**0.37, 0.15-0.87**	**0.016**
No	29 (19.9)	19 (25.3)	12 (11.4)				
No answer	1 (0.7)	2 (2.7)					
Time spent in freshwater							
Yes	85 (58.2)	34 (45.3)	57 (54.3)	**2.19, 1.25-3.84**	**0.007**	1.47, 0.71-3.11	ns
No	32 (21.9)	19 (25.3)	47 (44.8)				
No answer	29 (19.9)	22 (29.3)	1 (1.0)				
Hunting							
Yes	25 (17.1)	10 (13.3)	11 (10.5)	1.78, 0.80-4.21	ns	1.33, 0.48-3.69	ns
No	120 (82.8)	64 (85.3)	94 (89.5)				
No answer	1 (0.7)	1 (1.3)					
Smoking history							
Current smoker	12 (8.2)	1 (1.3)	3 (2.9)	3.06, 0.80-17.31	ns	0.47, 0.01-6.04	ns
Former smoker	30 (20.5)	25 (33.3)	10 (9.5)	**2.67, 1.19-6.47**	**0.014**	**4.85, 2.05-12.31**	**<.001**
Current and/or former	42 (28.8)	26 (34.7)	13 (12.4)	**2.87, 1.41-6.22**	**0.002**	**3.80, 1.71-8.84**	**<.001**
Never	103 (70.5)	48 (64.0)	92 (87.6)				
No answer	1 (0.7)	1 (1.3)					
Passive smoking history							
Yes	62 (58.5)	27 (55.1)	50 (54.3)	1.18, 0.64-2.17	ns	1.11, 0.51-2.41	ns
No	43 (40.6)	20 (42.6)	41 (44.6)				
No answer	1 (0.9)	2 (4.1)	1 (1.1)				
Oral contraceptive use							
Yes	60 (56.6)	27 (52.9)	35 (46.1)	1.59, 0.84-3.03	ns	1.43, 0.66-3.15	ns
No	44 (41.5)	22 (43.1)	41 (53.9)				
No answer	2 (1.9)	2 (3.9)					
Family history of IBD							
Yes	47 (32.2)	17 (22.7)		1.56[a], 0.79-3.20[b]	ns		ns
No	97 (66.4)	55 (73.3)					
No answer	2 (1.4)	3 (4.0)					

ABBREVIATIONS: CD, Crohn's disease; UC, ulcerative colitis; OR, odds ratio; CI, confidence interval; NZ, New Zealand; * statistically significant difference $p<0.05$; [a] and [b] = CD vs UC

DISCUSSION

Improvements to hygiene and sanitation as a result of urbanisation are strongly implicated in the aetiology of IBD and many other conditions that involve an aberrant immune response [31]. The observations that have led to this implication are largely thought to be explained by the hygiene hypothesis that surmises prenatal or early childhood infection may protect against allergic disease, while infection in older childhood may confer additional protection [32]. A variation of this concept, the 'old friends hypothesis', proposes that protection stems from exposure to harmless microorganisms [33]. In either scenario, the reduced abundance and biodiversity of microorganisms found in urban environments may be inadequate to prime the immune system against pathogens that are encountered [34].

In the present study, the small number of participants born in a rural location prohibited data analysis at this level. Analysis of sub-urban categories demonstrated a significant association between the risk of CD and UC with a major urban (≥100,000 residents) birthplace. These findings are in keeping with observations from other studies. In Canada, analysis of four decades of data from the University of Manitoba IBD Epidemiology Database found an urban birthplace to be a risk factor CD and UC [35]. The same association was confirmed in a larger birth cohort study comprising data collected in Alberta, Manitoba and Ontario [36]. An urban birthplace was also identified as a risk factor for CD in an Australian birth cohort study [37] and an Indian case-control study [38]. It is well documented that the particulars of events that occur at or around the time of birth; including birth method, maternal antibiotic use, and first feedings; greatly influence microbial exposure and subsequent colonisation of the neonatal gut [39]. Together, these findings could suggest that birth associated events have a rural or urban element that contributes to their influence on immune system establishment.

Several case–control studies have observed a higher risk of IBD [40], CD [41], and CD and UC [42–44] with urban residence during childhood, while others have reported no association [45,46]. In this study, growing up in a major urban NZ location was associated with a significantly increased risk of UC, but not CD. The absence of an association with CD is surprising given that two meta-analyses of urban and rural exposure and subsequent risk of IBD have reported a stronger association for CD than UC [30,47]. The reason for this observation is unknown, although, instances of location data comprising urban and rural categories were coded 'mixed'. This may have inadvertently resulted in underrepresentation of rural or urban exposure. An unanticipated result was the significantly higher risk of UC associated with small urban residence in the ten years prior to IBD symptom onset. It is difficult to explain this result, but considering the geographical location it might be due to a specific factor such as commute time to and from larger urban areas and related air pollution exposure, which has been linked to IBD [48].

The role of infectious microorganisms in IBD is controversial, particularly the notion that the aetiology of CD may involve MAP, the causative agent of Johne's disease in ruminants [49]. New Zealand has an increasing incidence of MAP infection among livestock [50] and a high prevalence with estimates of infection in 42% of beef cattle herds, 46% of deer herds, and 76% of sheep flocks [51]. In conjunction with the established link between MAP and CD, it was hypothesised that in a country with widespread livestock farming and a

high prevalence of both MAP and CD, that increased contact with potentially infected farm animals, the farms they reside, contaminated water, hunting, or consumption of unpasteurised milk [52] would be positively associated with CD. We instead did not observe any association with farm animal contact or hunting during childhood or in the ten years prior to IBD symptom onset. Similarly, Gearry et al. [43] did not observe any associations with farm animal contact in NZ IBD patients. These results differ from other studies that have reported a significantly reduced risk of IBD associated with regular farm animal contact during the first year of life [53] or living on a livestock farm from birth to five years [44]. The study was also unable to demonstrate an association between CD or UC and consumption of unpasteurised milk at either time point. Although, a small number of participants with CD or UC reported drinking unpasteurised milk in the ten years prior to IBD symptom onset, compared to no reports by controls. Few studies appear to have investigated this possible link and the limited findings are conflicting. While one study found that patients with CD or UC drank similar quantities of unpasteurised milk during childhood, as well as significantly less unpasteurised milk compared to controls [54], another found that during childhood and adolescence patients with CD consumed more unpasteurised milk than controls [55]. We did observe significant associations between risk of CD and both contact with freshwater and ≥5 days visiting a farm or farms in the ten years prior to IBD symptom onset. These findings support our hypothesis, although rather than MAP exposure, they could be explained by exposure to other microorganisms such as enteric bacteria [56] that may trigger the onset of IBD in individuals with impaired immunoregulation [57] or in genetically predisposed individuals [58].

An interesting finding was the negative association between risk of CD and drinking and cooking water sourced from a rainwater tank during childhood. This observation may be explained by the hygiene hypothesis on account of roof-collected rainwater potentially having a higher count of pathogenic microorganisms than treated public supply or supplied tank water, facilitating greater priming of the immune system. The potential for contamination of roof-collected rainwater is evident in a five-year NZ wide study that found low compliance with contamination prevention methods including cleaning, filtration, and sterilisation treatment [59]. Of the few published studies that have investigated sources of drinking water and risk of IBD, only one is comparable to the current study. In the French study by Baron et al. [60], tap water was protective against paediatric CD compared to bottled water or well water. Although the water source associated with protection is different, the authors also speculate that the microorganism profile is responsible for the protective effect. The occurrence of gastroenteritis in childhood has been found to be protective against CD and UC [42] and is potentially another explanation for this finding. Although the current study did not query childhood incidence of gastroenteritis, and this theory is purely speculative, it may be plausible on account of two earlier roof-collected rainwater studies that reported unacceptably high levels of coliform contamination in over 50% of samples from 685 NZ dwellings, as well as the presence of bacteria known to cause gastroenteritis such as *E.coli* [59,61].

In accordance with Strachan [32] and Rook's [33] hypotheses linking early childhood microorganism exposure to immunoregulation, contact with the abundance of diverse microorganisms found in dirt or soil likely enhances immune system priming. This view is supported by an observed protective effect between

having a vegetable garden during childhood and the risk of CD and UC [43]. The results of the present study did demonstrate a significantly protective effect of handling dirt or soil and UC development, though as this finding was confined to the ten years prior to IBD symptom onset it may instead reflect a decreased risk of IBD associated with healthier lifestyle choices including physical activity, open air and sunlight exposure [62].

Smoking tobacco is a well-established risk factor for CD, yet is protective against the development of UC [14]. In the present study, no association was observed between current smoking and the risk of IBD. This was unsurprising given the low proportion of current smokers. While active smoking elicits opposing effects on CD and UC risk, the risk of disease is greater in former smokers compared to never smokers, particularly UC [63–65]. This association was confirmed by results of the present study with an OR of 2.67 for risk of CD in former smokers, and 4.85 for risk of UC in former smokers. These values suggest a greater risk than seen in other studies, including a meta-analysis of 13 studies that reported a risk of UC in former smokers from 1.37 to 2.34 [66]. In a NZ case-control study, Gearry et al. [43] reported an OR for UC risk of 1.82 in former smokers, and no significant risk of CD. In another NZ case-control study, an OR of 1.94 was determined for CD in active or former smokers compared to never smokers [67]. It is unknown why the risk of CD and UC in the current study differs from other studies. Although, in comparison to earlier studies the number of former smokers may have been disproportionally high due to greater awareness of the health implications associated with smoking and increased pressure and support to quit smoking. The association between UC risk and smoking cessation, and between CD risk and ever smoking, is largely uncontested. However, whether or not passive smoking affects IBD risk though is uncertain. In this study there was no effect of passive smoking on CD or UC risk. These findings are in agreement with a meta-analysis of ten case-control studies that observed no significant association between passive smoke exposure during childhood and risk of CD or UC [68].

With regard to other risk factors associated with IBD, we found no difference between birth by caesarean section and risk of IBD. This is consistent with some [69,70] but not all studies [37,71]. Similarly, we were unable to demonstrate a protective effect of breastfeeding that has been seen for IBD [19,72], or the increased risk of CD associated oral contraceptive use [21,63].

Several limitations should be taken into account when considering the study findings. Retrospective questionnaires are prone to recall bias and missing data, potentially affecting the validity of the findings. The use of recruitment advertisements and consequently greater participation from individuals interested in IBD (self-selection bias) may have influenced the responses provided. Also, the number of recently diagnosed patients and patients with active disease was likely disproportionately high due to advertisement placement in gastroenterology clinics and community IBD support organisations. The urban and rural definitions defined by NZ's official data agency that take into consideration an individual's location of residence and proximity to where they work, shop, access healthcare and recreate [29], however they do not take into account some features that would typically be considered 'rural' such as livestock and waterways which are not uncommon in small urban areas of towns. As controls were unable to be age-matched with IBD patients, we deemed control data pertaining to the ten years prior to questionnaire

completion to be indicative of the ten years prior to IBD symptom onset for participants with IBD. Lastly, environmental factors such as stress or exposure to green space were not investigated, and the study findings may have been limited by the small sample size.

CONCLUSION

Our data suggest that from birth through to childhood, domicile in a large urban environment may be associated with a greater risk of IBD and drinking water from a rainwater tank during childhood may be protective against CD. These findings support the principle of future IBD development being heavily influenced by exposure events during early life. Furthermore, they add to evidence supporting the hygiene hypothesis and highlight possible health implications associated with urbanisation. The observations from the decade prior to IBD symptom development were more difficult to discern and may warrant further investigation.

There have been calls for future research to progress from the investigation of environmental factors to the effect of modifying exposure to environmental factors [9,73]. Given the variability between study outcomes and the importance of early life exposure events, it seems pertinent that future studies should continue to investigate the effect of a wide range of environmental factors while focussing more on disease outcomes, potential in-utero exposures, and the duration of exposure associated with an effect.

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CHAPTER SEVEN

VITAMIN D CONCENTRATIONS IN NEW ZEALANDERS WITH AND WITHOUT INFLAMMATORY BOWEL DISEASE: DO THEY DIFFER?

Low vitamin D status is implicated in gastrointestinal conditions and is linked to both Crohn's disease risk and poorer disease outcomes including greater disease activity. Low vitamin D status has also been reported for patients with ulcerative colitis. This study investigated whether the serum vitamin D levels of patients with Crohn's disease differs from controls and patients with ulcerative colitis. Further, the measurement of serum vitamin D concentration in New Zealand usually necessitates a venepuncture sample. An alternative method of sample collection, the blood spot method, is quick and less invasive. A sub-study involving blood sample by venepuncture was conducted to validate the blood spot method.

PAPER PUBLISHED IN THE "NEW ZEALAND MEDICAL JOURNAL".

Morton H, Pedley KC, Stewart RJC, Coad J. (2020).

Vitamin D concentrations in New Zealanders with and without inflammatory bowel disease: do they differ?

New Zealand Medical Journal 133:1511.

Note. The initial research proposal was a randomised double-blind controlled trial (see appendix 8) to determine supplementation efficacy in participants with inflammatory bowel disease and healthy controls, if vitamin D status correlates with disease activity, and if supplementation improves nutritional status and bone turnover markers and reduces disease activity. Regrettably, adequate funding could not be attained.

ABSTRACT

Background and Aim: Patients with inflammatory bowel disease (IBD), Crohn's disease (CD) or ulcerative colitis (UC), are at risk of low vitamin D owing to reduced absorption, medication-associated sunlight exposure restrictions, and/or increased requirements due to inflammation. This study aimed to determine if the serum vitamin D concentration of New Zealand (NZ) IBD patients relates to disease activity and differs from controls.

Methods: Data concerning demographics, sunlight exposure, vitamin D supplementation and disease activity were collected using a retrospective questionnaire. Serum vitamin D concentrations were measured in dried blood spots and validated against blood samples in a participant sub-group.

Results: Vitamin D concentration was significantly increased by supplementation (82.8 v 66.4nmol/L, $p<0.001$) and sunlight exposure whilst on holiday (75.2 v 63.7nmol/L, $p<0.001$). Patients with CD who reported active disease in the last year had significantly lower vitamin D concentrations (68.6 v 84.6nmol/L, $p=0.008$) than those who reported remaining in remission.

Conclusion: In this cohort of NZ residents, mean vitamin D of patients with IBD was not different from controls. In patients with CD, recent disease activity was significantly associated with lower vitamin D. The use of vitamin D supplementation may have implications for reducing disease activity occurrence in patients with CD.

INTRODUCTION

Vitamin D (25(OH)D or calcidiol) is a fat soluble steroid that plays a major role in bone health through the regulation of serum calcium and phosphate concentrations [1]. While the importance of vitamin D to bone health is well established, clear evidence of this hormone's involvement in immune function has come to light following the discovery of vitamin D receptor expression by a number of immune cell types [2–4], and, similarly, local production of the active vitamin D metabolite calcitriol by select immune cells [5]. Adequate vitamin D concentrations also appear to be important in the prevention of diseases such as cancer, heart disease and immune system mediated diseases [6,7].

Two such diseases are Crohn's disease (CD) and ulcerative colitis (UC), collectively termed inflammatory bowel disease (IBD) [8]. A link between IBD and vitamin D was proposed in response to the geographical distribution of IBD with the greatest incidences observed in regions furthest North and South of the equator [9], namely, New Zealand (NZ) [10], Canada [11], South Australia [12], Iceland [13], and Sweden [14]. This latitudinal trend is mirrored in other risk factors implicated in IBD including greater industrialisation, extent of development, and European ethnicity [9]. Moreover, in regions of these latitudes, the combined effect of a greater solar zenith angle, colder temperatures, and a conscious reduction in sunlight exposure to reduce skin cancer risk [1,15–17], led to significantly lower vitamin D concentrations than those observed closer to the equator.

New Zealand has one of the highest reported rates of IBD worldwide, and unlike many Western countries where the number of new diagnoses has plateaued, the incidence and prevalence continue to

rise [10,18]. In the last two decades researchers have identified a number of genes strongly associated with IBD, especially CD [19]. However, heritability does not account for all instances and the current consensus is that environmental and immune factors are also involved. New Zealand's high IBD incidence, elongated shape, and southern geographical position make it an ideal location to explore the possible relationship between vitamin D levels and IBD. The aim of this study was to determine the vitamin D concentration of NZ patients with IBD and to establish whether they differ from those of controls and relates to disease activity.

METHODS

Questionnaire

Participants aged 16 years and above, diagnosed with IBD, or controls with no family history of gastrointestinal disorders, responded to advertisements placed in gastroenterology clinics and at community IBD support organisations throughout NZ (*Figure 7.1*). Participants were asked to complete a self-administered retrospective questionnaire developed expressly for this study (*see Appendices*), concerning demographic data, residence location, holiday history in the previous 12 months, sunlight exposure habits, time spent outdoors per week, vitamin D testing in the previous 12 months and vitamin D supplementation in the previous 6 months. Residence location was coded by island (North, South), then by latitude (<37.5°S, 37.5-40°S, 40-42.5°S, 42.5-45°S, and >45°S). Sunlight exposure habits comprised sunblock use, protective clothing worn, shade seeking behaviour, sunbathing and sunburn. Patients with IBD were asked additional questions including the date of symptom onset, diagnosis date, family history of IBD, and disease activity experienced in the previous 12 months. Questionnaires were provided in hardcopy or via secure online survey platform (surveymonkey.com).

Sample Collection

Participants residing in or close to 10 major cities and towns throughout the length of NZ were invited to take part in the second part of the study by providing a small blood spot sample for serum vitamin D (25(OH)D) measurement. Exclusion criteria included individuals with a blood borne disease or not living predominantly in NZ in the previous 24 months. To obtain the sample, a finger lance was used and a minimum of three blood spots were collected from the second or third finger of the non-dominant hand.

The blood spot collection cards were air-dried and stored according to the manufacturer's instructions (ZRT Laboratory, Oregon, USA). Samples were collected by the same researcher over two calendar months to reduce potential sampling method disparities and to minimise seasonal differences in vitamin D concentration. Participants consenting to a blood spot sample, and residing in one of two cities, were also invited to take part in a sub-study where a 5ml blood sample was taken by venepuncture to measure serum vitamin D in order to validate the blood spot method.

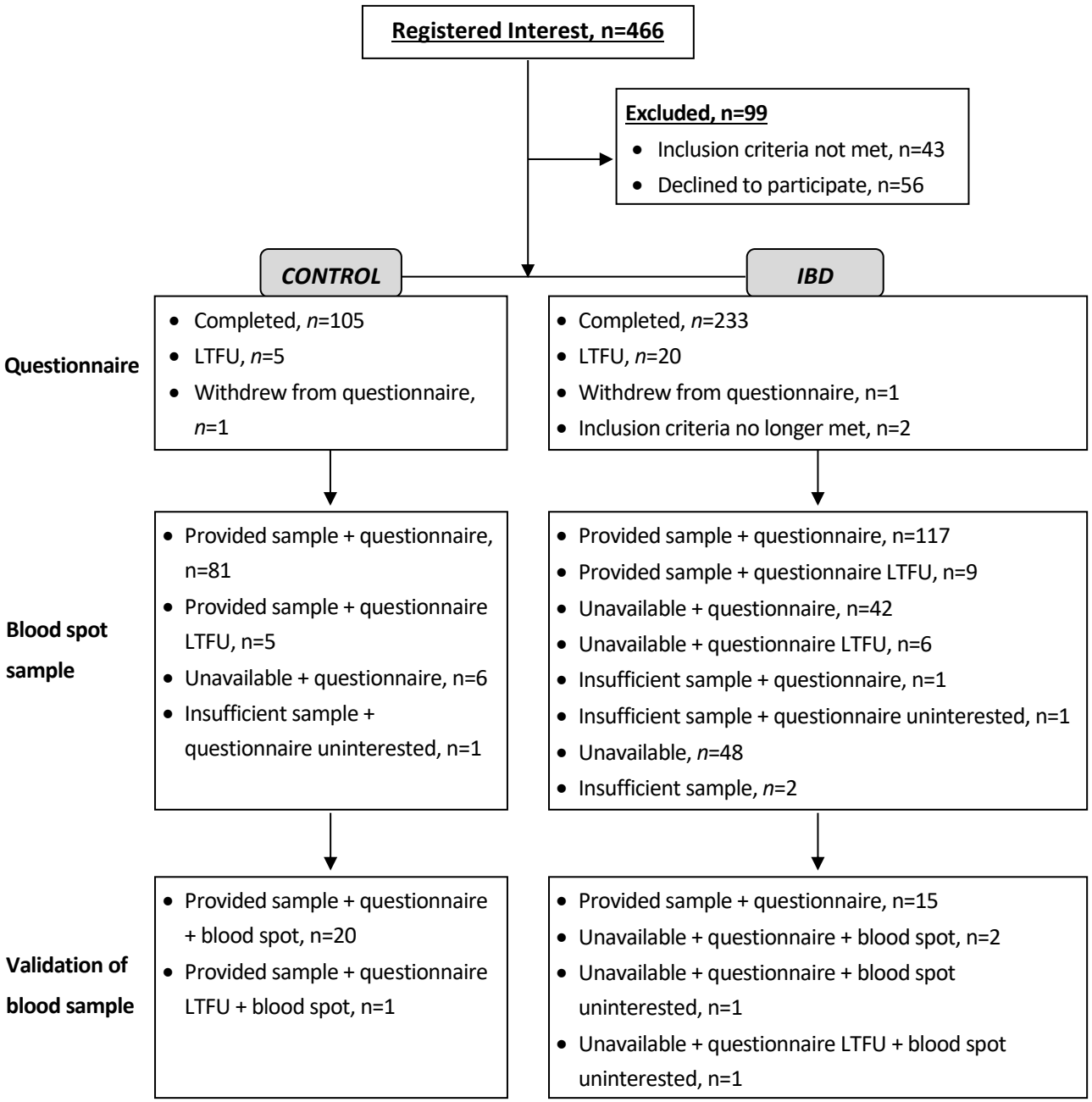

Figure 7.1 Inclusion and Completion of Controls and Patients with Inflammatory Bowel Disease

ABBREVIATIONS: IBD, inflammatory bowel disease; LTFU, loss to follow-up

Blood Spot Validation

Serum vitamin D were measured in dried blood spots using liquid chromatography tandem mass spectrophotometry (LC-MS/MS), and in venous blood samples using high-performance liquid chromatography (HPLC) tandem mass spectrometry (Canterbury Health Laboratories, Christchurch, NZ).

Ethics

The protocol for this study was approved by the Massey University Human Ethics Committee: Southern A, Palmerston North, NZ (MUHEC Reference 13/58). All study participants provided informed consent.

Statistical Analysis

Differences in vitamin D concentration between patients with IBD and controls, and between patients with CD and UC, were analysed using ANOVA test (SAS 9.2, North Carolina, USA). A *p*-value of <0.05 was considered statistically significant for all data analysis.

For analysis of vitamin D status, the ranges recommended by the Working Group of the Australian and New Zealand Bone and Mineral Society, Endocrine Society of Australia and Osteoporosis Australia, were applied; severe deficiency <12.5nmol/L, moderate deficiency 12.5-25nmol/L, mild deficiency 25-50nmol/L, and adequacy >50nmol/L [20]. Pearson's Chi-square test was used to test for association between vitamin D status and participant groups; patients with IBD and controls, and between IBD subgroups (CD and UC) (Minitab 17.3.1, Pennsylvania, USA).

A linear regression model was applied to vitamin D values from venous blood samples and their corresponding blood spot values (Minitab 17.3.1, Pennsylvania, USA). A correction factor was derived from the regression equation (y=10.36+1.1444x) and applied to all blood spot values.

RESULTS

Demographic and Participant Characteristics

A total of 198 participants completed the questionnaire and provided a blood spot sample, 81 controls, 79 patients with CD, 31 with UC, and seven with inflammatory bowel disease unclassified (IBDU) (*Table 7.1*).

Table 7.1 Baseline Characteristics of the 198 Participants

Characteristics	**Control, *n=81***	**IBD, n=117** (CD, UC, IBDU)	**CD, *n=79***	**UC, *n=31***
Mean age, years	42.4 ± 15.1	40.9 ± 14.1	39.0 ± 13.5	42.9 ± 14.5
Female, no. (%)	60, (74.1)	85, (72.6)	57, (72.2)	23, (74.2)
Location, North Island, no. (%)	54, (66.7)	72, (61.5)	49, (62.0)	19, (61.3)
Mean time since diagnosis, years	n/a	9.9 ± 0.9	9.4 ± 0.9	10.9 ± 2.1
Mean vitamin D (25(OH)D), nmol/L	70.1 ± 2.6	69.8 ± 2.1	72.5 ± 2.7	64.9 ± 3.8
<u>Supplementation</u>				
Current, no. (%)	5, (6.2)	22, (18.8)	16, (20.3)	3, (9.7)
In last 6 months, no. (%)	5, (6.2)	10, (8.5)	6, (7.6)	4, (12.9)
None, no. (%)	71, (87.7)	85, (72.6)	57, (72.2)	24, (77.4)
<u>Previous 12 months</u>				
Vitamin D measurement, no. (%)	3, (3.7)	12, (10.3)	6, (7.6)	5, (16.1)
Active disease, no. (%)	n/a	88, (75.2)	60, (75.9)	22, (71.0)

ABBREVIATIONS: CD, Crohn's disease; UC, ulcerative colitis; IBD, inflammatory bowel disease; IBDU, inflammatory bowel disease unclassified

Vitamin D

No significant difference was observed between the mean vitamin D concentration of patients with IBD and controls, or between IBD subgroups (CD and UC). More than three quarters of patients with IBD and controls had vitamin D concentrations considered adequate (>50nmol/L) (*Table 7.2*). Mild vitamin D deficiency (25-50nmol/L) percentages were comparable between patients with IBD and controls, however a greater difference was observed between IBD subgroups. Moderate vitamin D deficiency (12-25nmol/L) was only observed in patients with IBD, and no participants were considered severely vitamin D deficient (<12.5nmol/L). The difference in vitamin D status between patients with IBD and controls, and between IBD subgroups, was not significant.

Table 7.2 Vitamin D status of controls and patients with Inflammatory Bowel Disease

Vitamin D Status	**Control** ***n=81***	**IBD** (CD, UC, IBDU) ***n=117***	**CD** ***n=79***	**UC** ***n=31***
Moderate deficiency[1], no. (%)	0	1, (0.9)	1, (1.3)	0
Mild deficiency[2], no. (%)	15, (18.5)	26, (22.2)	14, (17.7)	10, (32.3)
Adequacy[3], no. (%)	66, (81.5)	90, (76.9)	64, (81.0)	21, (67.7)

[1] 12.5-25nmol/L, [2] 25-50nmol/L, [3] >50nmol/L; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IBDU, inflammatory bowel disease unclassified

Vitamin D concentration was significantly higher in patients with IBD, but not controls, who reported increased sunlight exposure on holiday in the previous 12 months (62.0 v 76.3, p=0.001), current supplementation (63.9 v 86.9, p<0.001) or supplementation within the previous 6 months (63.9 v 81.8, p<0.018). When the effect of increased sunlight exposure on holiday in the previous 12 months was analysed by IBD subgroup, the difference only remained significant in patients with CD (65.0 v 78.4, p=0.011). When the effect of supplementation was analysed by IBD subgroup, the difference associated with current supplementation remained significant in patients with CD (66.8 v 87.9, p<0.001) and in patients with UC (59.7 v 91.5, p=0.018), while the difference associated with previous supplementation only remained significant in patients with CD (66.8 v 85.5, p=0.046) (*Figure 7.2*). No differences were observed between vitamin D concentration and time spent in sunlight per week, sunlight exposure habits, location, or latitude.

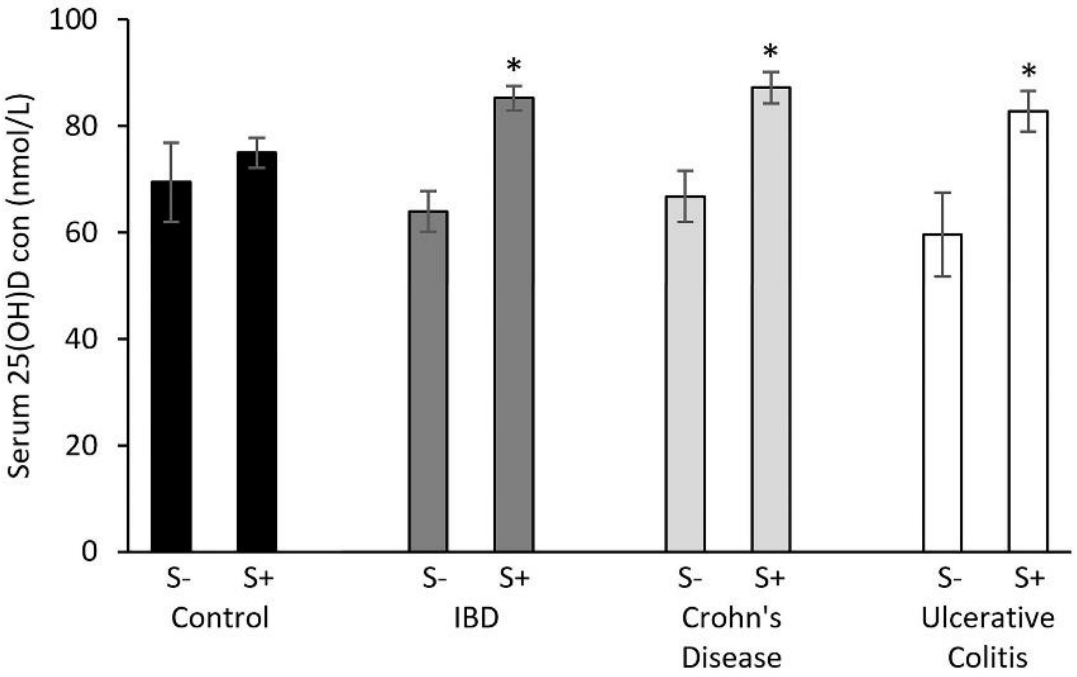

Figure 7.2 Effect of Supplementation (current or previous 6 months) on Serum Vitamin D Concentration by Participant Group

S- no supplementation, S+ supplementation; bars marked with* are significantly *(p*<0.05) different to no supplementation within the same participant group. NB Patients with both CD and UC, or IBDU were not analysed individually due to insufficient numbers, they were however included in IBD cohort analyses.

Disease Activity

A significant difference in vitamin D concentration was determined between patients with IBD that reported disease activity in the previous 12 months and those that reported remaining in remission only when supplementation was taken into account (*Table 7.3*).

When analysed by IBD subgroup, the mean vitamin D concentration remained significantly lower in patients with CD that had reported disease activity in the previous 12 months (68.6 v 84.6, *p*=0.008) compared to those that reported remaining in remission, irrespective of supplementation. There was no difference in patients with UC.

Table 7.3 Difference between Vitamin D (25(OH)D) Concentration and Disease Activity in the previous 12 months by Vitamin D Supplementation

Disease Activity	No supplementation (nmol/L)	Supplementation (nmol/L) (current or previous 6 months)	P value
Active Disease, *n=88*	62.2 ± 2.6	81.8 ± 4.1	0.001
Remission, *n=29*	68.5 ± 4.4	100.5 ± 8.6	<0.001

Blood Spot Method Validation

A subset of participants with and without IBD provided a venous blood sample for vitamin D measurement in addition to their blood spot sample (*n=36*). The mean vitamin D concentration in this subset was 69.9 ± 23.4nmol/L measured in blood spots and 75.0 ± 25.1nmol/L measured in venous blood samples. The blood spot values were lower than their corresponding venous blood sample values and were closely correlated (r^2=0.805) (*Figure 7.3*).

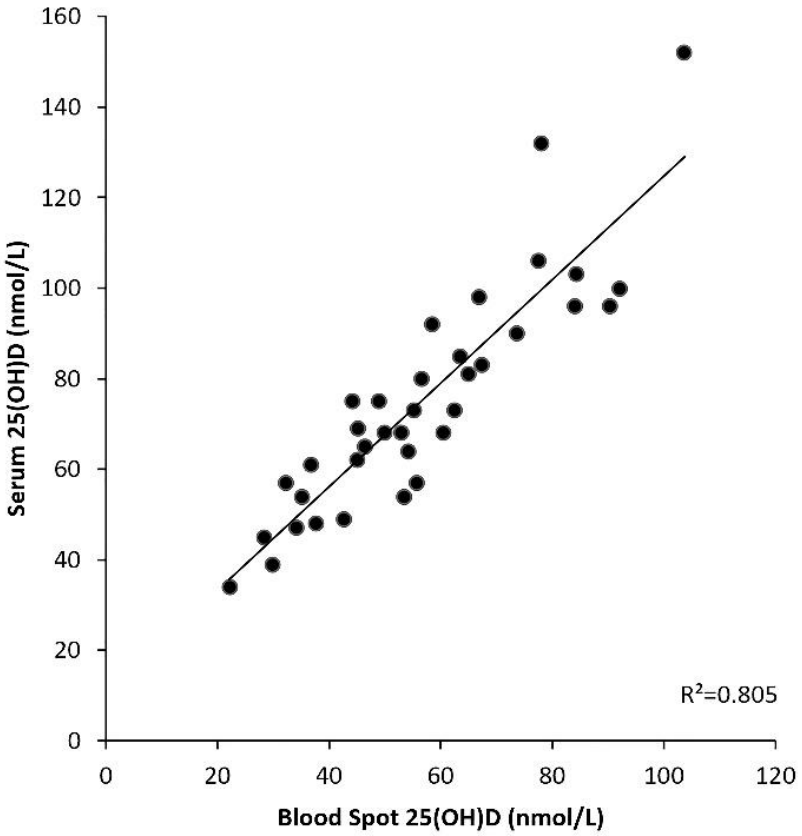

Figure 7.3 Correlation between Vitamin D measure in Blood Spot and Venous Blood Samples

DISCUSSION

The mean vitamin D concentration in this cohort, measured in blood spot samples collected over eight weeks (April-June), was 69.9nmol/L, a level that correlates well with the month standardised national mean of 63.0nmol/L as reported in the New Zealand Adult Nutrition Survey 2008/09 [21]. This value also falls between the mean concentrations reported in two other NZ studies; 53 and 63nmol/L for female and male participants aged 15 years and over, from samples collected nationwide during Autumn (March-May) 1997 [22], and 85nmol/L in South Island participants aged 18 years and over in samples collected during late summer (February) 2005 [23]. Overall, there was no significant difference between the mean vitamin D concentration of controls and patients with IBD, or between IBD subgroups. Accordingly, the percentage of participants considered to have adequate, mildly deficient, or moderately deficient vitamin D concentrations was not significantly different between participant groups or subgroups. As discrepancies exist between vitamin D status terminology and the 25(OH)D parameters associated with each status, it is difficult to compare these

findings with similar research. However, a meta-analysis of observational studies demonstrated that the odds ratio of being vitamin D deficient (≤50nmol/L) compared to controls is 1.63 in CD and 2.28 in UC [24]. In line with this data, a greater proportion of mild vitamin D deficiency was observed in patients with UC than those with CD, 32.3% and 17.7% respectively.

The percentage of patients with IBD who reported taking vitamin D supplementation was three-fold greater than controls, and two-fold greater in patients with CD than in patients with UC. Vitamin D concentrations were significantly higher in patients with IBD, but not controls, who reported supplementation currently or in the previous six months. This is unsurprising given the daily dose of 100-1,000IU reported by the controls, compared to 284-7,143IU reported by patients with IBD. While the total number of individuals reporting supplementation was too small to allow further statistical analysis, based on the variance in supplement use and dose, we might have expected a greater difference between the mean vitamin D concentrations. Comparison of the mean vitamin D concentration in patients with IBD, and in controls, reporting no supplementation revealed no significant difference, suggesting a considerable imbalance between oral intakes and ensuing serum concentration likely attributable to impaired nutrient absorption associated with inflammation, notably fat malabsorption [25,26], or having undergone small intestinal resection [27]. Researchers have also demonstrated impaired absorption of supplemental vitamin D in clinically quiescent patients with CD compared to controls, a 30% reduction across all patients, and 20% in those without a resection, thus another mechanism may be involved [28]. Genetic factors could influence vitamin D concentrations as polymorphisms of the genes that encode the vitamin D binding protein (VDBP) are reported to influence vitamin D concentrations [29], and expression of VDBP genetic variants has been demonstrated to differ between patients with IBD and controls [30].

Once adjusted for supplementation, a significant difference in vitamin D concentration was observed between patients with IBD who reported disease activity in the previous 12 months and those that reported remaining in remission. When analysed by IBD subgroup, the difference remained significant only in patients with CD irrespective of supplementation. This may reflect a stronger association between vitamin D concentration and CD, a relationship proposed in the Nurses' Health Study [31]. Alternatively, the present study may not have been adequately powered to investigate a difference in vitamin D concentration between patients with UC reporting active disease and patients with UC reporting remission. Differences in supplementation could potentially explain this observation as of the patients with UC in remission, 0% reported current supplementation and 25% reported supplementation in the previous six months, compared to 19% and 33% respectively of the equivalent patients with CD.

While the leading source of vitamin D is skin exposure to UV B radiation [5], the only UV exposure factor that had an effect on vitamin D concentration was seen in patients with IBD who reported increased sunlight exposure on holiday in the previous 12 months. Unlike other studies, no effect of latitude on vitamin D concentrations was observed [21,22,32], though a review of global vitamin D status demonstrates this relationship is less marked now than in earlier studies [15]. A discernible effect of latitude may also have been obscured by supplement use and dose. No effect was observed between vitamin D concentration and sunlight exposure score, and patients with IBD and controls produced similar scores. This is surprising as some routinely

prescribed IBD medications are associated with increased skin cancer risk, namely, Azathioprine and Mercaptopurine, thus patients prescribed these medications are advised to limit UV exposure [33]. Closer inspection revealed that while the patients with IBD reported greater sunscreen reapplication compliance and shade seeking, this was counteracted by lower sunhat use and a higher incidence of sunburn.

Finally, in the present study a close correlation was observed between the two measures of serum vitamin D: blood spot assay using LC-MS/MS and venous blood samples using HPLC. In agreement with other work, the vitamin D concentrations measured in blood spot LC-MS/MS assay were lower than their corresponding HPLC assay values [34,35]. This difference may in part be explained by variation between the assay methods, a recognised obstacle that led to the development of the Vitamin D External Quality Assessment Scheme (DEQAS), a scheme formed in 1989 to appraise vitamin D assay reliability [36,37]. It has also been suggested that assay variability may be attributable to either one or a combination of three factors; blood spot volume less than 50µl, differences in blood volume absorption due to variations in filter paper weight, and the location of the punched spot on the paper [38]. While obtaining adequate blood spot volume can be improved by collecting additional spots, the latter two factors may be more difficult to control.

CONCLUSION

Vitamin D concentrations were not different between NZ patients with IBD compared to controls irrespective of marked differences in supplement use and dose. This likely presents a challenge for reaching and maintaining adequate vitamin D concentrations in patients with IBD that have limited UV exposure or are not receiving regular supplementation. In patients with CD, a history of active disease in the previous 12 months was significantly associated with lower vitamin D concentration compared to patients who reported remaining in remission. Although this difference was not observed in patients with UC, vitamin D supplementation is safe, effective and inexpensive [39] and may have implications for reducing disease activity recurrence. Vitamin D supplementation should be recommended to patients with IBD by their healthcare providers, especially to patients with a recent history of active disease and those being treated with sun-sensitising medications.

Limitations

The present study had several limitations. Samples were collected from April through June when vitamin D concentrations are expected to be moderately high following summer and thus do not indicate nadir concentrations. Obtaining this information would have been useful for demonstrating the extent of both vitamin D inadequacy and deficiency in this at-risk population group. A retrospective questionnaire was used to collect data about sunlight exposure, vitamin D supplementation and disease activity. Due to the lack of a suitable validated questionnaire, sunlight exposure habits were allocated a score from 1-5 (almost never/never = 1, almost always/always = 5). The collection of disease activity history over a period of 12 months prevented the use of a validated disease activity index; however, using an index would have been time consuming and may have reduced the number of responses. Lastly, the sample size, particularly the number of patients with UC, may have limited the findings.

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CHAPTER EIGHT

THE INCIDENCE OF INFLAMMATORY BOWEL DISEASE IN NEW ZEALAND REMAINS HIGH, FINDINGS IN THE MANAWATŪ REGION

Recent research conducted in the South Island demonstrates that the incidence of inflammatory bowel disease (IBD) is increasing. Published research describing the incidence in the North Island is now more than 40 years out of date. In collaboration with a local gastroenterologist, and with the contribution of other clinicians in the region, this study determined the incidence of IBD between 2011 and 2015, and the 2013-point prevalence of IBD, for the Manawatū region, a region that to the best of our knowledge has not previously been studied. Additionally, the presenting symptoms and disease phenotype of the incident cohort were described, and the fulfilment of diagnostic criteria was assessed.

PAPER PUBLISHED IN "*DIGESTIVE DISEASE AND SCIENCES*".

Morton, H., Coad, J., Pedley, K.C., Irwin, J.R. (2023).

The Incidence of Inflammatory Bowel Disease in New Zealand remains high, findings in the Manawatū region.

Digestive Disease and Sciences (2023).

ABSTRACT

Background: New Zealand (NZ) has one of the world's highest rates of inflammatory bowel diseases (IBD), however available data are limited to southern, urban regions.

Aims: To determine the incidence and prevalence of IBD in the Manawatū region of NZ.

Methods: Patients in the Manawatū region, with a diagnosis of IBD made between 2011 to 2015 were identified. Demographic, diagnostic and disease data were collected, fulfilment of diagnostic criteria was assessed, and incidence rates were calculated. Comparison of disease phenotype and observed diagnostic criteria was made between diagnosis and 12-months following diagnosis. All resident patients with a diagnosis of IBD current on 5 March 2013 were identified, and prevalence rates were calculated.

Results: The mean annual age-standardised incidence rates of UC, CD, and IBD were 10.2, 17.0, and 27.2 per 100,000. IBD incidence was highest among those of European ethnicity (24.8 per 100,000), followed by Asian (1.4), and Māori (1.1). IBD incidence in the urban population was 34.0 per 100,000 (95% CI 24.1-46.0) compared to the rural population of 5.6 (95% CI 0.4-22.4). The age-standardised point prevalence of UC, CD, and IBD on 5 March 2013 was 157.7, 231.8, and 397.9 per 100,000, respectively.

Conclusions: The incidence and prevalence of IBD in the Manawatū region are comparable to those reported in other Australasian studies. Incidence was lower in Māori, and in the rural population. Follow-up is required to identify any changes in incidence and phenotype, and whether rural residence remains protective.

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD), the two inflammatory bowel diseases (IBD), are chronic inflammatory conditions of the gastrointestinal (GI) tract. Although similarities are seen between the symptoms and clinical features of UC and CD, each condition largely has a distinct presentation. In UC, mucosal inflammation affects the rectum and some or all of the colon in continuity [1], while in CD, inflammation may affect any part of the GI tract, distribution is typically patchy and separated by sections of normal mucosa, and transmural inflammation can give rise to abscesses, strictures, and fistulas [2].

Since the end of the 20th century, IBD incidence rates in Western countries have largely plateaued [3], while rates are rising rapidly in newly industrialised countries [4]. Stable and rising incidence rates, combined with population growth and low mortality in IBD, are causing prevalence rates to rise, increasing demand on already stretched healthcare services. The direct costs associated with IBD are substantial, contributed to by anti-tumour necrosis factor therapies [5] and the introduction of expensive newer monoclonal antibody treatments [6]. Given the significant implications for healthcare provision, there is an ongoing need to identify trends associated with IBD incidence and prevalence.

New Zealand (NZ), a country in the southwestern Pacific Ocean, has one of the highest reported rates of IBD worldwide. This has been well documented in the Canterbury region of the South Island, where between 2004 and 2014, the age-standardised incidence of IBD increased from 24.9 to 39.5 per 100,000 [7], and between 1996 and 2015 the incidence of paediatric IBD increased fourfold [8]. To the best of our knowledge, only two other published studies have examined the incidence of IBD in North Island locations of NZ. The first in 1962 described the incidence of UC in the capital city of Wellington [9], and the second described the incidence of IBD between 1969 and 1978 in Auckland [10]. The Manawatū differs from these previously studied regions. It is an agricultural area of NZ, with 23.5% of the population living in a rural location. It also has a higher proportion of Māori, who make up 20.6% of the population. The deprivation index[1] for the region is 6.6, compared to 5.4 nationally. The aims of this study were to determine the incidence and prevalence of IBD in the Manawatū region of the North Island, a region of NZ not previously studied, and to assess disease features and diagnostic criteria observed at diagnosis and 12-months following diagnosis.

MATERIALS AND METHODS

Study Design

A retrospective analysis was performed to identify all patients within the study area with a new diagnosis of IBD from 1 January 2011 to 31 December 2015. The study also aimed to identify all patients with an existing diagnosis of IBD who were residing in the study area on 5 March 2013.

Study Area and Population

The Manawatū region, and its health service provider MidCentral District Health Board (MidCentral DHB), are located in the lower central North Island of NZ. Dividing the region are the Tararua and Ruahine Ranges, a geographical feature that may result in some patients residing east of the ranges and being managed in neighbouring DHBs. To ensure any such patients were not missed from the cohort, the study area was restricted to the Manawatū region west of the ranges (*Figure 8.1*).

At the 2013 Census the study area population was 145,761 comprising 3.4% of the total NZ population. Ethnicities were European 77.6%, Māori 17.1%, Asian 6.3%, Pacific Peoples 3.8% and Middle Eastern/Latin American/African 0.8%. The proportion of European and Māori were higher than NZ as a whole (74.0% and 14.9%, respectively), and the proportion of Asian, Pacific Peoples, and Middle Eastern/Latin American/African were lower (11.8%, 7.4%, and 1.2%, respectively). Domicile distributions were urban 80.3% and rural 19.7% in the study area population, compared to urban 83.9% and rural 16.1% nationally.

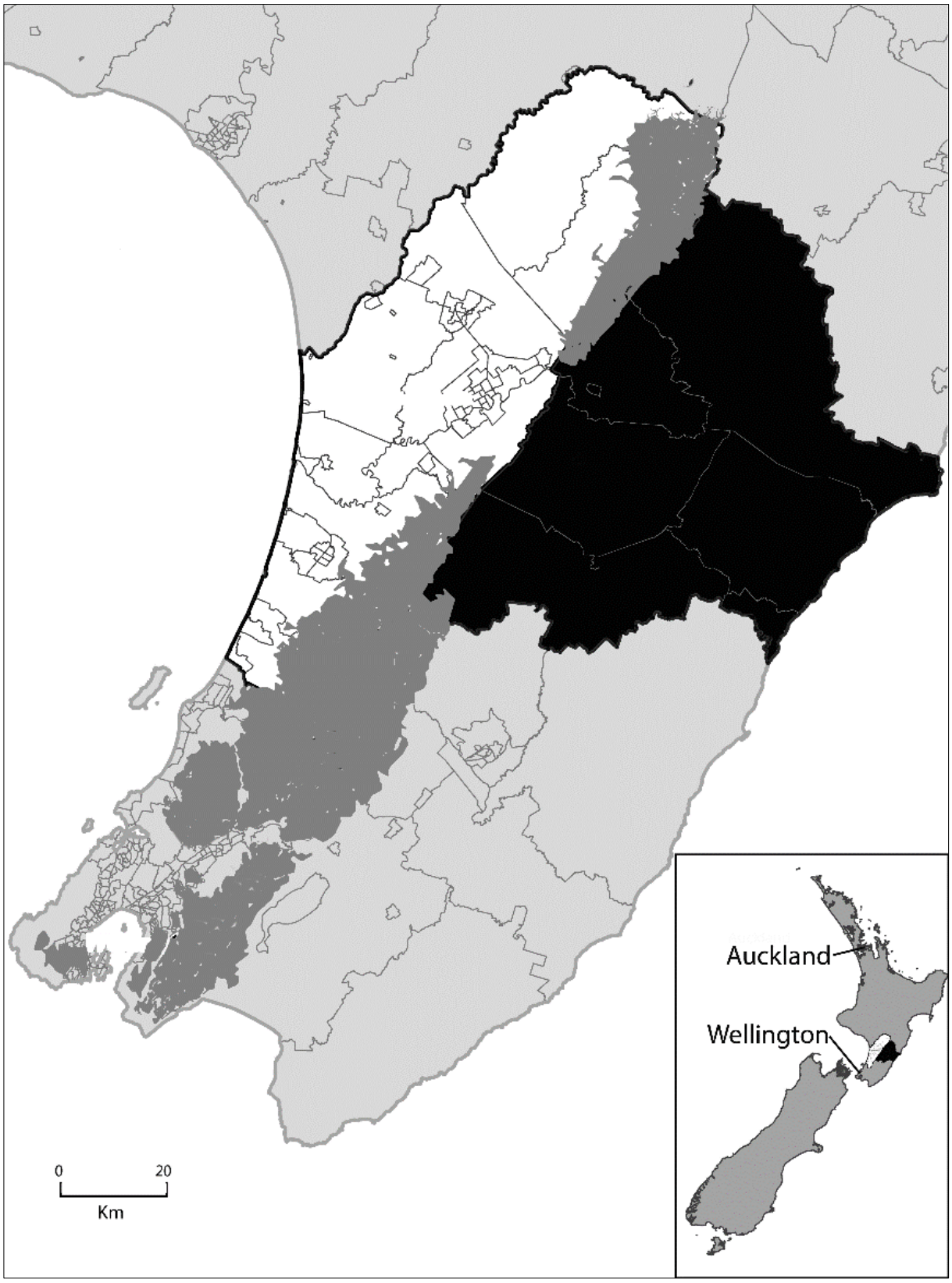

Figure 8.1 The Manawatū region

Study area (white), Tararua and Ruahine Ranges (dark grey), excluded area (black)

Diagnostic Criteria

Diagnostic criteria were based on the Lennard-Jones anatomic criteria for the diagnosis of CD and UC [11] and the European Crohn's and Colitis Organisation (ECCO) consensus diagnostic criteria [1,2]. The criteria to meet a diagnosis of IBD were presence of symptom chronicity, demonstration of chronic inflammation in the GI tract, exclusion of other diagnoses such as infective colitis or diverticular disease, and demonstration of the Lennard-Jones criteria (*Figure 8.2*). Symptom chronicity was defined as abdominal pain, diarrhoea (increased frequency of loose bowel motions), or per rectal (PR) bleeding for at least six weeks. Chronic inflammation was defined as at least one tissue sample of the GI tract showing histologic features of chronic inflammation: crypt architectural distortion, plasma cell infiltrate, lymphocyte infiltrate, or granulomata. In the absence of histological evidence, chronic inflammation was defined as two instances of demonstration of inflammation separated by at least six weeks. Demonstration of inflammation could be made at endoscopy, at surgery, on imaging (computed tomography enterography (CTE), magnetic resonance enterography (MRE)), or by a faecal calprotectin test (>50 μg/g).

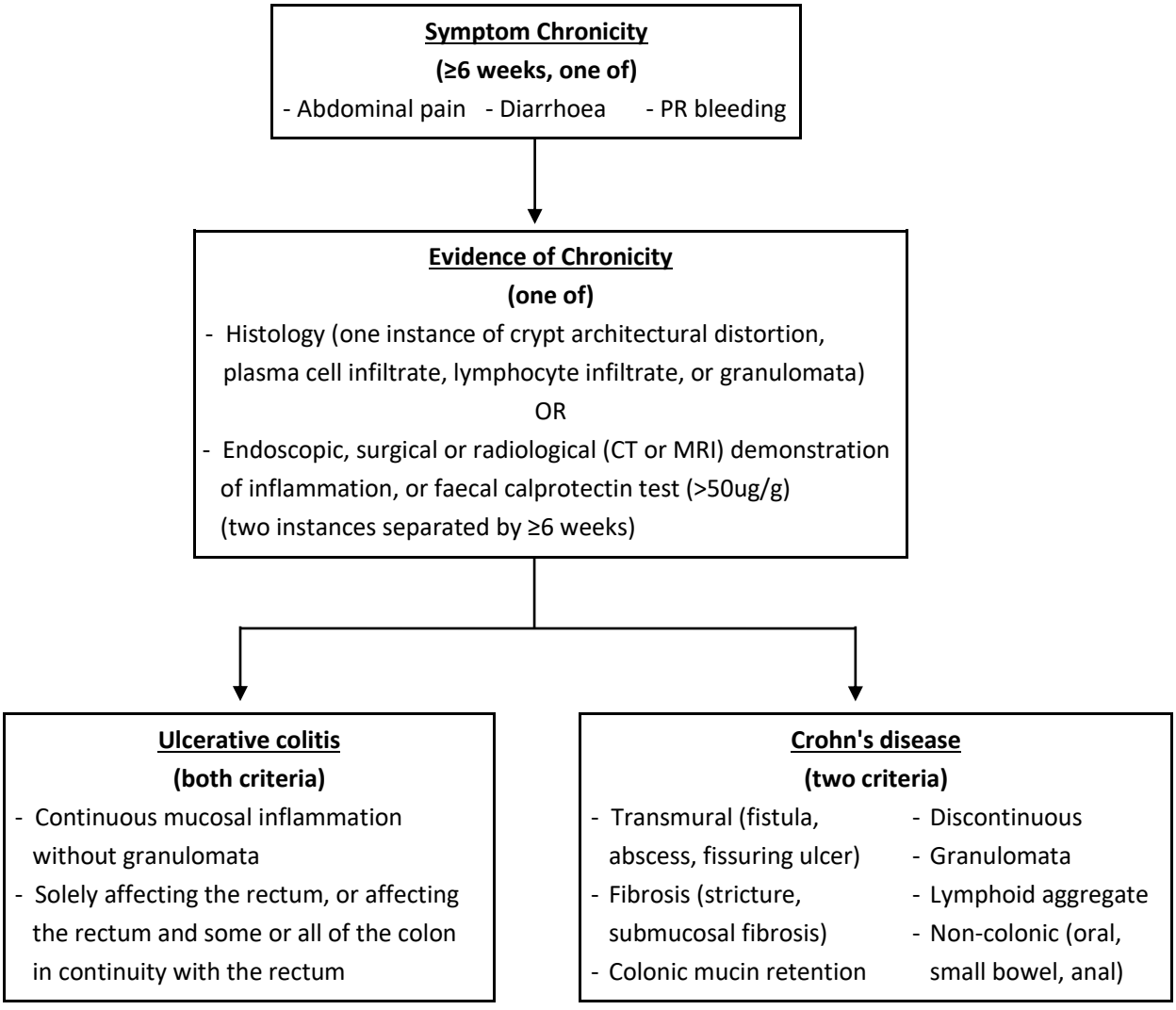

Figure 8.2 Diagnostic Criteria for the Inflammatory Bowel Disease Incident Cohort

The Lennard-Jones criteria for UC were the presence of continuous mucosal inflammation without granulomata, solely affecting the rectum, or affecting the rectum and some or all the colon in continuity with the rectum. The diagnostic criteria for CD were two or more of the following Lennard-Jones criteria: discontinuous distribution of inflammation in the GI tract (including rectal sparing), transmural inflammation (presence of fistula, abscess, or fissuring ulcer), fibrosis (presence of stricture, or of submucosal fibrosis on histological specimen), lymphoid aggregate in GI tissue, crypt architectural distortion in GI tissue, mucin retention in colonic tissue, non-caseating granulomata in tissue from the GI tract, or non-colonic disease location (upper GI tract, anal). Upper GI tract disease location was present if any of the following were present: gastric granulomata, inflammation in the duodenum or jejunum, or gastric ulceration not typical of reflux oesophagitis or peptic ulcer disease. The presence of granulomata was attributed greater diagnostic weight (x2) than other diagnostic criteria, namely fulfilment of two Lennard-Jones criteria while all other features each fulfilled one criterion. A diagnosis of inflammatory bowel disease unclassified (IBDU) was precluded in view of the absence of a comprehensive disease definition and established diagnostic criteria.

Diagnosis Algorithm

An algorithm-determined diagnosis and diagnosis date were derived from data in the six weeks preceding the leading clinician diagnosis date, through to 12 months post-diagnosis. Instances of the minimum diagnostic criteria not being met, disagreement between the algorithmic diagnosis and the diagnosis assigned by the leading clinician, or of disagreement between the date of diagnosis assigned by the algorithm and the date of diagnosis assigned by the leading clinician, were reviewed on a case-by-case basis. Resolution of algorithmic disagreement with leading clinician diagnosis centred on the diagnostic criteria observed.

Inclusion Criteria

Incidence criteria were assignment of a diagnosis of IBD by a gastroenterologist or general surgeon between 1 January 2011 and 31 December 2015 and domicile in the study area at the time of diagnosis. Exclusion criteria were diagnosis of IBD outside the incidence timeframe, domicile outside the study area at the time of diagnosis, incidental finding in an asymptomatic patient, or an alternative diagnosis eg ischaemic colitis. Prevalence criteria were assignment of a diagnosis of IBD prior to 6 March 2013, and domicile in the study area on 5 March 2013. Exclusion criteria were diagnosis of IBD after 5 March 2013, domicile outside the study area on 5 March 2013, or an alternative diagnosis.

Patient Identification

Patients were identified by searching events for IBD keywords within eight MidCentral DHB data repositories, from 1 January 2011 to 31 December 2015. The keywords and their potential misspellings were “inflammatory bowel,” “inflamatory bowel,” “crohn’s,” “crohns,” “chrons,” “chron's,” “ulcerative,” “colitis,” “indeterminate,” “indeterminite,” “ileitis,” “ilitis,” “enteritis,” “fistula,” “adalimumab,” “humira,” “asacol,” “mesalasine,” “mesalazine,” “pentasa,” “pentaza,” “azathioprine,” “azoathioprine,” “azathiaoprine,” “azoathioprine,” “infliximab,” “mercaptopurine,” “methotrexate,” “sulfa*,” and “sulph*”.

Clinic and procedure letters from all public and private gastroenterology clinics and general surgery practices in the Manawatū region were searched solely by keyword, MidCentral DHB endoscopy data were searched by keyword and by diagnosis coding of Crohn's or colitis, public and private radiology data were limited to cross-sectional abdominal and pelvic imaging and were searched by keyword, all public and private histology data were limited to the ileum and colon and searched by SNOMED inflammatory code (Supplementary Material *Table 8.5*), all public and private laboratory data were searched for testing of 6-thioguanine and thiopurine methyltransferase, the Medlab Central region wide prescription database were searched for adalimumab, azathioprine, mercaptopurine, mesalazine, and lastly, hospital admissions were searched for primary and secondary ICD codes for UC, CD, and IBDU (Supplementary Material *Table 8.6*).

Data Collection

Primary demographic data (date of birth, gender, and ethnicity) were collected from the MidCentral DHB patient management database (WebPAS) for all patients with a diagnosis of IBD. This database is routinely updated with Primary Health Organisation Enrolment Collection data: a national collection that holds Primary Healthcare System patient enrolment data.

For incident patients, additional demographic and diagnostic data were collected from clinic letters, procedure reports, and admission records. In the instance that data could not be obtained from electronic records, hard-copy case files were viewed. Additional demographic data comprised family history of IBD, smoking history, and domicile at date of diagnosis. Domicile data were coded in accordance with population counts from the 2013 Census and Statistics NZ classifications: large urban, 30,000–99,999 residents; medium urban, 10,000–29,999 residents; small urban, 1,000–9,999 residents; and rural <1,000 residents.

Diagnostic data comprised the leading clinician IBD diagnosis and date of diagnosis, pre- and 12 months post-diagnosis symptom history: abdominal pain, diarrhoea, PR bleeding, perianal fistula occurrence and atypical symptom occurrence. All IBD related endoscopy, radiology, and surgical procedures in the six weeks preceding diagnosis through to 12 months post-diagnosis were also reviewed and coded. The data collected were procedure date, procedure indication, location of macroscopic inflammation, stenosis presence and location, fistula presence and location, location of biopsies taken, and histological findings. Location of inflammation was coded as one or many of oesophagus, stomach, duodenum, jejunum, proximal ileum, distal ileum (distal 30cm), caecum, right colon (proximal to the splenic flexure), left colon, sigmoid colon, rectum, and anus. The histological findings recorded were architectural distortion, chronic inflammatory cell infiltrate in the lamina propria, crypt abscess (>1), granuloma (>1), deep fissuring ulceration, preservation of goblet cells, transmural or submucosal fibrosis, viral cytopathic inclusions in endothelial cells, helicobacter pylori organisms, low- or high- grade dysplasia, Paneth cell metaplasia, and normal/unremarkable.

A portion of the study data reported involvement of the rectum and left colon without reference to the transverse colon or splenic flexure. Consequently, a clear distinction between left sided and extensive UC sometimes could not be made. In order to prevent an over-estimation of patients with extensive disease, a

conservative approach was taken whereby classification of extensive UC was defined as involvement of the right colon, or of colon proximal to 60cm from the anus.

For all patients identified without a diagnosis of IBD, rudimentary demographic and diagnosis data were collected to prevent repeat identification and processing as different data sources were reviewed. Collected data were entered into a secure purpose-built Microsoft Access database.

Statistical Analysis

Data were analysed using JASP computer software (version 0.16.3.0). Categorical variables, including demographics and disease subtype, were assessed by Pearson's chi-square test and reported as absolute value and percentage. Continuous variables, namely age at diagnosis and duration of disease, were assessed by T test and reported as the mean. Significance was set at $p<0.05$.

Age was standardised using the World Health Organization's World Standard Population Distribution [12]. The incidence 95% confidence intervals (CI) were calculated assuming a Poisson distribution. Incidence and prevalence were defined per 100,000 persons in the study area population. Population data, and urban and rural population parameters, were obtained from Statistics New Zealand. Incident risk ratios (IRR) with 95% CI's were calculated for urban and rural domicile at date of diagnosis. Disease phenotype was described using the Montreal classification [13]. Algorithms to perform keyword searches, process raw data to identify cases, assign a date that minimum diagnostic criteria were met, and assign an algorithmic diagnosis of IBD, were performed in the R statistical computing environment (R Core Team, version 4.1.1).

For incident cases, the Montreal classification, procedure summary (endoscopy, radiology, surgery), and Lennard-Jones criteria, were determined at the time of diagnosis and 12-months post-diagnosis. Procedures at diagnosis were based upon data preceding the diagnosis date through to three days post-diagnosis. The Montreal classification and Lennard-Jones criteria at diagnosis were based upon data preceding the diagnosis date through to 30 days post-diagnosis to allow for possible procedure delays associated with high healthcare service demand. Observations at 12-month post-diagnosis were based upon subsequent data through to 365 days post-diagnosis date.

RESULTS

Annual Incidence of IBD in the Manawatū region

Between 1 January 2011 and 31 December 2015, 207 patients were diagnosed with IBD in the Manawatū region (UC 78, CD 129). The mean annual crude incidence rates per 100,000 were 10.7 (95% CI 6.5-17.6) for UC, 17.7 (95% CI 12.0-26.0) for CD, and 28.4 (95% CI 21.0-38.5) for IBD. Over the five-year study period, the highest annual incidence for IBD was 35.0 (95% CI 26.6-46.0) per 100,000 in 2012, and the lowest was 19.9 (95% CI 13.8-28.6) per 100,000 in 2015. After standardising the data for age, the mean annual incidence rates for UC, CD, and IBD were 10.2, 17.0, and 27.2 per 100,000, respectively.

Demographics of the IBD Incident Cohort

Female IBD incident cases (n=116, 56.0%) were greater than male (n=91, 44.0%), with similar gender distribution for UC (n=44, 56.4%) and CD (n=72, 55.8%). Analysis of gender distribution by age at diagnosis or year of diagnosis revealed some instances of male predominance, although no trend with either variable was observed. Age at diagnosis followed a bimodal distribution for both UC and CD (*Figure 8.3*). The primary peak in age was at 20-29 years, and a secondary peak at 40-49 years. The median age at diagnosis was 43.0 years for UC and 42.5 years for CD. Ethnicity was predominately European (n=181, 87.4%), followed by Asian (n=10, 4.8%), and Māori (n=8, 3.9%). The minority ethnicities were African (n=3, 1.4%), Latin American (n=2, 1.0%), and Pacific Peoples (n=1, 0.5%). Ethnicity data were unavailable for two patients.

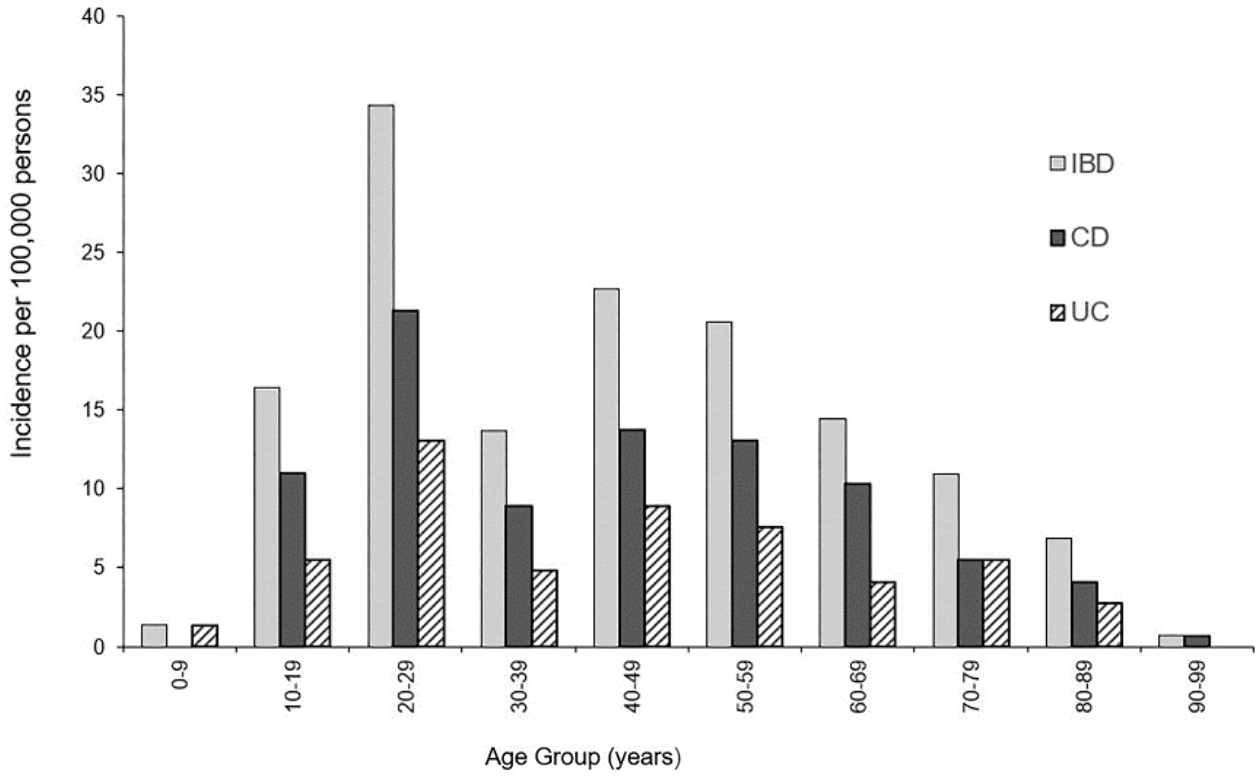

Figure 8.3 Incidence of Inflammatory Bowel Disease by Age Group

ABBREVIATIONS: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis

A greater proportion of patients with UC (n=12, 15.4%) reported a family history of IBD than patients with CD (n=14, 10.9%). Patients reported only one (n=21, 80.8%) or two (n=5, 19.2%) family members with a diagnosis of IBD. Crohn's disease (n=22, 71.0%) was the most frequently reported form of IBD diagnosed in a family member. This was true in both patients with UC (n=8, 61.5%) and patients with CD (n=14, 77.8%). At the time of diagnosis, more than half of patients with CD were previous or current cigarette smokers (n=66, 51.2%). The rate was lower in patients with UC (n=33, 42.3%) *(p*=0.261). The difference in current smoking rates was greater, twice as high in patients with CD (n=20, 15.5%) compared to UC (n=6, 7.7%) *(p*=0.115), while the rates of previous smoker were similar: UC 32.1% and CD 34.1%.

The proportion of rural and urban residents did not differ between patients with UC and patients with CD. Eight cases of IBD were identified among rural residents (n=28,707), and 199 among urban residents (n=117,054). The mean annual rural and urban IBD incidence rates were 5.6 (95% CI 0.4-22.4) and 34.0 (95% CI 24.1-46.0) per 100,000, respectively. The incidence rate did not differ significantly between small, medium, or large urban area (31.4, 32.5, and 35.1 per 100,000, respectively). Urban residence (≥1,000 residents) compared to rural residence was a risk factor for CD (IRR 6.08, 95% CI 2.5-19.1, p<0.001) and UC (IRR 6.13, 95% CI 2.0-30.4, p<0.001).

Presenting Symptoms of the IBD Incident Cohort

The most frequently observed presenting symptom in patients with UC was PR bleeding (n=67, 85.9%), and in patients with CD, diarrhoea (n=104, 80.6%) (*Table 8.1*). Less common symptoms were observed in 12 (5.8%) patients. These were anal pain in one patient with UC (1.3%), and in patients with CD these were vomiting (n=5, 3.9%), iron deficiency (ferritin <30 ug/l) (n=3, 2.3%), anal pain (n=1, 0.8%), persistent gum lesion and lip swelling (n=1, 0.8%) and mucus discharge and bloating (n=1, 0.8%). A perianal fistula was observed in four (3.1%) patients with CD in the six months prior to the date of diagnosis.

Table 8.1 Presenting Symptoms of the Manawatū region IBD Incident cohort

Symptom	Occurrence	UC, n=78	CD, n=129	IBD, n=207
≥1 (Diarrhoea, PR blood	Chronic	77 (98.7)	122 (94.6)	199 (96.1)
or Abdominal pain)	Acute	1 (1.3)	4 (3.1)	5 (2.4)
	None	0	3 (2.3)	3 (1.4)
Diarrhoea	Chronic	60 (76.9)	98 (76.0)	158 (76.3)
	Acute	2 (2.6)	6 (4.7)	8 (3.9)
	None	16 (20.5)	25 (19.4)	41 (19.8)
PR blood	Chronic	65 (83.3)	73 (56.6)	138 (66.7)
	Acute	2 (2.6)	5 (3.9)	7 (3.4)
	None	11 (14.1)	51 (39.5)	62 (30.0)
Abdominal pain	Chronic	40 (51.3)	92 (71.3)	132 (63.8)
	Acute	2 (2.6)	5 (3.9)	7 (3.4)
	None	36 (46.2)	32 (24.8)	68 (32.9)
PA fistula	Yes	0	4 (3.1)	4 (1.9)
	No	78 (100)	125 (99.6)	203 (98.1)
Other symptom	Yes	1 (1.3)	11 (8.5)	12 (5.8)
	No	77 (98.7)	118 (91.5)	195 (94.2)

Chronic = ≥6 weeks duration, Acute = < 6 weeks duration.

ABBREVIATIONS: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis

Investigative Procedures Performed at Diagnosis and 12 months Post-diagnosis

All 78 patients with UC, and 124 (96.1%) patients with CD, underwent diagnostic endoscopy, totalling 248

procedures (*Table 8.2*). Diagnostic imaging procedures (n=48) were performed on seven (9.0%) patients with UC and 32 (24.8%) patients with CD. While eight (3.9%) patients, all diagnosed with CD, underwent one surgical procedure. At 12 months post-diagnosis, an additional 47 endoscopies, 91 imaging procedures, and 18 surgeries had been performed. Of the five patients with CD that did not undergo diagnostic endoscopy, three were diagnosed during surgical resection, one patient during rectal examination under anaesthetic (EUA), and one following radiological investigation.

Table 8.2 Number of Patients that have Undergone Investigative Procedures at Diagnosis and 12 months Post-Diagnosis

	UC, n=78		**CD, n=129**	
Procedure	**Diagnosis, n (%)**	**12 months, n (%)**	**Diagnosis, n (%)**	**12 months, n (%)**
Endoscopy				
Gastroscopy	8 (10.3)	11 (14.1)	31 (24.0)	34 (26.4)
Sigmoidoscopy	18 (23.1)	24 (30.8)	18 (14.0)	25 (19.4)
Colonoscopy	61 (78.2)	66 (84.6)	107 (82.9)	116 (89.9)
Capsule			1 (0.8)	1 (0.8)
Any	*78 (100)*	*78 (100)*	*124 (96.1)*	*128 (99.2)*
Radiology				
MRI	3 (3.8)	8 (10.3)	18 (14.0)	56 (43.4)
CT	3 (3.8)	4 (5.1)	18 (14.0)	25 (19.4)
Abdominal US		3 (3.8)	2 (1.6)	5 (3.9)
Pelvic US			1 (0.8)	1 (0.8)
Barium enema	1 (1.3)	1 (1.3)		3 (2.3)
Barium enteroclysis		1 (1.3)	2 (1.6)	2 (1.6)
Linogram				2 (1.6)
Any	*7 (9.0)*	*15 (19.2%)*	*32 (24.8)*	*69 (53.5)*
Surgery				
Resection			4 (3.1)	5 (3.9)
Resection and ileostomy formation				4 (3.1)
Ileostomy reversal				2 (1.6)
Fistula surgery				2 (1.6)
Stricturoplasty				1 (0.8)
EUA			1 (0.8)	1 (0.8)
Diagnostic laparascopy			1 (0.8)	1 (0.8)
Surgical abscess drainage			2 (1.6)	6 (4.7)
Radiological abscess drainage				3 (2.3)
Re-look laparotomy for anastomotic leak				1 (0.8)
Any			*8 (6.2)*	*19 (14.7)*

ABBREVIATIONS: CD, Crohn's disease; UC, ulcerative colitis; US, ultrasound; EUA, examination under anaesthetic

Montreal Classification of the IBD Incident Cohort

Disease phenotype according to the Montreal Classification [13] is given in *Table 8.3*. In patients with UC, left-sided UC (E2) was observed most frequently at diagnosis (n=38, 48.7%). Patients with E1 disease extent at diagnosis were significantly younger at diagnosis than those with E2 (32.6 v 45.5, p=0.035) and E3 (32.6 v 46.0, p=0.043). In patients with CD, the colon (L2) was the most common disease location at diagnosis (n=57, 44.2%) and the majority (n=111, 86.0%) had inflammatory (B1) disease behaviour. One (0.8%) patient had isolated upper GI disease, perianal disease was observed in six (4.7%) patients, and one of the four B3 patients also presented with stricturing disease behaviour. The mean diagnosis age of patients with L2 disease location was significantly greater than L1 (51.6 v 40.2, p=0.01) and L3 (51.6 v 33.0, p<0.001). There was also a nonsignificant trend for L3 patients to be younger than L1 patients (33.0 v 40.2, p=0.09). Diagnosis age did not differ significantly by disease behaviour.

At 12 months post-diagnosis, disease extent remained stable in 76 (97.4%) patients with UC. The UC reclassifications included E1 to E2 (n=1), and E2 to E3 (n=1). Among patients with CD, disease location and behaviour remained stable in 123 (95.3%) and 120 (93.0%) of patients, respectively. Disease location reclassifications included L1 to L3 (n=1) and L2 to L3 (n=5), and disease behaviour reclassifications included B1 to B2 (n=1), B1 to B3 (n=3), and B2 to B3 (n=3). Perianal location increased from six (4.7%) to ten (7.8%) patients, and seven (5.4%) of the ten B3 patients also presented with stricturing disease behaviour.

Table 8.3 Phenotypic Presentation of the Manawatū region IBD Incident cohort at Diagnosis and 12 months Post-Diagnosis

Montreal classification of IBD	Diagnosis, n (%)	12 months, n (%)
UC Extent		
EI: ulcerative proctitis	17 (21.8)	16 (20.5)
E2: left-sided UC	38 (48.7)	38 (48.7)
E3: extensive UC	23 (29.5)	24 (30.8)
CD Location		
L1: terminal ileum	30 (23.3)	29 (22.5)
L2: colon	57 (44.2)	52 (40.3)
L3: ileocolon	41 (31.8)	47 (36.4)
L4 upper GI	3 (2.3)	3 (2.3)
Isolated upper GI	1 (0.8)	1 (0.8)
CD Behaviour		
B1: inflammatory	111 (86.0)	107 (82.9)
B2: stricturing	14 (10.9)	12 (9.3)
B3: penetrating	4 (3.1)	10 (7.8)
Perianal	6 (4.7)	10 (7.8)

ABBREVIATIONS: IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis

Symptom Chronicity, Chronic Inflammation and Lennard-Jones Diagnosis Criteria

Diagnosis criteria were assessed in the 219 patients with sufficient diagnostic data. 12 (5.5%) did not meet the minimum symptom duration of six weeks at the leading clinician diagnosis date. Following individual case review, four patients were excluded from the cohort: two instances of acute symptoms, and two of self-limiting colitis. Eight of these patients remained in the cohort. Three patients presented with acute symptom onset requiring admission: one with an inflammatory mass and retroperitoneal abscess, one with acute abdominal pain and a thickened terminal ileum on MRE, and one with acute abdominal pain who underwent subsequent hemicolectomy. Symptoms observed in the remaining five patients were: 4-5 weeks PR bleeding; 4-5 weeks abdominal pain, diarrhoea, and unintentional weight loss; chronic occasional diarrhoea, urgency and tummy upset, and chronic iron deficiency; six months perianal discomfort and excoriation; and seven months of mucus discharge and bloating with a history of perianal abscesses.

Evidence of chronic inflammation (one histological instance, or two non-histological instances separated by six weeks) could not be demonstrated for eight of the remaining 215 patients. Specifically, two cases were of non-histological evidence separated by fewer than six weeks, four cases were of a sole instance of non-histological evidence, and two cases had no evidence of chronic inflammation. These eight patients were excluded from the cohort.

The required Lennard-Jones criteria were demonstrated in all 78 patients with UC at diagnosis (*Table 8.4*). At 12 months post-diagnosis, diagnostic criteria for CD were observed in three (3.8%) of these patients. Among the 129 patients with CD, five cases (2.4%) were reviewed on account of not meeting the minimum number of two Lennard-Jones CD criteria at the date of diagnosis. Non-colonic location was observed in four patients, and discontinuous distribution of inflammation in one patient. All five patients remained in the cohort.

Application of the Diagnosis Algorithm

The leading clinician diagnoses of the 207 confirmed patients were IBDU 15, UC 79, and CD 113. Application of the diagnosis algorithm identified 41 instances of disagreement with the leading clinician diagnosis (IBDU 15, UC 17, CD 10). A considered diagnosis of UC was assigned to six patients with a leading clinician diagnosis of IBDU, and to ten patients with a leading clinician diagnosis of CD. These changes were made on the basis of demonstrated continuous mucosal inflammation, rectal inflammation, and lymphoid aggregate. A considered diagnosis of CD was assigned to nine patients with a leading clinician diagnosis of IBDU, and 17 with a leading clinician diagnosis of UC. These changes were made on the basis of demonstrated lymphoid aggregate along with one or two of: non-colonic disease location, discontinuous distribution of inflammation, submucosal fibrosis, colonic mucin retention, or non-caseating granulomata.

Table 8.4 Lennard-Jones Diagnosis Criteria demonstrated in the Manawatū region IBD Incident cohort at Diagnosis and 12 months Post-Diagnosis

	CD, n=129		UC, n=78	
Lennard-Jones Diagnosis Criteria	**Diagnosis, n (%)**	**12 months, n (%)**	**Diagnosis, n (%)**	**12 months, n (%)**
UC				
a) Continuous mucosal inflammation without granulomata[ab]			78 (100)	78 (100)
b) Affecting solely the rectum or the rectum and colon in continuity[ab]	53 (41.1)	45 (34.9)	78 (100)	75 (96.2)
CD				
a) Mouth to anus				
- Non-colonic disease location[ab]	73 (56.6)	77 (59.7)		3 (3.8)
b) Discontinuous				
- Discontinuous[ab]	60 (46.5)	69 (53.5)		2 (2.6)
- Rectal sparing[ab]	32 (24.8)	33 (25.6)		
Both	*76 (58.9)*	*84 (65.1)*		
c) Transmural				
- Abscess[b]	2 (1.6)	9 (7.0)		
- Fistula[b]	2 (1.6)	4 (3.1)		
- Fissuring ulceration[a]	2 (1.6)	7 (5.4)		
All	*5 (3.9)*	*12 (9.3)*		
d) Fibrosis				
- Submucosal Fibrosis[a]	1 (0.8)	1 (0.8)		
- Stenosis[b]	15 (11.6)	19 (14.7)		
Both	*15 (11.6)*	*19 (14.7)*		
e) Lymphoid				
- Lymphoid Aggregates or	117 (90.7)	119 (92.2)	76 (97.4)	76 (97.4)
- Crypt Architectural Distortion[a]				
f) Mucin				
- Colomucin[a]	2 (1.6)	3 (2.3)		
g) Granulomata				
- Granulomata[a]	35 (27.1)	40 (31.0)		1 (1.3)

ABBREVIATIONS: CD, Crohn's disease; UC, ulcerative colitis

[a] Histologic finding, [b] macroscopic finding, [ab] histologic or macroscopic finding

IBD Incident Patient Identification and Diagnosis Confirmation

Keyword search within the eight data repositories identified 5,146 unique patients (Supplementary Material *Table 8.7*). A total of 219 patients remained after exclusion of invalid National Health Index numbers, patients with no diagnosis or diagnosis other than IBD, patients diagnosed outside the study timeframe or outside the study area, incidental findings, and patients with insufficient data to confirm IBD diagnosis. The final incident cohort comprised 207 patients, 78 patients with UC and 129 with CD. The greatest number of incident cases were identified from histology data, followed by MidCentral endoscopy data, then Medlab Central prescription data.

Point Prevalence of IBD in the Manawatū region

At March 2013, the crude prevalence rate of IBD per 100,000 in the Manawatū region was 456.9 (95% CI 423.6-492.9). The leading clinician diagnoses were IBDU 16, UC 285, and CD 365, equating to 11.0 (95% CI 6.7-17.9), 195.5 (95% CI 174.1-219.6), and 250.4 (95% CI 226.0-277.4) per 100,000, respectively. After age-standardisation the prevalence rates per 100,000 were 8.4 for IBDU, 157.7 for UC, 231.8 for CD, and 397.9 for IBD. Of the 666 patients identified with evidence of an IBD diagnosis, the greatest number of prevalence cases were identified from Medlab Central prescription data (n=496), followed by MidCentral gastroenterology clinic data (n=381), then MidCentral endoscopy data (n=324).

Demographics of the IBD Prevalence Cohort

A greater proportion of patients with IBD were female (n=359, 53.9%). Gender analysis by disease subtype demonstrated a significantly greater number of female patients with CD (211 vs 154, p=0.003), while UC was slightly more common among males (51.6%).

The median age was 51.8 years for patients with IBD, 56.2 for UC, 47.2 for CD, and 59.9 for IBDU. Patients with UC were significantly older than CD (55.9 vs 48.2, p<0.001). Prevalence rate was highest in the 60-69 years age group, followed by 40-49 years. Ethnicity was predominantly European (n=620, 93.1%), followed by Māori (n=21, 3.2%), and Asian (n=12, 1.8%). The remaining ethnic groups each comprised less than 1% of the prevalent cohort.

The domicile of prevalence patients on 13 March 2013 was predominately small, medium or large urban (485, 651, and 513 per 100,000, respectively) with fewer patients residing in a rural area (94 per 100,000). Urban or rural residence did not differ between disease subtype *(p*=0.392).

The date of IBD diagnosis was identified for 541 patients. In this group the median disease duration at March 2013 is 7.1 years for patients with IBD, 8.2 for UC, 6.9 for CD, and 1.8 for IBDU. Disease duration was significantly greater in patients with CD compared to IBDU (9.2 vs 2.5, p=0.004), and UC compared to CD (11.6 vs 9.2, p=0.005) and IBDU (11.6 vs 2.5, p<0.001).

DISCUSSION

The mean annual age-standardised incidence rates for UC, CD, and IBD were 10.2, 17.0, 27.2 per 100,000, respectively, from 2011-2015 in the Manawatū region. These rates correspond well to the 2004 Canterbury UC, CD, and IBD rates of 7.5, 16.3, and 25.2 per 100,000, respectively [14], as well as the 2012 UC, CD, and IBD rates of 6.3, 21.8, and 29.8 per 100,000, respectively, in the neighbouring Otago region [15]. Although, the follow-up Canterbury study revealed incidence rates 1.6-fold greater than ten years earlier [7], and the identification methods employed in the Otago study may have resulted in underestimation of some cases [15], suggesting the incidence rates in the current study are lower than expected. One possible explanation is regional variability. Alternatively, this observation is in agreement with greater prevalence rates of paediatric IBD in the South Island compared with the North [16]. Given the geographical location of the South Island, and the existence of a north to south IBD gradient in the Northern Hemisphere, particularly for CD [17], this may be evidence of a south to north IBD gradient in the Southern Hemisphere.

In Australia, the IBD incidence rates reported from 2005-2011 almost mirror the rates seen in NZ. Two prospective population-based studies in Victoria reported annual IBD incidence rates of 29.3 (crude) per 100,000 between 2007-2008 [18], and 24.7 (age-standardised) per 100,000 between 2010-2011 [19]. In north Brisbane, Queensland, an earlier study in 2005 reported an IBD incidence of 30.3 per 100,000 [20]. Given the parallels between Australian and NZ, it is probable that genetic backgrounds and environmental risk factors are similar across both populations and largely explain the agreement between incidence rates.

Data on the prevalence of IBD is scarce, and rates may be underestimated due to difficulties associated with case identification and data acquisition. However, the observed IBD prevalence of 456.9 per 100,000 is higher than rates previously reported in NZ and Australia [14,19]. In terms of global estimates, the highest reported prevalence rates are for North America and Europe: ranging from 340.0 to 505.0 per 100,000 for UC, and 262.0 to 322.0 per 100,000 for CD [3]. This places the observed prevalence of CD (250.4 per 100,000) alongside some of the highest reported rates worldwide.

Among prevalent cases, the ratio of CD:UC was 1.3:1, higher than the 1.1:1 seen in the 2004 Canterbury study [14], and both rates are lower than their corresponding CD:UC incidence ratios of 1.7:1 and 2.2:1, respectively. These findings align with the decrease or plateauing of UC incidence, and ongoing rise in CD incidence observed in many other westernised countries [3,21]. In fact, recent findings suggest that the division between UC and CD rates in NZ may increase further. The research by Lopez et al. [16] demonstrated a CD:UC ratio of 5:1 among paediatric prevalent cases in 2015, while the incidence of paediatric IBD between 1996 and 2015 equates to a CD:UC ratio of 8.4:1 [8].

Urban residence at the time of diagnosis was associated with a six-fold greater incidence of IBD compared to rural residence. In a meta-analysis of 40 studies by Soon et al. [22], a positive association was found between living in an urban environment and risk of both CD and UC irrespective of disparities between urban population definitions, or even the absence of a definition. Similarly, in a large Canadian study, 14 different definitions of rural/urban were assessed and an association between rural residence and

reduced risk of paediatric IBD persisted for most definitions [23]. The higher incidence of IBD in urban settings is thought to stem from increased hygiene, leading to a decrease in exposure to immune system priming and impairment of immunoregulatory mechanisms [24]. Whereas rural environments provide greater opportunity for microorganism exposure through contact with pets, livestock, soil, and untreated water. Another potentially contributing factor is an imbalance between urban and rural healthcare and implications for time to diagnosis. Correspondingly, in a recent study of NZ rural patients with IBD, time and financial constraints were identified as a significant challenge in accessing urban-based healthcare, and privacy concerns associated with living in smaller communities led to avoidance of rural IBD services [25].

Disease location in patients with UC was generally consistent with other reports for left-sided and extensive UC, while proctitis was seen in a small proportion of patients (n=17, 21.8%) at diagnosis compared to 30-49% reported in other Australasian and European studies [7,18,19,26,27]. An unexpected finding was the significantly younger diagnosis age of patients with proctitis, contrary to reports in the Canterbury study and a large Swedish study with extensive UC being associated with a significantly younger diagnosis age [26,28]. Disease extent was stable in the majority of patients with UC in the 12 months following diagnosis with only two (2.6%) reclassifications. In patients with CD, the proportion of ileal disease location (n=30, 23.3%) was at the lower end of those seen in comparable studies, ileocolonic and upper GI disease were in accord, and a high proportion of patients had colonic disease (n=57, 44.2%) [7,15,18,19,26,27]. With regard to disease behaviour, the low rate of perianal disease (n=6, 4.7%) was the sole atypical observation. Tarrant et al. [26] demonstrated that the presence of perianal disease can predict an enhanced risk of extensive and complicated disease progression. This was not evident in the current study as of the six location reclassifications, no patients had perianal disease, and of the nine behaviour reclassifications, only one patient had perianal disease. Ileal and upper GI disease has also been associated with a greater risk of disease progression [29,30]. Instead, in this study disease progression was observed in patients with ileal, ileocolonic, and colonic disease. These different outcomes likely reflect the small cohort size and short follow-up period.

Early diagnosis and intervention markedly improve disease prognosis, yet the diagnostic definition of IBD (particularly for CD) remains vague, and usage of the few well-defined definitions is scant. Among the 219 patients with a leading clinician diagnosis of IBD, 25 patients (IBDU 4, UC 2, CD 16) did not meet the criteria for symptom chronicity, demonstration of chronic inflammation, or demonstration of Lennard-Jones criteria. This suggests such criteria are too rigid to diagnose mild or early-stage IBD, most notably CD. We believe a consensus is required on minimum diagnostic criteria that allow for earlier diagnosis of this patient subset. With regard to CD, most patients did not formally meet Lennard-Jones criteria at diagnosis due to a lack of demonstration of disease beyond the mucosa of the bowel. Diagnostic criteria that do not rely on demonstration of transmural involvement of bowel tissue would allow description of the natural history of early CD, and study of early therapeutic intervention. Until this is achieved, our knowledge of the management of CD is unfortunately limited to those who have already suffered transmural complications.

This study has several limitations. Firstly, the collection of some data was compromised by the retrospective study design. Specifically, demographic and diagnosis data could not be located for some prevalent cases which restricted the scope of analysis and may have led to the inclusion of cases where the diagnosis of IBD has since been revoked. Secondly, it is possible that some incident cases were not identified. Although we anticipate this number is low due to the inclusion of additional data sources that only record information for patients managed in the study area, specifically, histology reports and prescription data. Similarly, it is possible that prevalent cases in remission for an extended period of time; managed without prescription of adalimumab, azathioprine, mesalazine or methotrexate; or managed outside the study area; were not identified. Thirdly, instances of sigmoidoscopy, or premature colonoscopy cessation, may have resulted in underrepresentation of UC extent and CD location. Study strengths include robust data collection through extensive utilisation of data repositories. Further, data in this study have been presented in an objective and detailed format. This will allow accurate comparison of this cohort against other cohorts. It will also allow accurate comparison of future cohorts, even if the diagnostic criteria for IBD change in the future.

CONCLUSION

In the Manawatū region the mean annual age-adjusted IBD incidence from 2011 to 2015 was 27.2 per 100,000, and the 2013 age-adjusted prevalence was 397.9 per 100,000. Rates of IBD were significantly reduced in the rural community, an observation that warrants further research. Comparison of findings between IBD cohorts in different locations is compromised by a lack of uniformity in assigning a diagnosis of IBD, usually at the mild end of the disease severity spectrum. We consider that an objective diagnostic definition of early stage IBD, particularly CD, is needed in order to reduce inter-observer variability, and to better standardise cohorts of patients with IBD.

Supplementary Material

Supplementary Table 8.5 Patient Identification: Inflammatory SNOWMED Codes

Code	Description
D6214001	Inflammatory_bowel_disease_NOS
D6216001	Crohn's_disease
D6255001	Ulcerative_colitis_NOS
M4000001	Inflammation_NOS
M4000001	Colitis_NOS_(T-67000)
M4000001R001	Colitis_NOS_(T-67000)
M4100001	Inflammation_acute_NOS
M4300001	Inflammation_chronic_NOS
M4717001R001	Lymphocytosis_NOS

Supplementary Table 8.6 Patient Identification: ICD Codes

Code	Description
K50	Crohn's disease [regional enteritis]
K50.0	Crohn's disease of small intestine
K50.1	Crohn's disease of large intestine
K50.8	Crohn's disease of both small and large intestine
K50.9	Crohn's disease, unspecified
K51	Ulcerative colitis
K51.0	Ulcerative (chronic) pancolitis
K51.2	Ulcerative (chronic) proctitis
K51.3	Ulcerative (chronic) rectosigmoiditis
K51.5	Left sided colitis
K51.8	Other ulcerative colitis
K51.9	Ulcerative colitis, unspecified
K52.3	Indeterminate colitis

Supplementary Table 8.7 Data Sources: Cases of IBD in the Manawatū region, 2011-2015

Data Source	Data Records Reviewed	Unique Patients	Keyword hit	Incident Patients	Prevalent Patients
Histology (public and private)	7,223	5,748	1,027	193	284
MDHB Endoscopy	7,167	5,664	1,049	174	324
Prescription Data (Medlab Central)	7,026,718	186,125	1,405	166	496
MDHB Gastroenterology Clinic	17,762	6,849	1,380	145	381
Laboratory Data (public and private)	NA	NA	652	101	253
MDHB Surgery Clinic	49,849	18,307	1,606	90	236
MDHB Radiology	NA	NA	1,036	70	158
MDHB Inpatient Admissions	647,838	83,948	265	64	149
Private Clinic and Private General Surgery	7,166	4,179	838	38	128
Private Radiology	NA	NA	150	5	15
TOTAL	NA	NA	5,146	207	666

ABBREVIATIONS: MDHB, MidCentral District Health Board

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CHAPTER NINE

DISCUSSION

Crohn's disease (CD), one of two inflammatory bowel diseases (IBD), is a complex condition that is thought to be caused by an interplay of immune dysregulation, environmental factors, and genetic predisposition [1]. The highest incidence rates are observed in Western industrialised countries, particularly Canada which reported 22.6 per 100,000 in Nova Scotia from 1996 to 2009 [2]. Data on the incidence of CD in New Zealand (NZ) are limited, however, in Canterbury the 2004 and 2014 rates per 100,000 were 16.5 [3] and 26.4 [4], respectively, and 21.8 per 100,000 in 2012 in Otago [5]. A 2005 CD prevalence of 155.2 was also reported for Canterbury [3]. In addition to having one of the highest rates of CD worldwide that is clearly increasing, a 4-fold increase in the paediatric incidence of CD between 1996 and 2015 [6] demonstrates that CD poses a significant public health concern in NZ.

In order to gauge the number of people affected by CD in the Manawatū Region, the incidence and prevalence of CD, and also the other main form of IBD, ulcerative colitis (UC), were determined (chapter nine). The mean annual age-standardised incidence of CD between 2011 to 2015, and 2013 crude point prevalence were 16.9 and 250.4 per 100,000, respectively. These rates are lower than those observed for the South Island at the same time, and unfortunately no recent North Island data are available for comparison. Geographical variability of IBD rates is well documented [7] and could explain these differences. Further, some incident cases were excluded for the study as minimum diagnostic criteria could not be confirmed.

The global burden of CD is growing, not only on account of an increasing incidence, but also due to longer life expectancy and a younger age of onset which is also associated with a higher risk of disease complications [8]. As CD is a lifelong condition of unknown aetiology, the provision of requisite patient healthcare is influenced evidence of current trends associated with incidence and prevalence and other disease attributes such as phenotype and progression patterns. This study yielded epidemiological, clinical, and demographic data in a region not previously studied that can be used by NZ healthcare stakeholders. The determination of incidence and prevalence data provides a baseline for future research in the region, and the comprehensive assessment and presentation of diagnostic criteria will permit comparison with future cohorts.

One of the findings of the Manawatū Region incidence and prevalence study was a six-fold greater incidence of IBD associated with urban residence at the time of diagnosis compared to rural residence. This is consistent with the findings of a systematic review that demonstrates an association between living in an

urban environment and a higher risk of IBD [9]. An urban environment, specifically a major urban (≥100,000 residents) birthplace and major urban residence during childhood were also positively associated with IBD in our study of NZ environmental factor exposure (chapter six). Other factors that were independently positively associated with IBD were farm exposure and freshwater contact prior to pre-diagnosis symptom onset, while negative associations were observed with drinking and cooking water sourced from rainwater during childhood, and handling soil or dirt prior to pre-diagnosis symptom onset.

We hypothesised that CD would be positively associated with increased exposure to potential sources of Mycobacterium avium subspecies paratuberculosis (MAP) as a consequence of exposure to ruminant farm animals, the farms on which they reside, contaminated water, hunting, or consumption of unpasteurised milk. This hypothesis was not supported, however the associations with farm exposure, freshwater contact, drinking and cooking water source, and soil or dirt handling suggest that microorganism exposure could be a factor.

The pathophysiology of CD is complex and only partially understood, so therapeutic approaches are limited to those that target the inflammation caused by CD and consequent symptoms. The efficacy of currently available therapies is also variable with many patients still experiencing spontaneous relapses, and biological therapies, which are typically only prescribed when other therapies have failed, can become ineffective after an extended period of time due to patient development of antidrug antibodies [10]. Patients with IBD often believe that certain foods influence their symptoms and experiment with dietary modification in an attempt to improve symptom management [11,12]. Investigation of these beliefs found that the proportion of patients that associate dietary elements (certain foods, additives, or cooking methods) with symptom onset or exacerbation were 55% and 70%, respectively (chapter three). This is consistent with the 51-88% seen in other studies [11–20]. Only 35% of patients associated dietary elements with symptom reduction, though this also falls within the range reported by others 24-57% [12,16,20,21].

Self-directed dietary modification, especially the exclusion of certain foods or food groups without understanding the particulars of a balanced diet and nutrient sources, may lead to inadequate energy or nutrient intake. The results of our study demonstrated that the intake of certain fruits and vegetables is associated with an adverse effect on symptoms. With the exception of a poor overall intake of fruits and vegetables, avoidance of certain fruits and vegetables is unlikely to have a discernible effect on dietary provision of fibre and micronutrients. Although, this may be complicated by malabsorption occurrence. Adverse effects of dairy products were also frequently reported, specifically, cheese, yoghurt, and milk, as well as bread, which together contribute approximately half of dietary calcium in NZ [22]. Avoidance of these foods can compromise intake of calcium and protein and have a negative effect on bone health. This may be particularly detrimental for patients with CD as even low-grade inflammation activates bone resorption and inhibits bone formation [23], and corticosteroid therapy, which is effective at inducing remission [24], can substantially decrease bone mineral density [25].

Extensive dietary restriction creates concerns about adequate intake of energy and essential nutrients. Although this was not evident in our study as many of the foods identified, with the possible

exception of select breads and cereals, are not typically relied on as a primary source of energy. Exclusion of many of the dietary elements associated with adverse effects would be healthful, specifically, fried and deep-fried foods, alcohol, coffee, and cream. Similarly, several dietary elements associated with symptom reduction, and their increased inclusion in a patient's diet, would improve diet quality. Dietary elements that were frequently reported to reduce symptoms were baking or grilling foods, lean sources of protein, oily and non-oily fish, eggs, and yoghurt. These dietary modifications would have the potential to lower the intake of saturated animal fats, improve the ratio of omega-3 to omega-6 fatty acids, and increase the intake of vitamins that are only found in a limited number of foods including vitamin D and vitamin B12.

The study demonstrates that a high proportion of NZ patients with IBD associate dietary elements with their symptoms. The dietary elements most associated with an adverse effect on symptoms, including specific fruits and vegetables, dairy-products, spicy foods, nuts and seeds, alcohol, and foods with a high fat content, are in agreement with previous and recent research findings [19,20,26–28]. Several limitations may have compromised the utility of study findings. Whether the foods associated with adverse effects led to restricted intake or total avoidance was not determined. It would have also been of interest to establish if patients had received diet advise at any time since their diagnosis, and the source of this advice.

Patient beliefs concerning diet and symptoms has been extensively studied [11,12,14–18,21,29–36], however, little work has explored how dietary components have an effect. Advances in cell culture methods have produced a method for the isolation and culture of small intestinal crypts into organoids [29]. The research objective was to explore the potential of small intestinal organoids to investigate the effect of food components and environmental agents, such as MAP, on gut cell function and barrier integrity. Although the crypt isolation and culture methodology were established, the restricted access to apical cell membranes which are internally located around the organoid lumen requiring macrophage microinjection had a serious impact on progression of the organoid work. This limitation has recently been addressed by the introduction of techniques to produce single cell 'apical out' monolayers from organoids grown in culture which allow easy access to both the apical and basolateral surfaces. These 'opened up 2-dimensional organoids' [30] would have avoided the need for microinjection but this novel approach was not published until after the organoid work was abandoned.

As an alternative to the organoid model, the Caco-2 cell model was used to investigate the effect of food components on gut cell function and barrier integrity. *In vitro* digestion (described in appendix 3) of a selection of foods associated with adverse symptom effects (chapter three), and cell exposure to the supernatant of these foods resulted in tight junction disruption and a reduction in monolayer integrity. This could explain the association between certain foods and symptoms as even a brief compromise in barrier integrity can permit increased paracellular transport of luminal microorganisms, and the ensuing immune response may be excessive in patients with IBD [31]. The potential of vitamin D to mitigate dietary instigated damage was also investigated. Treatment with vitamin D did minimise changes in monolayer integrity, although this was not observed for all foods.

As vitamin D status is inversely associated with CD risk and disease activity [32–34], a case-control study was conducted to determine the serum vitamin D concentration of patients with CD and controls. In this study, there was no difference between the mean vitamin D concentration of the patient and control group. This was initially surprising, although it is likely explained by large differences between supplementation dose, 100-1,000 IU/day in the control group, and 284-7,143 IU/day in the patient group. An association was also observed between recent disease activity and lower vitamin D concentration. The main study limitation was not collecting the samples at the end of winter when vitamin D levels are generally low, and the occurrence of insufficiency and deficiency could have been more accurately determined. The findings of the sub-study showed that serum vitamin D concentrations measured from blood spot samples were slightly lower, but closely correlated with concentrations measured from venous blood samples. This less-invasive and convenient alternative to venepuncture could be used in the clinical setting to screen population groups at risk of vitamin D deficiency, such as the elderly and children.

CONCLUSIONS

The findings of this research suggests that environmental factor exposure, specifically urbanisation, increases the risk of CD in NZ. The longstanding hypothesis that MAP can cause CD was explored by investigating exposure to probable MAP sources, though our findings do not support this theory. Consistent with other research, investigation of patient's beliefs about diet and symptoms demonstrated that symptom onset or exacerbation is frequently associated with specific fruits and vegetables, dairy products, alcohol, and coffee. This effect may be attributable to an intermittent compromise of intestinal barrier integrity.

The measurement of serum vitamin D concentrations demonstrated no significant differences between patients with IBD and healthy controls despite a higher supplementation dose in the patient group, however, recent disease activity was associated with lower vitamin D. The epidemiological study results demonstrated the incidence and prevalence of IBD in the Manawatū Region are comparable to high rates reported in other locations within Australasia, and further corroborated the association between urbanisation and IBD.

In summary, these findings demonstrate an association between urbanisation, diet, and vitamin D status in NZ patients with CD. It is important to be mindful of the fact that association does not necessarily indicate causation, although a greater understanding of environmental factors, especially modifiable factors, could provide opportunities for reducing CD risk, managing symptoms, or slowing disease progression.

FUTURE RESEARCH RECOMMENDATIONS

The burden of CD in NZ is growing rapidly, and the impact on patients, their family, the healthcare system, and society is immense. Robust epidemiological data are required to be informed of trends in IBD incidence, prevalence, and phenotype. Research should be conducted in regions that have not been studied previously or where data are markedly out of date to determine current IBD trends, and in previously researched regions to identify changing trends. A prospective study design will minimise the risk of bias and confounding factors.

The high proportion of patients with IBD that associate their symptoms with dietary elements warrants future investigation. The use of a self-administered questionnaire for data collection saves time and any costs are low, however, this method is prone to low accuracy [43] and respondent cognitive burden [44]. An interview method should be considered for future research. A well-trained interviewer can probe for extra detail regarding implicated foods, preparation methods and intake patterns, as well as the extent of any post-diagnosis diet modification, and the time between consumption and the development or exacerbation of symptoms attributed to a food. Further, the nutritional status and bone health of patients should be assessed soon after diagnosis and monitored at regular intervals. This will facilitate earlier identification of deficiencies or low bone mineral density and generate valuable data to further explore the effects of inflammation on nutrient absorption and retention, and bone health.

Despite the difficulties experienced with the intestinal organoid model, this model has enormous advantages. In particular, organoids can be visualised at the cellular level using confocal imaging, cultured from human biopsy material, and can be grown and maintained in *in vitro* culture for weeks to months while maintaining normal cell function. Differences in the gut health of patients with IBD and healthy controls, such as barrier integrity, cell turnover and differentiation, should be investigated in organoids cultured from human biopsies. The organoid model should be utilised to explore interactions between environmental components and gut cells. Furthermore, the ability to store organoids prepared from patient biopsies for an extended period of time in liquid nitrogen would allow these samples to be re-examined as our understanding of the possible mechanisms involved in IBD becomes clearer.

A substantial body of evidence demonstrates an inverse association between vitamin D status and both the risk of CD and disease activity in patients with CD [32–34]. The effect of vitamin D supplementation on disease activity has been investigated, although comparison of study findings is compromised by disparities in vitamin D status terminology and the corresponding serum concentrations between countries. Future research should further investigate if vitamin D status correlates with disease activity, the efficacy of supplementation in patients with CD in comparison with healthy controls, and if supplementation reduces disease activity and improves nutritional status and bone health (see appendix 8 for a proposal to robustly test the role of vitamin D in a randomised placebo-controlled trial).

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APPENDICES

1. Published Papers

FIRST AUTHOR

Morton H, Pedley KC, Stewart RJC, Coad J (2020). Inflammatory Bowel Disease: Are Symptoms and Diet Linked? *Nutrients* 12(10):2975

Morton H, Pedley KC, Stewart RJC, Coad J (2020). Vitamin D concentrations in New Zealanders with and without inflammatory bowel disease: do they differ? *New Zealand Medical Journal* 133:1511

Morton H, Coad J, Pedley KC, Irwin JR (2023). The Incidence of Inflammatory Bowel Disease in New Zealand remains high, findings in the Manawatū region. *Digestive Disease and Sciences (2023).*

SECOND AUTHOR

Stewart RJC, Morton H, Coad J, Pedley KC. (2019). *In vitro* digestion for assessing micronutrient bioavailability: the importance of digestion duration. *International Journal of Food Sciences and Nutrition*, 70:1

2. Presentations

CONFERENCE

Morton H, Pedley KC, Stewart RJC, Coad J. *Dietary Factors and Gut Integrity: Implications for Crohn's Disease.* Poster presentation at the Nutrition Society of New Zealand Annual Scientific Conference, Auckland 2012.

Morton H, Pedley KC, Coad J. *25-hydroxyvitamin D Levels in New Zealanders with and without active Inflammatory Bowel Disease*. Oral presentation at the New Zealand Society of Gastroenterology Annual Scientific Meeting, Rotorua 2015.

Morton H, Pedley KC, Coad J. *25-hydroxyvitamin D Levels in New Zealanders with and without active Inflammatory Bowel Disease.* Oral presentation at the Nutrition Society of New Zealand Annual Scientific Conference, Wellington 2015.

Morton H, Pedley KC, Coad J (2020). *Inflammatory Bowel Disease: Are Symptoms and Diet Linked?* Oral presentation at the Nutrition Society of New Zealand Annual Scientific Conference, Napier 2019.

Morton H, Coad J, Pedley KC, Irwin JR. *The Incidence and Prevalence of Inflammatory Bowel Disease in the Manawatū region, 2011-2015.* Oral presentation at the New Zealand Society of Gastroenterology Annual Scientific Meeting, Dunedin 2018.

OTHER

Morton H, Coad J, Pedley KC, Irwin JR. *The Incidence and Prevalence of Inflammatory Bowel Disease in the Manawatū region, 2011-2015.* Oral presentation at MidCentral DHB Clinical Audit and Research Week, Palmerston North 2019.

Morton H, Coad J, Pedley KC, Irwin JR. *The Incidence and Prevalence of Inflammatory Bowel Disease in the Manawatū region, 2011-2015.* Oral presentation at the Palmerston North Medical Research Foundation Colloquium, Palmerston North 2019.

Morton H, Pedley KC, Coad J. *Vitamin D, Bone Health, and the Immune System*. Oral presentation at Crohn's and Colitis New Zealand, support group meetings; Auckland, Hawkes Bay, Dunedin, Invercargill, Timaru, Christchurch, 2014

3. *In vitro* digestion for assessing micronutrient bioavailability: the importance of digestion duration

PUBLISHED IN THE "INTERNATIONAL JOURNAL OF FOOD SCIENCES AND NUTRITION"

Stewart RJC, Morton H, Coad J, Pedley KC. (2019).

In vitro digestion for assessing micronutrient bioavailability: the importance of digestion duration.

International Journal of Food Sciences and Nutrition, 70:1

ABSTRACT

Static digestion *in vitro* is a commonly used technique for investigating micronutrient availability which allows the nutrients or foods of interest to be exposed to conditions that simulate those found within the stomach and small intestine. The activity of these digestive enzymes throughout their respective simulated digestion phases has been reported to decline due to the autolytic activity of the proteases and therefore incomplete digestion may result. The degree of protease inactivation under commonly simulated digestion conditions requires further quantification. Pepsin and pancreatic protease activities were assessed throughout a simulated digestion protocol *in vitro* over multiple time points using stop-rate spectroscopy. The protease activity of both pepsin and pancreatin decreased significantly during their respective digestion phases. Results suggest that gastric and intestinal proteases are destroyed or inactivated during their respective digestive phase. For this reason, prolonged digestion protocols may require protease supplementation throughout digestion to correctly simulate physiological conditions.

INTRODUCTION

Human trials are regarded as the gold standard for investigating micronutrient bioavailability in humans [1]; however, these trials are limited by cost and/or ethical considerations and are often not suitable for screening multiple nutrient–micronutrient interactions within the human gastrointestinal tract. To overcome these limitations, a number of sophisticated animal models [2–6] have been developed to investigate nutrient micronutrient-interactions and micronutrient bioavailability. Although animal models provide further opportunity to investigate the role of digestion and mechanisms of nutrient bioavailability, their use is often limited by specific technical requirements, infrastructure, and ethical considerations. Alternatively, *in vitro* models provide a rapid, cost-effective screening tool which have been used extensively to investigate multiple nutrient–nutrient interactions [7–9]. *In vitro* studies also overcome many of the ethical issues associated with *in vivo* research, through replacing animals completely or reducing the number of animals required. The *in vitro* digestion model is important as a screening tool in micronutrient research and should be constantly assessed and refined to accurately simulate physiological processes.

In vitro models often include a digestion phase, developed to simulate gastrointestinal processes within the stomach and proximal small intestine. This process is usually coupled with measurements of micronutrient solubility and dialysability alone [10–14] with measurements of mucosal micronutrient transport in biological tissues such as ligated sections of small intestine [15] or with measurements of mucosal micronutrient transport in cultured enterocytes [5,7,16–18]. Combining measures of nutrient solubility during digestion with measures of nutrient transport in biological tissues may be the most accurate *in vitro* model to predict micronutrient bioavailability in humans from a range of food items [19–21].

The validity of various *in vitro* digestion protocols as a model for nutrient bioavailability has been reviewed extensively elsewhere [22–25]. Collectively, these reviews suggest that in order for an *in vitro* system to accurately simulate human digestive processes, the digestion protocol should include both a gastric phase and an intestinal digestion phase, both of which should be performed under specific physiological conditions. The gastric digestive phase is suggested to be performed at pH 1–3 in the presence of pepsin for a duration of 1–2 hours at 37 °C. The intestinal phase is suggested to be performed at pH 6–7.5 in the presence of pancreatin and bile salts for a duration of 1–2 hours at 37 °C at approximately 300 mOsm.

Despite being performed under similar conditions, the hydrolytic activity of mammalian digestive proteases has been previously reported to decrease both within the simulated gastric phase and the simulated intestinal phase [26,27]. These studies collectively suggest that pepsin activity is decreased under prolonged gastric digestion simulations, and trypsin and chymotrypsin activities are significantly decreased under prolonged intestinal digestion simulations. As a consequence, this reduction in protease activity may affect the ability of the *in vitro* digestion to accurately simulate human digestion if the duration of digestion is prolonged or if proteases are activated in advance to their addition to the digestate.

The duration of an *in vitro* digestion designed to simulate digestion in the stomach and small intestine varies markedly by up to 4 hours within the micronutrient bioavailability literature [28–30]. The most common *in vitro* digestion duration range for each digestion phase is used to investigate micronutrient

bioavailability spans between 1 and 2 hours [5,7,14,30–33]. The stability and activity of pepsin or pancreatic proteases at the cessation of a simulated gastrointestinal digestion within this digestion duration is unknown.

The activity of pepsin and pancreatic proteases during simulated gastric and intestinal digestion phases requires further investigation over a digestion duration similar to that used for micronutrient bioavailability assays. This is required to characterise and quantify any reduction in protease activity and propose possible resolutions to this reduction if necessary. We expect that the hydrolytic potential of proteases commonly used to perform digestion *in vitro* will decrease within their respective digestive phase under simulated *in vitro* conditions over the duration of 1–2 hours at a quantifiable rate.

MATERIALS AND METHODS

Chemicals, Enzymes and Hormones

All chemicals, enzymes and hormones unless otherwise stated were purchased from Sigma Aldrich (St. Louis, MO, USA).

In Vitro Digestion: Gastric Digestion

The digestion method was modified from Glahn et al. [34]. Immediately before use, porcine pepsin (P7000) was activated by combination with 0.1 M HCL. The final pepsin concentration was 25mg/mL. For the simulated gastric digestion, 5 mL saline solution (120mM NaCl and 5mM KCl) was titrated to pH 2.5 with 1M HCl prior to the addition of 0.5mL activated pepsin. Samples were further adjusted to pH 2.0, made up to a final volume of 6mL and incubated at 37°C on a rocking shaker for 60 minutes. Six separate gastric digestion phases were performed.

In Vitro Digestion: Intestinal Digestion

Immediately before use, porcine pancreatin (P1625) and bile salts (B-8631) were combined with 0.1M NaHCO3 to a final concentration of 9.26 and 55.5mg/mL, respectively. For the simulated intestinal digestion, the simulated gastric digestion samples (prepared as described above) were titrated to pH 6.0 with 1M NaHCO3 and combined with 0.5mL activated pancreatin/bile solution in triplicate. All digests were adjusted to 300mOsm and made up to a final volume of 15mL with saline solution, pH 7.0, before incubation at 37°C on a rocking shaker for 180 minutes.

Pepsin Activity Assay

The gastric phase of the *in vitro* digestion system was set up as described above. The pepsin activity of each gastric digest was analysed immediately after the addition of the activated pepsin and after 30, 60, 90, and 120 minutes incubation.

Pepsin activity was measured by spectrophotometric stop-rate determination modified from Chow and Kassel [35]. Bovine haemoglobin was combined with deionised water (2.5% w/w) and filtered through

glass wool. The haemoglobin filtrate was diluted 4:1 with 0.3M HCl and incubated at 37°C for 5 minutes. Aliquots (1mL) of gastric digest were removed from the digestate without volume replacement and immediately combined with 5mL acidified haemoglobin and incubated for 10 minutes at 37°C. Pepsin activity was stopped by the addition of 5mL of 5% trichloroacetic acid solution (TCA). For the blank, 5 mL TCA solution was combined with 1mL gastric digest prior to the addition of 5 mL of acidic haemoglobin. All samples were separated by centrifugation at 450xg for 7 minutes and the supernatant was passed through 0.22µm Minisart syringe filters (Sartorius AG, Goettingen Germany). The TCA-soluble peptide concentration of each sample was analysed at 280nm by spectrophotometer (Cary 300, Varian, CA, USA). The concentration of pepsin units per millilitre of gastric digest was calculated from Equation (1):

$$\text{Pepsin U/mL} = (\text{ABS test} - \text{ABS blank}) * \text{DF} / (0.001\ \Delta\text{ABS} * 0\ \text{minutes} * 1\text{mL} \quad (1)$$

Pancreatin Protease Assay

The intestinal phase of the *in vitro* digestion system was set as described above. The protease activity of the intestinal digests was analysed immediately after the addition of activated pancreatin, and after 60, 120, and 180 minutes incubation. The pancreatic protease activity assay was extended to 180 minutes to simulate prolonged *in vitro* simulations of the intestinal phase as proposed by Minekus et al. [25].

Protease activity was measured by spectrophotometric stop-rate determination using the substrate casein, a method modified from Cupp-Enyard [36]. Bovine casein was combined with potassium phosphate buffer (0.65% w/w) (50mM potassium phosphate dibasic, trihydrate in deionised water), heated to 80°C ± 5°C for 10 minutes, cooled to 37°C and corrected to pH 7.5 with 1M NaOH. Aliquots of intestinal digest (1mL) were removed from the digestate without volume replacement and immediately combined with 5mL casein solution in duplicate and the reaction was incubated for 10 minutes at 37°C.

Protease activity was stopped by the addition of 5mL of 1.6% TCA. For the blank, 5mL TCA solution was combined with 5mL of casein solution prior to the addition of the intestinal digest. All samples were separated by centrifugation at 335xg for 7 minutes. Supernatant aliquots (2mL) were combined with 5mL sodium carbonate (5.3g/100mL deionised water) followed immediately by 1mL of Folin's reagent (0.5M). All samples were incubated for 30 minutes at 37°C and passed through disposable 0.22µm Minisart syringe filters.

Samples and blanks were measured by spectrophotometry at 660nm and the concentration of TCA-soluble peptides were calculated from a tyrosine standard curve ranging from 0 to 0.5µM tyrosine in deionised water. The concentration of pancreatic protease units per 1mL of intestinal digest was calculated from Equation (2):

$$(\mu\text{M tyrosine equivalents} * 11\text{mL}) / (1\text{mL digestate} * 10\ \text{minutes} * 2\text{mL}) \quad (2)$$

Statistical Analysis

Changes in pepsin or pancreatin activity per ml of digestate at each time point during the digestion were calculated as a percentage of the initial protease activity at time zero. Statistical analysis was performed using SAS 9.2 statistical software (SAS Institute Inc., Cary, NC, USA). Changes in pepsin and pancreatin activity were analysed by repeated measures ANOVA and linear regression. All analyses were undertaken using the general linear model and regression procedures. Where appropriate, post-hoc analysis was carried out using least square difference analysis.

RESULTS

Pepsin protease activity during the simulated gastric digestion phase is illustrated in *Figure 1*. Pepsin activity declined over the duration of the gastric digestion at a rate of 0.551% per minute (R^2 = 0.91, *p*<.0001). Compared to the initial activity of pepsin at time zero, pepsin activity was significantly decreased by 10% after 30 minutes incubation (*p* =.004), 18.5% by 60 minutes (*p* <.0001), 49% by 90 minutes (*p*<.0001) and 63% by 120 minutes incubation (*p*<.0001).

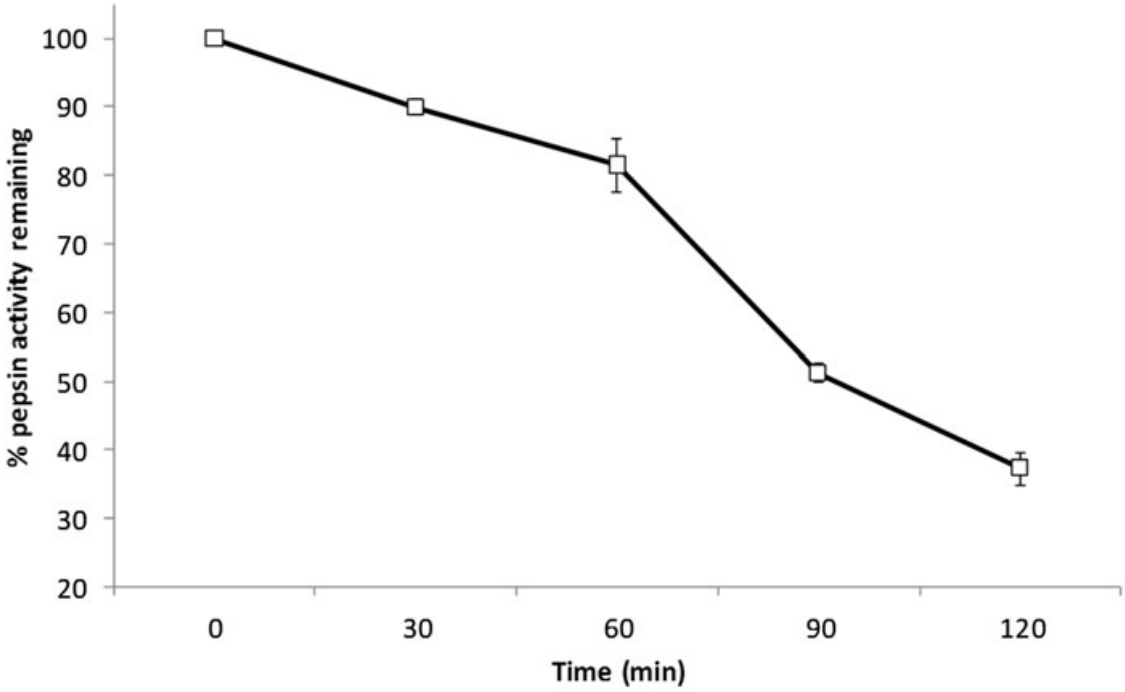

Figure 1 Pepsin Protease Activity During Simulated Gastric Digestion

Pepsin activity was measured by stop-rate determination in 1mL samples of gastric digestates. Samples were taken immediately after the addition of pepsin to the digest and after incubation at 37°C on a rocking shaker for 30, 60, 90, and 120 minutes. Data points represent mean values ± SEM. All data points are significantly different from one another *(p*<.05).

Pancreatin protease activity during the simulated intestinal digestion phase is illustrated in *Figure 2*. Pancreatic protease activity declined over the duration of the intestinal digestion at a rate of 0.188% per minute (R^2 = 0.951, p<.0001). Compared to the initial activity of pancreatin protease at time zero, pancreatin protease activity significantly decreased by 6% after 60 minutes incubation (p=.015), 21.5% by 120 minutes (p<.0001) and 32.5% by 180 minutes (p<.0001).

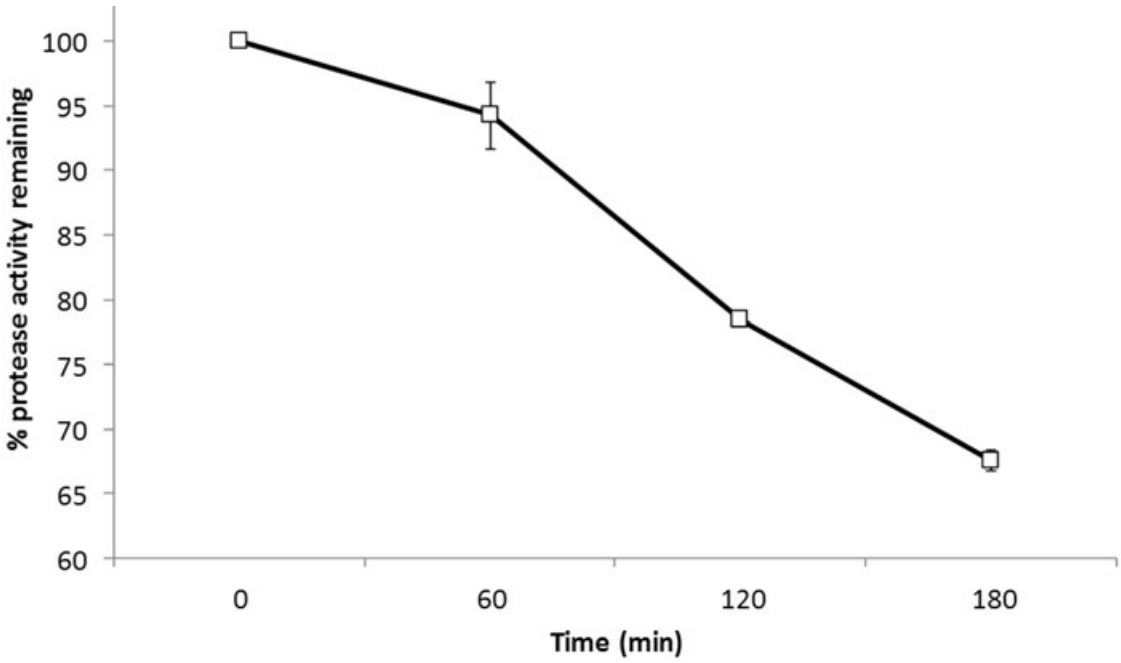

Figure 2 Pancreatin Protease Activity During Simulated Intestinal Digestion

Protease activity was measured by stop-rate determination in 1mL samples of intestinal digestates. Samples were taken immediately after the addition of pancreatin to the digest and after incubation at 37°C on a rocking shaker for 60, 120, and 180 minutes. Data points represent mean values ± SEM. All data points are significantly different from one another *(p*<.05).

DISCUSSION

In vitro digestion protocols developed for micronutrient bioavailability research typically pre-activate and combine pepsin with an acidified food slurry once at the initiation of the gastric phase of digestion [7,12,14,30]. This activation step may be performed immediately prior to digestion, or may be preceded by an intermediary step designed to remove non-specifically bound iron from the pepsin solution prior to combination with the food slurry [34].

Once activated, pepsin hydrolyses peptide bonds at the C-terminus of phenylalanine, leucine, tyrosine and tryptophan forming a range of polypeptide sequences which vary in length and composition to initiate protein digestion prior to gastric emptying [37]. In order for pepsin to hydrolyse proteins throughout the gastric phase of digestion *in vitro*, pepsin is continuously maintained at a pepsin: protein ratio of

approximately 1:20 weight for weight, respectively [38]. This ratio is also recommended when using commercial sources of pepsin *in vitro* [37].

The stomach maintains a pepsin concentration of approximately 0.08–0.8mg/mL during the consumption of a meal [38]. This is achieved by sequestering pepsinogen within chief cells during periods of fasting, and secreting pepsinogen during gastric distention, sensory or chemical cues within the proximal gastrointestinal tract [39]. By regulating pepsinogen secretion, the optimal pepsin: protein ratio can be maintained or exceeded during the process of digestion to facilitate gastric protein digestion.

However, the catalytic activity of pepsin has been previously reported to decrease under simulated gastric conditions *in vitro* [26]. This inhibitory effect has been reported to occur within 3 hours digestion duration over a range of pepsin concentrations between 0.05 and 0.2mg/mL in both the absence and presence of dietary protein. The authors reported that the decrease in protease activity was directly proportional to the number of hydrolytic products formed in the absence of dietary protein, suggesting that significant pepsin autolysis had occurred.

Porcine pepsin A contains up to 90 potential autolysis cleavage sequences [40]. Although many of these cleavage sequences will not be accessible for hydrolysis due to the enzyme's tertiary structure, the significant reduction in pepsin protease activity observed both in the current study and by Qiao and Gumpertz [26] suggests that substantial pepsin autolysis may be likely.

The gastric digestion conditions were specifically performed in the current study to simulate digestion conditions typically used for micronutrient bioavailability research [13,14,34,41]. The results also strongly suggest that pepsin autolysis occurs soon after it is activated when subjected to the digestion conditions commonly used for micronutrient bioavailability research, and decreased by over 100 enzyme units per milligram of anhydrous protease. Collectively, these results clearly indicate that the optimal pepsin–protein ratio may not be maintained over the duration of a simulated gastric digestion *in vitro*.

When simulating the intestinal phase of digestion *in vitro*, pancreatin is typically activated with bile salts under alkaline conditions prior to combination with the neutralised gastric digesta once at the initiation of the intestinal phase of digestion [5,14,34,41]. Pancreatin is a crude preparation of pancreatic secretions consisting of proteases, lipase, amylase and ribonuclease. Of all proteases within pancreatin, trypsin appears to be most abundant [27]. In order for pancreatic proteases to hydrolyse proteins throughout the intestinal phase of digestion *in vivo*, proteases are secreted from the pancreas at a rate of approximately 5 and 10kU per 30 minutes during the consumption of a meal [42], suggesting that the physiological pancreatin:protein ratio should be maintained at approximately 1:25 (w/w).

However, like pepsin, the catalytic activity of pancreatic proteases has been reported to significantly decrease under simulated gastric conditions *in vitro* over 180 minutes digestion duration [27]. The authors reported a decrease in protease activity over a range of pancreatin concentrations between 0.05 and 0.4mg/mL – a protease concentration range commonly used within the micronutrient bioavailability literature [13,14,34,41]. Like pepsin, the decrease in pancreatin protease activity also appears to

be directly proportional to the number of hydrolytic products formed, suggesting that significant pancreatin autolysis had occurred [27].

Porcine trypsin and chymotrypsin each contain up to 14 potential autolytic cleavage sequences [40]. As similarly described above for pepsin, although many of these cleavage sequences will not be accessible for hydrolysis due to the enzymes tertiary structure, the significant reduction in protease activity observed during the intestinal phase of digestion suggests that substantial pancreatin autolysis may be likely.

The intestinal digestion conditions were specifically performed in the current study to simulate digestion conditions typically used for micronutrient bioavailability research. The reduction in protease activity was less severe in the current study compared to the results of Qiao et al. [27] by approximately 20% at each time point; however, this may be due to a difference in digestion pH between the two studies. The intestinal phase of the current study was performed at pH 7 rather than pH 8 to simulate digestion conditions recommended for nutrition research [25]. These results indicate that like pepsin, the optimal pancreatic protease: protein ratio may not be maintained over the duration of a simulated intestinal digestion *in vitro*.

CONCLUSION

Overall, the results of this study suggest that both pepsin and pancreatic protease activities are significantly reduced under the simulated gastrointestinal conditions currently defined in the micronutrient bioavailability literature. Due to the quantifiable linear relationship between protease activity and time for both pepsin and pancreatin, the decrease in protease activity per minute can be predicted and corrected at allocated time points throughout the digestion to maintain physiological protease:protein ratios. The practicality of this suggestion requires further investigation over a range of *in vitro* digestion durations and protease concentrations.

Limitations

This study has several limitations. First, the number of time points for sampling could be increased to further investigate the relationship between protease activity and time both within the gastric and intestinal phases. Second, the effect of time on protease activity in the presence of food samples requires further characterisation to determine the rate of autolysis in the presence of other protein sources.

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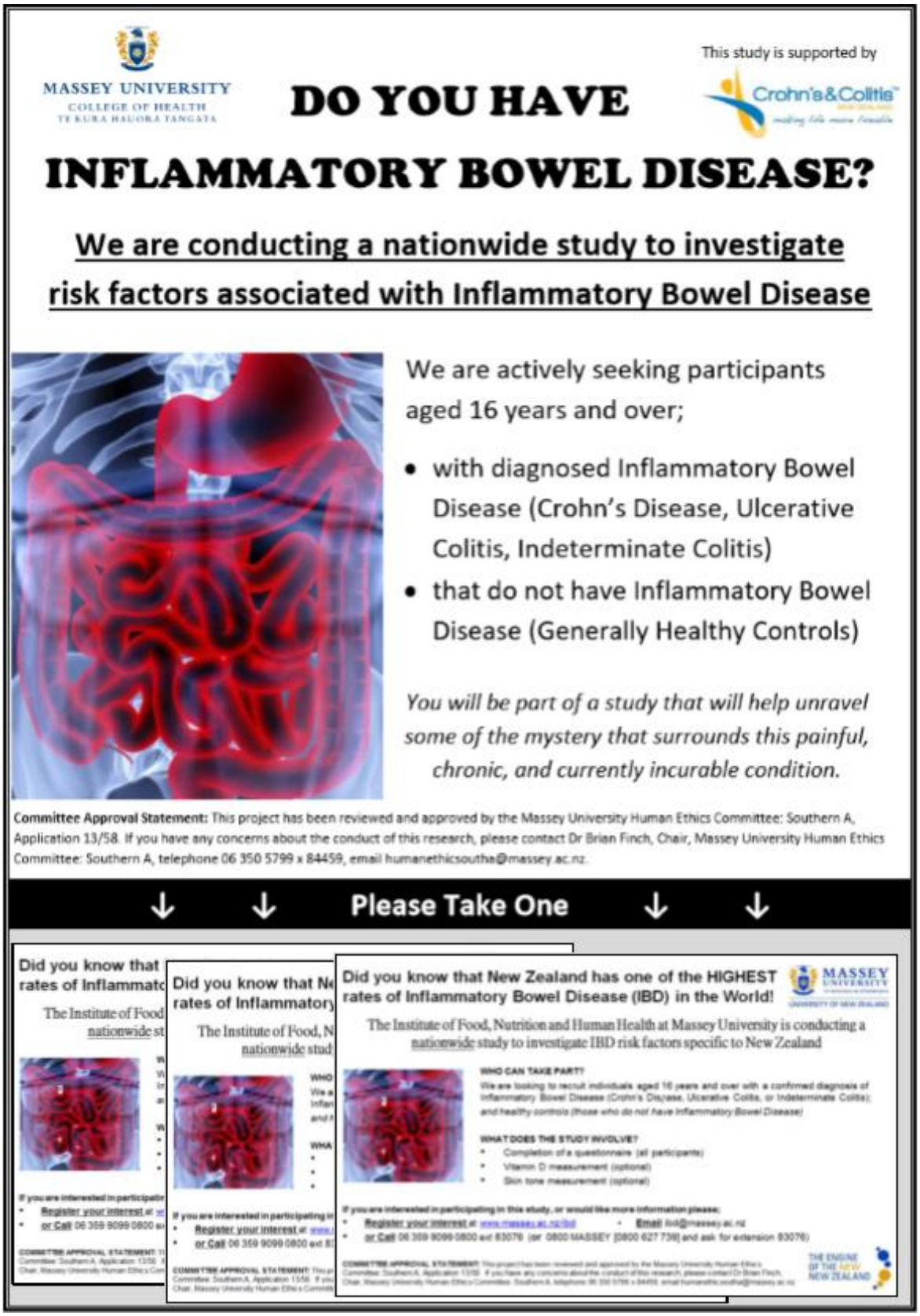
4. Participant Recruitment Advertisement



5. Participant Information Sheet

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Inflammatory Bowel Disease and Environmental Factor Study

INFORMATION SHEET

RESEARCHERS INTRODUCTION

This is a staff and student research project being conducted by myself, Hannah Morton (a nutrition PhD research student) and my supervisor Dr Jane Coad (an Associate Professor in human nutrition). We are both nutritionists working in the Institute of Food, Nutrition and Human Health, at Massey University.

The research study has three main aims;

1. To investigate the potential associations between risk of exposure to environmental factors (including a common bacteria and vitamin D level) in New Zealand residents with Inflammatory Bowel Disease (IBD) and healthy controls.
2. To identify foods associated with the triggering or exacerbation of IBD symptoms.
3. To determine the vitamin D level of New Zealand residents with Inflammatory Bowel Disease and healthy controls.

The secondary aims of the study are;

1. To compare two methods of determining skin tone; the Von Luschan's Chromatic Scale matched by the participant, and measurements obtained using a dermal spectrophotometer.
2. To compare measured vitamin D level to reported sun exposure behaviour and measured skin tone.

PROJECT DESCRIPTION AND INVITATION

Crohn's disease (CD) and ulcerative colitis (UC), the two major forms of Inflammatory Bowel Disease (IBD), are chronic diseases that affect the gastrointestinal tracts of humans.

IBD is often very difficult to manage and the effects are not only physical but social too. The impact of living with IBD was very recently investigated***** in a large group of New Zealanders and revealed the following;

- "One in four of those living with IBD have lost or quit a job due to IBD
- 64% of IBD patients need emergency care before they are diagnosed
- 57% of people with IBD report they have been hospitalised in the last 5 years due to IBD symptoms
- 51% of people describe their disease as not in remission"

For reasons which are unclear, the prevalence of IBD in New Zealand is one of the highest in the world and is continuing to increase. The cause of the disease is unknown but genetic, environmental, dietary, infectious and immunological factors are all thought to be involved.

The study investigating IBD is comprised of three parts; a questionnaire (*all participants*), vitamin D measurement and matching your skin tone to a colour chart (optional), and skin tone measurement by the researcher chart (optional).

Part A: Questionnaire (*All Participants*)

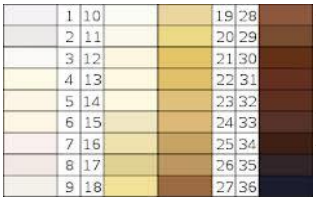
Using a self-administered questionnaire, we would like to investigate the possible associations between risk of exposure to environmental factors - including a common bacteria and estimated low vitamin D status - in New Zealand residents with IBD and healthy controls. The questionnaire will also be used to investigate the second primary aim, to identify foods associated with the triggering or exacerbation of IBD symptoms.

***** *IMPACT is a nationwide survey undertaken from May 2012 to April 2013 to reveal the impact of living with the chronic disease IBD*

Part B: Vitamin D Measurement and Matching Your Skin Tone (*Optional*)

Vitamin D is an essential nutrient which is acquired mainly from exposure of skin to sunlight and may have a role in regulation of the immune system. As the physical symptoms of IBD often suggest impaired immune system function we would like to determine whether vitamin D levels differ in New Zealand residents with IBD compared to healthy controls. Vitamin D levels will be measured using the blood spot method which is quick, easy and minimally invasive.

An individual's skin tone affects their ability to absorb the sunlight necessary for vitamin D production. If skin tone and reported sunlight exposure are known their vitamin D level can be predicted more accurately than on reported sunlight exposure alone. As skin tone varies we are asking all participants taking part in the measurement component of the study to also match their skin tone on the underside of both forearms to the closest corresponding colour on Von Luschan's Chromatic Scale.



Von Luschan's Chromatic Scale

Part C: Skin Tone Measurement (*Optional*)

I would like to compare two methods of determining skin tone in a subgroup of participants. The first, the Von Luschan's Chromatic Scale, is based on matching skin tone on the underside of both forearms to the corresponding colour on a colour card (as in Part B). The second, the dermal spectrophotometer, electronically measures colour by shining a small light on the skin.

We are inviting individuals with a confirmed IBD diagnosis, and generally healthy controls (who do not have IBD or a history of gastrointestinal complaints), who currently reside in New Zealand, to take part in this research study.

Participant Identification and Recruitment

Advertisements for this research study have been placed in New Zealand based gastroenterology departments/clinics, General Practitioner clinics and Māori Health Providers. Crohn's and Colitis New Zealand will also advertise the research study through their regional support groups.

We would like to invite two groups of participants to take part in this research study; ***Individuals with diagnosed Inflammatory Bowel Disease***, and generally ***Healthy Controls***.

Inflammatory Bowel Disease (IBD) group	*Healthy Control group*
Selection criteria: Individuals with diagnosed* Inflammatory Bowel Disease (Crohn's disease, ulcerative colitis, or IBD unclassified), who have predominantly grown up in New Zealand and/or have lived in New Zealand for the previous two years. Exclusion criteria: under 16 years of age or suspected (undiagnosed) Inflammatory Bowel Disease * by an appropriately qualified medical specialist eg Gastroenterologist, General Practitioner.	Selection criteria: Healthy individuals who have predominantly grown up in New Zealand and/or have lived in New Zealand for the previous two years. Exclusion criteria: family history of Inflammatory Bowel Disease or other conditions that affect the intestine such as colon cancer, coeliac disease, diverticular disease or Irritable Bowel Syndrome; or under 16 years of age.

If you decide to participate in this research project we encourage you to tell as many friends, family and colleagues about the research study so we can try and meet our participant target and obtain results That represent New Zealand's current population.

The research team consulted a Māori representative whilst planning this study. If you have any concerns please contact a member of the research team (contact details on final page under 'Project Contacts') who will be happy to discuss your concerns and undertake further consultation where applicable.

PROJECT PROCEDURES

Questionnaire

You will be asked to complete a questionnaire that contains general questions such as age and gender, questions specific to your childhood (0 – 12 years) such as milk consumption habits, and questions about your sun exposure in the last 12 months. Participants with diagnosed IBD will be asked to answer some additional questions about their condition such as when diagnosis took place, and whether they associate any foods with the triggering or exacerbation of their IBD symptoms.

The questionnaire also contains questions that some participants may consider to be of a personal nature. However participation in this research study is voluntary; if you decide to participate you have the right to decline to answer any question; and all information will automatically be assigned a unique study specific ID number and only identified by this number.

You can choose to complete the questionnaire in hard copy or via a secure online survey platform link. If you choose the 'hardcopy' you will be posted the questionnaire and a return addressed pre-paid envelope. The questionnaire has been pretested, we estimate the time involved to be; 15 - 35 minutes for the IBD participant group, and 10 – 25 minutes for the healthy control group.

Part B: Vitamin D Measurement and Matching Your Skin Tone (*Optional*)

You will be asked to provide a very small non-fasted blood sample using the blood spot method. This method involves cleaning the fingertip with an alcohol wipe, pricking the fingertip and spotting/blotting the blood onto two circles of a spot card. The small puncture will then be covered with a plaster. This method is much quicker and easier than the usual venous blood sample method, and the sample size required is significantly smaller (approximately quarter of a ml or less).

Blood Spot Card

Blood spot samples will be obtained in one of three locations depending on where you live;

- **Participants in the Manawatū** will be invited to have their blood spot sample taken at the Human Nutrition research unit at Massey University Palmerston North by the researcher Hannah Morton.
- **Participants outside the Manawatū** will be invited to have their blood spot sample taken at their work-place (where appropriate) or their nearest participating pharmacy by the researcher or a pharmacy staff member. The researcher will schedule pharmacy blood spot collection appointments.
- **Participants attending their Crohn's and Colitis support group meeting (where applicable**) will be invited to have their blood spot sample taken at the venue of their support group meeting by the researcher.

The finger prick can be done by the researcher/pharmacy staff member, who are fully trained in the procedure, or you can perform the finger prick with their assistance. The researcher/pharmacy staff member collecting the sample will wear sterile disposable gloves during the procedure, sterile disposable wipes will be used to clean the fingertip before the finger prick is done, and a sterile plaster will be placed on the fingertip where the blood spot was obtained from.

*** You are welcome to have a support person (whanau or friend) accompany you during your blood spot sample ***

You will be required to complete a consent form prior to providing your blood spot sample, which can be given to the researcher or pharmacy staff member at your appointment. Participants providing their sample at their nearest participating pharmacy will be provided with an envelope to seal their consent form in.
Along with your consent form you will be provided with a Von Luschan's Chromatic Scale card and user instructions. You will be asked to match your skin tone on the underside of both forearms to the closest corresponding colour on Von Luschan's Chromatic Scale, then place the completed card in the same envelope as your consent form to bring along to your blood spot sample appointment.

Part C: Skin Tone Measurement (*Optional*)

If you are able to have your blood spot sample taken you will also be invited to take part in the secondary aim of this research study – comparing two methods of determining skin tone. Determining skin tone will involve matching the skin tone on the underside of both your forearms to Von Luschan's Chromatic Scale. The researcher will then measure the skin tone in the same places using a portable dermal spectrophotometer which electronically measures colour by shining a small light on the skin. This process is painless and risk free. Both measurements will be carried out under light controlled conditions.

COMPENSATION AND TIME INVOLVED

Participants can choose to go in a random draw for 1 of 5 $100 dollar gift vouchers (the final question of the questionnaire contains this option). This will be drawn in February 2015 after data collection has been completed.
The time involved for each part of the research study is as follows;

- Part A Questionnaire: approximately 10 - 35 minutes
- Part B Blood spot sample approximately 3-5 minutes, your skin tone approximately 1-2 minutes
- Part C Measurement of skin tone: approximately 1 - 3 minutes

DISCOMFORTS OR RISKS AS A RESULT OF PARTICIPATION

The researcher/pharmacy staff member performing or advising about the finger prick will be fully trained to take blood spot samples using this method. The lancet used to prick the fingertip may cause mild discomfort during the time of application (less than one second), there is also a small possibility that the area where the sample was taken from will be slightly tender for a short time after the sample is taken. Participants will be offered a heat pad if they have cold hands as warming up cold hands will increase the blood flow and will reduce the risk of mild discomfort associated with use of the lancet. There is a small chance that the blood spots do not adequately cover the target areas on the collection card and a second blood spot will need to be obtained. We consider the likelihood of needing to perform a second collection very low.

DATA USE, STORAGE, AND DISPOSAL

All participants will be given a study specific ID number, and any reference to the data will be by this ID number only. Information received from participants will be stored in a locked filing cabinet in a locked office and will only be available to the researcher and primary supervisor. The electronic data will be stored on computers and servers, which are protected by passwords and will only be available to the researcher and primary supervisor. Data will be transferred to an official secure archive after 5 years and destroyed by them after the 10 year period. The data stored at this archive is identified by barcode and is accessible by nominated people who have pin numbers. Where blood spot samples are taken at a pharmacy, the pharmacy staff will also return your consent form, Von Luschan's Chromatic Scale card and questionnaire (if received by post) to the researchers. A separate envelope will be provided. Where blood spot samples are taken at the Human Nutrition research unit or the venue of your Crohn's and Colitis support group meeting, only the researcher will have access to the information provided on the consent form. All blood spot samples will be sent to the manufacturer (ZRT Laboratory, http://www.zrtlab.com) in the USA for vitamin D analysis. **PARTICIPANTS MAY REQUEST THE RETURN OF THEIR BLOOD SPOT CARD, OTHERWISE ALL BLOOD SPOT CARDS WILL BE STORED AT -20˚C FOR 90 DAYS THEN DISPOSED OF BY ZRT LABORATORY.** The blood spot cards will be labelled with the participant's unique study specific ID number and date of birth (as the laboratory analysing the cards requires two points of reference). No other identifying information will be sent to the manufacturer.

METHOD FOR ACCESSING A SUMMARY OF THE PROJECT FINDINGS

All participants will be posted a summary of the study results at the completion of the study. Information resulting from the project will also be shared through a report made available to Crohn's and Colitis New Zealand as well as peer-reviewed publications and seminars (for colleagues, professionals and the general public).

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

Researcher, PhD student Hannah Morton, h.morton1@massey.ac.nz or 06 350-4336 ext 83076
Primary supervisor, Associate Professor Jane Coad, j.coad@massey.ac.nz or 06 350-4336
If you have any questions about the research project please contact either of us.

Committee Approval Statement: This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 13/58. If you have any concerns about the conduct of this research, please contact Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799 x 84459, 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, travel 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.

6. Participant Information Sheet - Vitamin D Validation substudy

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Inflammatory Bowel Disease and Environmental Factor Study – Vitamin D Validation substudy

INFORMATION SHEET

This information sheet is supplementary to the information provided in the 'Inflammatory Bowel Disease and Environmental Study' information sheet and is being provided to a subset of participants based in the Manawatū and Auckland region that have expressed interest in providing a blood spot sample for analysis of vitamin D.

Description and Invitation

The blood spot method was been chosen to measure vitamin D levels because it is quick, easy and minimally invasive. As this method is not widely used we would like to undertake a small validation study, in a subset of participants, to compare vitamin D levels determined using the blood spot method with those determined using traditional venous blood sample.

We are inviting participants taking part in the Inflammatory Bowel Disease and Environmental Factor Study to also take part in this Vitamin D validation substudy.

Project Procedures

You will be asked provide a single 5ml non-fasted blood sample via venepuncture from a vein in your forearm. This sample will be taken at the Human Nutrition research unit at Massey University Palmerston North or Albany by a phlebotomy trained staff member.

You will be required to complete a consent form prior to providing your blood sample which can be given to the researcher immediately prior to the blood sample being taken, or to the phlebotomist taking the sample. You will also be provided with an envelope to seal their consent form in.
NOTE: this consent form is separate from the consent form relating to your blood spot sample.

*** You are welcome to have a support person (whanau or friend) accompany you during your blood sample ***

Exclusion Criteria

History of difficult veins.

Time Involved

The time involved for providing a blood sample is approximately 3-5 minutes.

Discomforts or Risks as a result of Participation

Having a blood sample taken via venepuncture can sometimes cause discomfort and there is a small chance of bruising.

DATA USE, STORAGE, AND DISPOSAL

All participants will be given the same specific ID number as in the main study, and any reference to the data will be by this ID number only. Information received from participants will be stored in a locked filing cabinet in a locked office and will only be available to the researcher and primary supervisor. The electronic data will be stored on computers and servers, which are protected by passwords and will only be available to the researcher and primary supervisor. Data will be transferred to an official secure archive after 5 years and destroyed by them after the 10 year period. The data stored at this archive is identified by barcode and is accessible by nominated people who have pin numbers.

The blood samples will be stored at -80˚C at Massey University until the minimum number of required samples (30) has been reached, they will then be sent to Canterbury Health Laboratories for vitamin D analysis. The blood samples will be labelled with the participant's unique study specific ID number only, no other identifying information will be sent to the Laboratory. Participants may request the return of their unused blood after vitamin D analysis has taken place, otherwise all blood samples will be disposed of by Canterbury Health Laboratories.

METHOD FOR ACCESSING A SUMMARY OF THE PROJECT FINDINGS

Participants that take part in this vitamin D validation substudy will be posted the outcome of their vitamin D test as determined from their venous blood sample. Participants with a vitamin D level below 12.5 nmol/L, recognised by the Ministry of Health as severe deficiency, will be referred to their medical practitioner.

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 during participation;*
- *provide information on the understanding that your name will not be used unless you give permission to the researcher;*

PROJECT CONTACTS

Researcher, PhD student Hannah Morton, h.morton1@massey.ac.nz or 06 350-4336 ext 83076
Primary supervisor, Associate Professor Jane Coad, j.coad@massey.ac.nz or 06 350-4336
If you have any questions about the research project please contact either of us.

Committee Approval Statement: This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 13/58. If you have any concerns about the conduct of this research, please contact Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799 x 84459, 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, travel 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.

7. Participant Screening Sheet

Study ID number

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Inflammatory Bowel Disease and Environmental Factor Study

SCREENING SHEET

	HEALTHY CONTROL GROUP	
1.	Where did you see the study advertised?	
2.	Are you 16 years of age or older?	**Yes** / No
3.	Did you predominantly live in New Zealand during your childhood (0-12 years)?	Yes / No
4.	Have you predominantly lived in New Zealand for the previous two years?	**Yes** / No
5.	Do you have a family history of Inflammatory Bowel Disease or any other condition that affects the intestine such as: - colon cancer, - coeliac disease, - diverticular disease, -or Irritable Bowel Syndrome?	Yes / **No**
6.	Do you carry any blood-borne diseases?	Yes / **No**
7.	Above criteria met?	Yes / No
8.	Participation outlined; - purpose of study - provision of information sheet - participation is voluntary - completion and consent of questionnaire implies consent	
9.	Invitation to participate accepted?	Yes / No

Or

	IBD GROUP	
1.	Where did you see the study advertised?	
2.	Are you 16 years of age or older?	**Yes** / No
3.	Did you predominantly live in New Zealand during your childhood (0-12 years)?	Yes / No
4.	Have you predominantly lived in New Zealand for the previous two years?	**Yes** / No
5.	Have you been diagnosed with IBD by a medical specialist?	**Yes** / No
6.	How was your IBD diagnosis made? (by an appropriately qualified medical specialist eg Gastroenterologist). _____	**Yes** / No
7.	Do you carry any blood-borne diseases?	Yes / **No**
8.	Above criteria met?	Yes / No
9.	Participation outlined; - purpose of study - provision of information sheet - participation is voluntary - completion and consent of questionnaire implies consent	
10.	Invitation to participate accepted?	Yes / No

Name:				Address:		
Email:						
Phone Number/s:						
Preferred contact method:		Phone		Email		Text message
Participant interested in:		Questionnaire				Vitamin D measurement
		Skin tone measurement				
Preferred Questionnaire Format:		Email				Post

8. Questionnaire – Participants with IBD

Study ID number

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Inflammatory Bowel Disease (IBD) and Environmental Factor Study Questionnaire

PARTICIPANT WITH INFLAMMATORY BOWEL DISEASE

First Name: Family Name:

Part 1: General Information and Outdoor Exposure

1. What is your gender? ☐ Male ☐ Female

2. What is your age? Years: _____

3. What is your height and weight? *(please list and tick (√) the relevant units)*
   Height: _____ cm ☐ ft ☐ in ☐
   Weight: _____ Kg ☐ lb ☐

4. What is your Ethnicity?
   NOTE: this information will only be used to describe the ethnic makeup of the study population. No analysis of this information will take place, nor will any specific numbers be disclosed.
   ☐ European ☐ Maori
   ☐ Pacific Peoples ☐ Asian
   ☐ Middle Eastern ☐ African
   ☐ Other (please specify) _____

5. Where was your place of birth?
   Rural Area/Town/City: _____
   Country (if outside NZ): _____

6. What was your method of birth?
   ☐ Natural (vaginal) ☐ Caesarean Section
   ☐ Not sure

7. As a baby (0-3 months), were you fed breast milk solely, formula solely, mixed (breast milk and formula), or cow's milk?
   ☐ Breast milk (solely) ☐ Pasteurised Cow's milk (solely)
   ☐ Formula Fed (solely) ☐ Non-Pasteurised Cow's milk (solely)
   ☐ Not sure
   ☐ Mixed (please specify)

The next 6 questions all refer to your childhood; 0 - 12 years of age inclusive

8. What were your place/s of residence during childhood (0-12 years)

(please start with the earliest place of residence)

(if you require more space please see the final pages of this

Rural Area/Town/City:
Country (if outside NZ):
Duration (months/years):
Farm? ☐ Yes ☐ No
Farm type (if applicable) eg dairy:

Rural Area/Town/City:
Country (if outside NZ):
Duration (months/years):
Farm? ☐ Yes ☐ No
Farm type (if applicable) eg dairy:

Rural Area/Town/City:
Country (if outside NZ):
Duration (months/years):
Farm? ☐ Yes ☐ No
Farm type (if applicable) eg dairy:

Rural Area/Town/City:
Country (if outside NZ):
Duration (months/years):
Farm? ☐ Yes ☐ No
Farm type (if applicable) eg dairy:

9. During your childhood (0-12 years), **excluding** your place/s of residence, did you spend a total of 5 or more days on farms? ☐ Yes ☐ No

- If **YES**, please complete the adjacent fields

(if you require more space please see the final pages of this questionnaire)

Farm type eg dairy
Duration (days/weeks per year):
Closest Town:
Country (if outside NZ):

Farm type eg dairy
Duration (days/weeks per year):
Closest Town:
Country (if outside NZ):
Farm type eg dairy

Duration (days/weeks per year):

Closest Town:

Country (if outside NZ):

10. In any 1 year during your childhood (0-12 years) did you have contact with farm animals on 5 or more occasions?
Eg petting, feeding, playing in areas the farm animals reside?
- If **YES**, please select which (if any) of animals from the adjacent list only

☐ Yes ☐ No

☐ Cattle (dairy) ☐ Goats
☐ Cattle (non-dairy) ☐ Deer
☐ Sheep ☐ Rabbits

11. In any 1 year during your childhood (0-12 years) did you spend time handling or playing in dirt/soil on 5 or more occasions?
- If **YES**, please select the activities you took part in from the adjacent list

☐ Yes ☐ No

☐ Digging ☐ Hand washing potatoes
☐ Gardening ☐ Clay modelling
☐ Mud slide ☐ Other (please specify)

12. In any 1 year during your childhood (0-12 years) did you spend time in fresh water creeks, rivers, dams, lakes, ponds, or waterways on 5 or more occasions?
- If **YES**, please select the activities you took part in from the adjacent list

☐ Yes ☐ No

☐ Swimming ☐ Paddling
☐ Fishing ☐ Playing eg dam building
☐ Other (please specify)

13. During your childhood (0-12 years), did you participate in hunting where any form of animal handling took place?
- If **YES**, please select which (if any) animals from the adjacent list

☐ Yes ☐ No

☐ Cattle (dairy) ☐ Goats
☐ Cattle (non-dairy) ☐ Deer
☐ Sheep ☐ Rabbits

14. What form/s of Inflammatory Bowel Disease have you been diagnosed with?

☐ Crohn's Disease ☐ Ulcerative Colitis
☐ Indeterminate Colitis ☐ Other (please specify)

15. When were you diagnosed with IBD? Month: Year:

16. When did your IBD symptoms begin? Month: Year:

17. Do you have a family history of IBD? ☐ Yes ☐ No

- If **YES**, please list the number of family members affected, the form of IBD (Crohn's Disease, Ulcerative Colitis, or Indeterminate Colitis), and tick (√) the relevant box for immediate family (IF) or extended family (EF).
  Immediate Family (IF): Parent, child, sibling
  Extended Family (EF): Grandparent, Aunt, Cousin, Nephew

Number	Form of IBD	IF	EF

The next 6 questions all refer to the 10 years before your Inflammatory Bowel Disease symptoms started

18. In the 10 years before your IBD symptoms started, where were your place/s of residence?
(please start with the most recent place of residence)
(if you require more space please see the final pages of this questionnaire)

Rural Area/Town/City:
Country (if outside NZ):
Duration (months/years):
Farm? ☐ Yes ☐ No
Farm type (if applicable) eg dairy:

Rural Area/Town/City:
Country (if outside NZ):
Duration (months/years):
Farm? ☐ Yes ☐ No
Farm type (if applicable) eg dairy:

Rural Area/Town/City:
Country (if outside NZ):
Duration (months/years):
Farm? ☐ Yes ☐ No
Farm type (if applicable) eg dairy:

Rural Area/Town/City:
Country (if outside NZ):
Duration (months/years):
Farm? ☐ Yes ☐ No
Farm type (if applicable) eg dairy:

19. In the 10 years before your IBD symptoms started, **excluding** your place/s of residence, did you spend a total of 5 or more days on farms? ☐ Yes ☐ No

- If **YES**, please complete the adjacent fields
  (if you require more space please see the final pages of this questionnaire)

Farm type eg dairy
Duration (days/weeks per year)
Closest Town
Country (if outside NZ)
Farm type eg dairy
Duration (days/weeks per year)
Closest Town
Country (if outside NZ)
Farm type eg dairy
Duration (days/weeks per year)
Closest Town
Country (if outside NZ)

20. In any <u>1 year</u> during the 10 years before your IBD symptoms started did you have any contact with farm animals on 5 or more occasions? Eg petting, feeding, working in areas the farm animals reside

- If **YES**, please select which (if any) animals from the adjacent list

☐ Yes ☐ No

☐ Cattle (dairy) ☐ Goats
☐ Cattle (non-dairy) ☐ Deer
☐ Sheep ☐ Rabbits

21. In any <u>1 year</u> during the 10 years before your IBD symptoms started did you spend time handling or playing in dirt/soil on 5 or more occasions?

- If **YES**, please select which activities you took part in from the adjacent list

☐ Yes ☐ No

☐ Digging ☐ Hand-washing potatoes
☐ Gardening ☐ Clay modelling
☐ Mud slide ☐ Other (please specify)

22. In any <u>1 year</u> during the 10 years before your IBD symptoms started did you spend time in fresh water creeks, rivers, dams, lakes, ponds, or waterways on 5 or more occasions?

- If **YES**, please select the activities you took part in from the adjacent list

☐ Yes ☐ No

☐ Swimming ☐ Paddling
☐ Fishing ☐ Playing eg dam building
☐ Other (please specify)

23. In the 10 years before your IBD symptoms started, did you participate in any hunting where any form of animal handling took place?

- If **YES**, please select which (if any) animals were handled from the adjacent list

☐ Yes ☐ No

☐ Cattle (dairy) ☐ Goats
☐ Cattle (non-dairy) ☐ Deer
☐ Sheep ☐ Rabbits

24. Do you currently smoke (cigarettes or any other form of tobacco)? ☐ Yes ☐ No

- If **YES**, approximately how long have you been smoking? Months: _____ Years: _____

25. During the 10 years before the onset of your IBD symptoms, did you smoke (cigarettes or any other form of tobacco)? ☐ Yes ☐ No

- If **YES**, approximately how long before the onset of your IBD symptoms did you start smoking? Months: _____ Years: _____

  When did you stop smoking? Month: _____ Year: _____

- If **NO**, during the 10 years before the onset of your IBD symptoms, were you exposed to smoke? (cigarette or other tobacco related) ie passive smoking ☐ Yes ☐ No

  On average how would you rate this exposure? *(please select one option only)*

  ☐ Very infrequent (< 1 exposure per month)
  ☐ Infrequent (> 1 exposure per month)
  ☐ Frequent (> 1 exposure per week)
  ☐ Very frequent (> 1 exposure per day)

26. Have you experienced active IBD during the previous 12 months? ☐ Yes ☐ No (I have remained in remission)

- If **YES**, please tick (√) the active disease category/categories that apply, and the number of occurrences for each disease state selected

	√	Number of distinct occurrences during the last 12 months	Total duration during the last 12 months (eg xx days or weeks or months)
- Fluctuating-Active	☐		
- Flare-ups	☐		
- Constant-Active	☐		
- Other (please specify)	☐		

_____

Question 27 is for females participants only, male participants please turn the page and proceed to Part 2

27. <u>Female participants only</u>: During the 10 years before the onset of your IBD symptoms, did you take the oral contraceptive? ☐ Yes ☐ No

- If **YES**, please list duration of each occasion

	Months	Years
	Months	Years
	Months	Years

Part 2: UV Exposure and Diet

28. Have you been on holiday in the last 12 months? ☐ Yes ☐ No

If **YES**, please complete the adjacent fields including whether or not you think you think you had increased sun exposure than you would have if you had not been been on holiday

(if you require more space please see the final pages of this questionnaire)

Rural Area/Town/City:	
Duration (days/weeks):	
Country (if outside NZ)	
Increased sun exposure?	☐ Yes ☐ No
Rural Area/Town/City:	
Duration (days/weeks):	
Country (if outside NZ)	
Increased sun exposure?	☐ Yes ☐ No

29. In the **last year**, on average, how much time did you spend **outdoors** (minutes or hours) per **week** in; (please consider hobbies, work, gardening, etc)

	Minutes	Or	Hours
- **Winter** (June – August)			
- **Summer** (December – February)			

30. In the **last year** have you used a sunbed?

☐ No ☐ Yes, 10 sessions or less ☐ Yes, 11-20 sessions ☐ Yes, 20 – 30 sessions ☐ Yes, 30 sessions or more

31. When I am **outside** in **winter** I; (please tick (√) the relevant box)

	Almost never / never	A few times	Sometimes	Most of the time	Almost always / always
- Wear a sunhat?					
- Wear sunscreen?					
- Spend time in the sun? (but do not consider it sunbathing)					

32. When I am outside in **summer** I; (please tick (√) the relevant box)

	Almost never / never	A few times	Sometimes	Most of the time	Almost always / always
- Wear a sunhat?					
- Wear sunscreen?					
- Wear sunscreen AND reapply it according to the label instructions?					
- Avoid sun exposure by seeking shade?					
- Spend time in the sun? (but do not consider it sunbathing)					
- Sunbathe?					
- Get sunburnt?					

33. Do you currently take, or in the last 6 months have you taken, supplements that contain Vitamin D?

- ☐ Yes, I currently take supplements that contain Vitamin D
- ☐ Yes, in the last 6 months I have taken supplements that contained Vitamin D
- ☐ No, I do not currently take, or in the last 6 months I have not taken, supplements contain Vitamin D

If **YES**, please supply the following details from the product packaging:

- Manufacturer? _____
- Recommended dosage? eg xx IU _____
- How many do you take? _____
- How often do you take them? eg daily _____
- How long have you been taking them? Weeks: Months: Years:
- Why did you begin taking them? _____
- Other information? _____

34. Has your vitamin D level been measured in the past year? ☐ Yes ☐ No

- If **YES**, when (please list the date of the most recent measurement) and the outcome (if known) Month: Year:

Outcome (eg xx $25(OH)D_3$ / xx $1,25(OH)D_3$ / deficient / insufficient / adequate)

The next 3 questions all refer to your childhood; 0 - 12 years of age inclusive

35. During your childhood (0-12 years), what type of cow's milk did you predominantly consume?
(please select <u>one</u> option only)

- ☐ Pasteurised
- ☐ Un-Pasteurised
- ☐ Organic
- ☐ None (please proceed to question **37**)

36. During your childhood (0-12 years), approximately how much cow's milk did you drink **weekly**?
(please select <u>one</u> option only)

- ☐ > 6 glasses
- ☐ 4-6 glasses
- ☐ 1-3 glasses
- ☐ < 1 glass

37. During your childhood (0-12 years), what was your predominant source of drinking and cooking water?
(please select <u>one</u> option only)

- ☐ Main water supply
- ☐ Tank (rain water)
- ☐ Bore
- ☐ Tank (supplied)
- ☐ Other (please specify) _____

The next 3 questions all refer to the 10 years before your Inflammatory Bowel Disease symptoms started

38. In the 10 years before you IBD symptoms started, what type of cow's milk did you predominantly consume?
(please select one option only)

- ☐ Pasteurised
- ☐ Un-Pasteurised
- ☐ Organic
- ☐ None (please proceed to question **40**)

39. In the 10 years before you IBD symptoms started, approximately how much cow's milk did you drink **weekly**?
(please select one option only)

- ☐ > 6 glasses
- ☐ 4-6 glasses
- ☐ 1-3 glasses
- ☐ < 1 glass

40. In the 10 years before you IBD symptoms started, what was your predominant source of drinking and cooking water?
(please select one option only)

- ☐ Main water supply
- ☐ Tank (rain water)
- ☐ Bore
- ☐ Tank (supplied)
- ☐ Other (please specify)

41. Do you associate any foods or food combinations (excluding allergies or intolerances **that cause discomfort DISTINCT from your IBD symptoms**) with;

- **onset** of your IBD symptoms? (appendix **1**) ☐ Yes ☐ No
- **worsening** of your IBD symptoms? (appendix **2**) ☐ Yes ☐ No
- **reducing** the severity of your IBD symptoms? (appendix **3**) ☐ Yes ☐ No

If **YES**, to any of these questions, please refer to the associated appendix at the back of this questionnaire

42. Would you be happy for the researcher to contact you to clarify any answers you have provided (if required)? ☐ Yes ☐ No

43. Would you like your name to be put in the random draw (only for participants in this study) for 1 of 5 $100 gift vouchers? ☐ Yes ☐ No thank you

THANK YOU FOR TAKING THE TIME TO COMPLETE THIS QUESTIONNAIRE

APPENDIX 1: Foods or Food Combinations you associate with the **ONSET** of your IBD symptoms

Please tick (√) the food/s you associate with the **onset** of your IBD symptoms (excluding allergies/intolerances that cause discomfort **DISTINCT** from your IBD symptoms) and rate the effect of the food/s (1 = little effect 2 = moderate effect 3 = definite effect), and/or list other foods and/or food combinations below this table.

√	FRUIT	1	2	3
	Apple			
	Apricot			
	Avocado			
	Banana			
	Cherry			
	Grapes			
	Grapefruit			
	Kiwifruit			
	Mandarin			
	Nectarine			
	Orange			
	Peach			
	Pear			
	Pineapple			
	Plum			
	Strawberry			
√	VEGETABLES	1	2	3
	Asparagus			
	Beans			
	Beetroot			
	Broccoli			
	Brussels Sprouts			
	Cabbage			
	Carrot			
	Capsicum			
	Cauliflower			
	Celery			
	Chickpeas			
	Chilli			
	Corn			
	Courgette			
	Cucumber			
	Garlic			
	Kumara			
	Leek			
	Lentils			
	Lettuce			
	Mushroom			
	Onion			
	Parsnip			
	Pumpkin			
	Spinach			
	Tomato			

√	NUTS/SEEDS/ DRIED FRUIT	1	2	3
	Almond			
	Apricot			
	Brazil			
	Cashew			
	Cranberry			
	Date			
	Fig			
	Hazelnut			
	Macadamia			
	Peanut			
	Pine nut			
	Pistachio			
	Pumpkin			
	Raisin			
	Sunflower			
	Walnut			
√	GRAINS	1	2	3
	Barley			
	Oats			
	Popcorn			
	Rice			
	Wheat			
√	BREAD	1	2	3
	Brown			
	Full-grain			
	Gluten-free			
	White			
	Wholemeal			
√	CEREALS	1	2	3
	Bran based			
	Corn based			
	Rice based			
	Wheat based			
	Muesli			
√	MISC	1	2	3
	Eggs			
	Pastry			
	Tobacco			

√	DAIRY PRODUCTS	1	2	3
	Butter			
	Cheese – hard			
	Cheese – soft			
	Cream			
	Ice-cream			
	Cow's Milk – light			
	Cow's Milk – whole			
	Yoghurt – fruit			
	Yoghurt – dairy food eg caramel			
√	MEAT	1	2	3
	Beef			
	Chicken			
	Fish – non oily eg hoki, snapper			
	Fish – oily eg salmon, tuna			
	Lamb			
	Pork			
	Processed eg salami, luncheon			
	Seafood eg mussels			
	Turkey			
	Veal			
√	SAUCES	1	2	3
	Barbeque			
	Chilli			
	Chutney			
	Mayonnaise			
	Maple syrup			
	Salad dressing			
	Tomato			
√	SPREADS	1	2	3
	Honey			
	Jam			
	Margarine			
	Marmalade			
	Peanut Butter			
	Marmite or Vegemite			

√	SWEETS/SNACKS	1	2	3
	Biscuits			
	Cake			
	Chocolate			
	Crackers			
	Corn chips			
	Dips			
	Lollies			
	Muesli bars			
	Pizza			
	Potato chips			
√	BEVERAGES	1	2	3
	Alcohol – any			
	Beer			
	Coffee			
	Energy drinks			
	Fruit juice			
	Hot Chocolate			
	Milo			
	Soft drinks - any			
	Soft drinks – sugar free			
	Spirits			
	Tea – black			
	Tea – herbal			
	Wine			
√	ADDITIVES	1	2	3
	Artificial Sweetener			
	Food colouring			
	Herbs			
	Pepper			
	Salt			
	Spices			
	Sugar			
√	COOKING METHODS	1	2	3
	Baked			
	Deep Fried			
	Fried			
	Grilled			

Other Foods and/or Food Combinations:

APPENDIX 2: Foods or Food Combinations you associate with the **WORSENING** of your IBD symptoms

Please tick (√) the food/s you associate with the **worsening** of your IBD symptoms (excluding allergies/intolerances that cause discomfort **DISTINCT** from your IBD symptoms) and rate the effect of the food/s (1 = little effect 2 = moderate effect 3 = definite effect), and/or list other foods and/or food combinations below this table.

√	FRUIT	1	2	3
	Apple			
	Apricot			
	Avocado			
	Banana			
	Cherry			
	Grapes			
	Grapefruit			
	Kiwifruit			
	Mandarin			
	Nectarine			
	Orange			
	Peach			
	Pear			
	Pineapple			
	Plum			
	Strawberry			

√	VEGETABLES	1	2	3
	Asparagus			
	Beans			
	Beetroot			
	Broccoli			
	Brussels Sprouts			
	Cabbage			
	Carrot			
	Capsicum			
	Cauliflower			
	Celery			
	Chickpeas			
	Chilli			
	Corn			
	Courgette			
	Cucumber			
	Garlic			
	Kumara			
	Leek			
	Lentils			
	Lettuce			
	Mushroom			
	Onion			
	Parsnip			
	Pumpkin			
	Spinach			
	Tomato			

√	NUTS/SEEDS/ DRIED FRUIT	1	2	3
	Almond			
	Apricot			
	Brazil			
	Cashew			
	Cranberry			
	Date			
	Fig			
	Hazelnut			
	Macadamia			
	Peanut			
	Pine nut			
	Pistachio			
	Pumpkin			
	Raisin			
	Sunflower			
	Walnut			

√	GRAINS	1	2	3
	Barley			
	Oats			
	Popcorn			
	Rice			
	Wheat			

√	BREAD	1	2	3
	Brown			
	Full-grain			
	Gluten-free			
	White			
	Wholemeal			

√	CEREALS	1	2	3
	Bran based			
	Corn based			
	Rice based			
	Wheat based			
	Muesli			

√	MISC	1	2	3
	Eggs			
	Pastry			
	Tobacco			

√	DAIRY PRODUCTS	1	2	3
	Butter			
	Cheese – hard			
	Cheese – soft			
	Cream			
	Ice-cream			
	Cow's Milk – light			
	Cow's Milk – whole			
	Yoghurt – fruit			
	Yoghurt – dairy food eg caramel			

√	MEAT	1	2	3
	Beef			
	Chicken			
	Fish – non oily eg hoki, snapper			
	Fish – oily eg salmon, tuna			
	Lamb			
	Pork			
	Processed eg salami, luncheon			
	Seafood eg mussels			
	Turkey			
	Veal			

√	SAUCES	1	2	3
	Barbeque			
	Chilli			
	Chutney			
	Mayonnaise			
	Maple syrup			
	Salad dressing			
	Tomato			

√	SPREADS	1	2	3
	Honey			
	Jam			
	Margarine			
	Marmalade			
	Peanut Butter			
	Marmite or Vegemite			

√	SWEETS/SNACKS	1	2	3
	Biscuits			
	Cake			
	Chocolate			
	Crackers			
	Corn chips			
	Dips			
	Lollies			
	Muesli bars			
	Pizza			
	Potato chips			

√	BEVERAGES	1	2	3
	Alcohol – any			
	Beer			
	Coffee			
	Energy drinks			
	Fruit juice			
	Hot Chocolate			
	Milo			
	Soft drinks - any			
	Soft drinks – sugar free			
	Spirits			
	Tea – black			
	Tea – herbal			
	Wine			

√	ADDITIVES	1	2	3
	Artificial Sweetener			
	Food colouring			
	Herbs			
	Pepper			
	Salt			
	Spices			
	Sugar			

√	COOKING METHODS	1	2	3
	Baked			
	Deep Fried			
	Fried			
	Grilled			

Other Foods and/or Food Combinations:

APPENDIX 3: Foods or Food Combinations you associate with **REDUCING** the severity of your IBD symptoms

Please tick (√) the food/s you associate with **reducing** your IBD symptoms and rate the effect of the food/s (**1 = little effect 2 = moderate effect 3 = definite effect**), and/or list other foods and/or food combinations below this table.

√	FRUIT	1	2	3
	Apple			
	Apricot			
	Avocado			
	Banana			
	Cherry			
	Grapes			
	Grapefruit			
	Kiwifruit			
	Mandarin			
	Nectarine			
	Orange			
	Peach			
	Pear			
	Pineapple			
	Plum			
	Strawberry			
√	VEGETABLES	1	2	3
	Asparagus			
	Beans			
	Beetroot			
	Broccoli			
	Brussels Sprouts			
	Cabbage			
	Carrot			
	Capsicum			
	Cauliflower			
	Celery			
	Chickpeas			
	Chilli			
	Corn			
	Courgette			
	Cucumber			
	Garlic			
	Kumara			
	Leek			
	Lentils			
	Lettuce			
	Mushroom			
	Onion			
	Parsnip			
	Pumpkin			
	Spinach			
	Tomato			

√	NUTS/SEEDS/ DRIED FRUIT	1	2	3
	Almond			
	Apricot			
	Brazil			
	Cashew			
	Cranberry			
	Date			
	Fig			
	Hazelnut			
	Macadamia			
	Peanut			
	Pine nut			
	Pistachio			
	Pumpkin			
	Raisin			
	Sunflower			
	Walnut			
√	GRAINS	1	2	3
	Barley			
	Oats			
	Popcorn			
	Rice			
	Wheat			
√	BREAD	1	2	3
	Brown			
	Full-grain			
	Gluten-free			
	White			
	Wholemeal			
√	CEREALS	1	2	3
	Bran based			
	Corn based			
	Rice based			
	Wheat based			
	Muesli			
√	MISC	1	2	3
	Eggs			
	Pastry			
	Tobacco			

√	DAIRY PRODUCTS	1	2	3
	Butter			
	Cheese – hard			
	Cheese – soft			
	Cream			
	Ice-cream			
	Cow's Milk – light			
	Cow's Milk – whole			
	Yoghurt – fruit			
	Yoghurt – dairy food eg caramel			
√	MEAT	1	2	3
	Beef			
	Chicken			
	Fish – non oily eg hoki, snapper			
	Fish – oily eg salmon, tuna			
	Lamb			
	Pork			
	Processed eg salami, luncheon			
	Seafood eg mussels			
	Turkey			
	Veal			
√	SAUCES	1	2	3
	Barbeque			
	Chilli			
	Chutney			
	Mayonnaise			
	Maple syrup			
	Salad dressing			
	Tomato			
√	SPREADS	1	2	3
	Honey			
	Jam			
	Margarine			
	Marmalade			
	Peanut Butter			
	Marmite or Vegemite			

√	SWEETS/SNACKS	1	2	3
	Biscuits			
	Cake			
	Chocolate			
	Crackers			
	Corn chips			
	Dips			
	Lollies			
	Muesli bars			
	Pizza			
	Potato chips			
√	BEVERAGES	1	2	3
	Alcohol – any			
	Beer			
	Coffee			
	Energy drinks			
	Fruit juice			
	Hot Chocolate			
	Milo			
	Soft drinks - any			
	Soft drinks – sugar free			
	Spirits			
	Tea – black			
	Tea – herbal			
	Wine			
√	ADDITIVES	1	2	3
	Artificial Sweetener			
	Food colouring			
	Herbs			
	Pepper			
	Salt			
	Spices			
	Sugar			
√	COOKING METHODS	1	2	3
	Baked			
	Deep Fried			
	Fried			
	Grilled			

Other Foods and/or Food Combinations:

Extra space if required (please list the question number your answer relates to)

QUESTION	ANSWER

9. Questionnaire – Participants without IBD (Healthy Controls)

Study ID number

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Inflammatory Bowel Disease (IBD) and Environmental Factor Study Questionnaire

HEALTHY CONTROL

First Name: Family Name:

Part 1: General Information and Outdoor Exposure

1. What is your gender? ☐ Male ☐ Female

2. What is your age? Years: _____

3. What is your height and weight? *(please list and tick (√) the relevant units)*
   Height: _____ cm ☐ ft ☐ in ☐
   Weight: _____ kg ☐ lb ☐

4. What is your Ethnicity?
   NOTE: this information will only be used to describe the ethnic makeup of the study population. No analysis of this information will take place, nor will any specific numbers be disclosed.
   ☐ European ☐ Maori
   ☐ Pacific Peoples ☐ Asian
   ☐ Middle Eastern ☐ African
   ☐ Other (please specify)
   _____

5. Where was your place of birth?
   Rural Area/Town/City: _____
   Country (if outside NZ): _____

6. What was your method of birth?
   ☐ Natural (vaginal) ☐ Caesarean Section
   ☐ Not sure

7. As a baby (0-3 months), were you fed breast milk solely, formula solely, mixed (breast milk and formula), or cow's milk?
   ☐ Breast milk (solely) ☐ Pasteurised Cow's milk (solely)
   ☐ Formula Fed (solely) ☐ Non-Pasteurised Cow's milk (solely)
   ☐ Not sure
   ☐ Mixed (please specify)

The next 6 questions refer to your childhood; 0 - 12 years of age inclusive

8. What were your place/s of residence during childhood (0-12 years)
(please start with the earliest place of residence)
(if you require more space please see the final pages of this questionnaire)

Rural Area/Town/City:
Country (if outside NZ):
Duration (months/years):
Farm? ☐ Yes ☐ No
Farm type (if applicable) eg dairy:

Rural Area/Town/City:
Country (if outside NZ):
Duration (months/years):
Farm? ☐ Yes ☐ No
Farm type (if applicable) eg dairy:

Rural Area/Town/City:
Country (if outside NZ):
Duration (months/years):
Farm? ☐ Yes ☐ No
Farm type (if applicable) eg dairy:

9. During your childhood (0-12 years), **excluding** your place/s of residence, did you spend a total of 5 or more days on farms?
If **YES**, please complete the adjacent fields
(if you require more space please see the final pages of this questionnaire)

☐ Yes ☐ No

Farm type eg dairy:
Duration (days/weeks per year):
Rural Area:
Country (if outside NZ):

Farm type eg dairy:
Duration (days/weeks per year):
Rural Area:
Country (if outside NZ):

Farm type eg dairy:
Duration (days/weeks per year):
Rural Area:
Country (if outside NZ):

10. In any 1 year during your childhood (0-12 years) did you have contact with farm animals on 5 or more occasions?

☐ Yes ☐ No

Eg petting, feeding, playing in areas the farm animals reside?
If **YES**, please select which (if any) animals from the adjacent list.

- ☐ Cattle (dairy)
- ☐ Cattle (beef)
- ☐ Sheep
- ☐ Goats
- ☐ Deer
- ☐ Rabbits

11. In any 1 year during your childhood (0-12 years) did you spend time handling or playing in dirt/soil on 5 or more occasions?
If **YES**, please select the activities you took part in from the adjacent list

- ☐ Yes
- ☐ No

- ☐ Digging
- ☐ Gardening
- ☐ Mud slide
- ☐ Hand-washing potatoes
- ☐ Clay modelling
- ☐ Other (please specify)

12. In any 1 year during your childhood (0-12 years) did you spend time in fresh water creeks, rivers, dams, lakes, ponds, or waterways on 5 or more occasions?
If **YES**, please select the activities you took part in from the adjacent list

- ☐ Yes
- ☐ No

- ☐ Swimming
- ☐ Fishing
- ☐ Other (please specify)
- ☐ Paddling
- ☐ Playing eg dam building

13. During your childhood (0-12 years), did you participate in hunting where any form of animal handling took place?
If **YES**, please select which (if any) animals were handled from the adjacent list

- ☐ Yes
- ☐ No

- ☐ Cattle (dairy)
- ☐ Cattle (non-dairy)
- ☐ Sheep
- ☐ Goats
- ☐ Deer
- ☐ Rabbits

The next 6 questions refer to the last 10 years eg 2003 – 2013

14. During the last 10 years, where were your place/s of residence?
(please start with your current place of residence)
(if you require more space please see the final pages of this questionnaire)

Rural Area/Town/City:				
Country (if outside NZ):				
Duration (months/years):				
Farm?	☐	Yes	☐	No
Farm type (if applicable) eg dairy:				

Rural Area/Town/City:				
Country (if outside NZ):				
Duration (months/years):				
Farm?	☐	Yes	☐	No
Farm type (if applicable) eg dairy:				

	Rural Area/Town/City:
	Country (if outside NZ):
	Duration (months/years):
	Farm? ☐ Yes ☐ No
	Farm type (if applicable) eg dairy:
	Rural Area/Town/City:
	Country (if outside NZ):
	Duration (months/years):
	Farm? ☐ Yes ☐ No
	Farm type (if applicable) eg dairy:
15. During the last 10 years, **excluding** your place/s of residence have you spent a total of 5 or more days on farms? If **YES**, please complete the adjacent fields *(if you require more space please see the final pages of this questionnaire)*	☐ Yes ☐ No Farm type eg dairy: Duration (days/weeks per year): Rural Area: Country (if outside NZ): Farm type eg dairy: Duration (days/weeks per year): Rural Area: Country (if outside NZ): Farm type eg dairy: Duration (days/weeks per year): Rural Area: Country (if outside NZ):
16. In any 1 year during the last 10 years have you had contact with farm animals on 5 or more occasions? Eg petting, feeding, playing in areas the farm animals reside If **YES**, please select which (if any) of animals from the adjacent list.	☐ Yes ☐ No ☐ Cattle (dairy) ☐ Goats ☐ Cattle (non-dairy) ☐ Deer ☐ Sheep ☐ Rabbits
17. In any 1 year during the last 10 years have you spent time handling or playing in dirt/soil on 5 or more occasions? If **YES**, please select the activities you took part in from the adjacent list	☐ Yes ☐ No ☐ Digging ☐ Hand-washing potatoes ☐ Gardening ☐ Clay modelling

	☐ Mud slide	☐ Other (please specify)
18. In any 1 year during the last 10 years have you spent time in fresh water creeks, rivers, dams, lakes, ponds, or waterways on 5 or more occasions?	☐ Yes	☐ No
If **YES**, please select the activities you took part in from the adjacent list	☐ Swimming ☐ Fishing ☐ Other (please specify)	☐ Paddling ☐ Playing eg dam building
19. During the last 10 years, have you participated in any hunting where any form of animal handling took place?	☐ Yes	☐ No
If **YES**, please select which (if any) animals from the adjacent list	☐ Cattle (dairy) ☐ Cattle (non-dairy) ☐ Sheep	☐ Goats ☐ Deer ☐ Rabbits
20. Do you currently smoke (cigarettes or any other form of tobacco)?	☐ Yes	☐ No
- If **YES**, approximately how long have you been smoking?	Months:	Years:
21. During the last 10 years did you smoke (cigarettes or any other form of tobacco)?	☐ Yes	☐ No
- If **YES**, approximately how long did you smoke for?	Months:	Years:
- If **NO**, during the last 10 years have you been exposed to smoke (cigarette or other tobacco related)? ie passive smoking	☐ Yes	☐ No
On average how would you rate this exposure? *(please select one option only)*	☐ Very infrequent (< 1 exposure per month) ☐ Infrequent (> 1 exposure per month) ☐ Frequent (> 1 exposure per week) ☐ Very frequent (> 1 exposure per day)	
Question 22 is for females participants only, male participants please turn the page and proceed to Part 2		
22. Female participants only: During the last 10 years, have you taken the oral contraceptive?	☐ Yes	☐ No
- If **YES**, please list the duration of each occasion	Months:	Years:
	Months:	Years:
	Months:	Years:

Part 2: UV Exposure and Diet

23. Have you been on holiday in the last 12 months? ☐ Yes ☐ No

If **YES**, please complete the adjacent fields including whether or not you think you had increased sun exposure than you would have if you had not been on holiday *(if you require more space please see the final pages of this questionnaire)*

Rural Area/Town/City:		
Duration (days/weeks):		
Country (if outside NZ)		
Increased sun exposure?	☐ Yes	☐ No

Rural Area/Town/City:		
Duration (days/weeks):		
Country (if outside NZ)		
Increased sun exposure?	☐ Yes	☐ No

24. In the **last year**, on average, how much time did you spend **outdoors** (minutes or hours) per **week** in; (please consider hobbies, work, gardening, etc)

- **Winter** (June – August)
- **Summer** (December – February)

Minutes	Or	Hours

25. In the **last year** have you used a sunbed?

☐ No ☐ Yes, 10 sessions or less ☐ Yes, 11-20 sessions ☐ Yes, 20 – 30 sessions ☐ Yes, 30 sessions or more

26. When I am **outside** in **winter** I; (please tick (√) the relevant box)

	Almost never / never	A few times	Sometimes	Most of the time	Almost always / always
- Wear a sunhat?					
- Wear sunscreen?					
- Spend time in the sun? (but do not consider it sunbathing)					

27. When I am outside in **summer** I; (please tick (√) the relevant box)

	Almost never / never	A few times	Sometimes	Most of the time	Almost always / always
- Wear a sunhat?					
- Wear sunscreen?					
- Wear sunscreen AND reapply it according to the label instructions?					
- Avoid sun exposure by seeking shade?					
- Spend time in the sun? (but do not consider it sunbathing)					
- Sunbathe?					
- Get sunburnt?					

28. Do you currently take, or in the last 6 months have you taken, supplements that contain Vitamin D?

- ☐ Yes, I currently take supplements that contain Vitamin D
- ☐ Yes, in the last 6 months I have taken supplements that contained Vitamin D
- ☐ No, I do not currently take, or in the last 6 months I have not taken, supplements contain Vitamin D

If **YES**, please supply the following details from the product packaging:

Manufacturer? _____

Recommended dosage? eg xx IU _____

How many do you take? _____

How often do you take them? eg daily _____

How long have you been taking them? Week: _____ Months: _____ Years: _____

Why did you begin taking them? _____

Other information? _____

29. Has your vitamin D level been measured in the past year? ☐ Yes ☐ No

If **YES**, when (please list the date of the most recent measurement) and the outcome (if known)

Month: _____ Year: _____

Outcome (eg xx 25(OH)D_3 / xx 1,25(OH)D_3 / deficient / insufficient / adequate _____

The next 3 questions refer to your childhood; 0 - 12 years of age inclusive

30. During your childhood (0-12 years), what type of cow's milk did you predominantly consume?
(please select <u>one</u> option only)

- ☐ Pasteurised
- ☐ Un-Pasteurised
- ☐ Organic
- ☐ None (please proceed to question **31**)

31. During your childhood (0-12 years), approximately how much cow's milk did you drink **weekly**?
(please select <u>one</u> option only)

- ☐ > 6 glasses
- ☐ 4-6 glasses
- ☐ 1-3 glasses
- ☐ < 1 glass

32. During your childhood (0-12 years), what was your predominant source of drinking and cooking water?
(please select <u>one</u> option only)

- ☐ Main water supply
- ☐ Tank (rain water)
- ☐ Bore
- ☐ Tank (supplied)
- ☐ Other (please specify) _____

The next 5 questions refer to the last 10 years eg 2003 – 2013

33. During the last 10 years, what type of cow's milk did you predominantly consume? *(please select one option only)*

- ☐ Pasteurised
- ☐ Un-Pasteurised
- ☐ Organic
- ☐ None (please proceed to question **34**)

34. During the last 10 years, approximately how much cow's milk did you drink **weekly**? *(please select one option only)*

- ☐ > 6 glasses
- ☐ 4-6 glasses
- ☐ 1-3 glasses
- ☐ < 1 glass

35. During the last 10 years, what was your predominant source of drinking and cooking water? *(please select one option only)*

- ☐ Main water supply
- ☐ Tank (rain water)
- ☐ Bore
- ☐ Tank (supplied)
- ☐ Other (please specify)

36. Would you be happy for the researcher to contact you to clarify any answers you have provided (if required)?

- ☐ Yes
- ☐ No

37. Would you like your name to be put in the random draw (only for participants in this study) for 1 of 5 $100 gift Vouchers

- ☐ Yes
- ☐ No thank you

THANK YOU FOR TAKING THE TIME TO COMPLETE THIS QUESTIONNAIRE

Extra space if required (please list the question number your answer relates to)

QUESTION	ANSWER

10. Vitamin D Intervention Study Proposal

Before you start filling out this form, read carefully the information regarding the Lottery Health Research on www.communitymatters.govt.nz to help you decide whether you are eligible for a grant and that your translational research project meets the priorities of Lottery Health Research.

Please answer every question. Incomplete or late applications are unlikely to be accepted. An application will be incomplete if all additional information requested is not attached. Do not alter margins. Please observe word and page restrictions. Submit your application in MS Word or editable PDF format attached to an email to lhr@dia.govt.nz by **4.00pm on Wednesday 11 September 2013**.

SECTION A: SUMMARY OF THE TRANSLATIONAL RESEARCH PROJECT

A1. Host institution

Massey University

A2 Translational research location

Palmerston North

A3. Name of the project leader

Name	Position	Department/Organisation
Jane Coad	**Associate Professor**	**Institute of Food Nutrition and Human Health**
	Director of Human Nutrition and Dietetics Division	

A4. Translational research title

Young New Zealanders with Inflammatory Bowel Disease – the beneficial role of Vitamin D?

A5. Abstract (short summary of the research proposal) of the translational research proposal

The prevalence of Inflammatory Bowel Disease (IBD) in New Zealand is one of the highest in the world and is increasing especially in children and adolescents for whom many conventional treatments are not appropriate. The health consequences include malnutrition, reduced bone mineral density and nutritional deficiencies such as anaemia. Hypovitaminosis D is higher amongst patients with IBD compared to healthy individuals. Optimising vitamin D status has the potential to improve the nutritional status, bone health and disease activity of individuals with IBD. Very few studies have assessed whether vitamin D supplementation will improve the health risks associated with IBD in children and young people. This randomised double-blind controlled trial aims to investigate the impact of vitamin D supplementation (4,000IU/day) on disease activity and inflammatory markers in participants with IBD, aged 8-30 years, and vitamin D status, bone health and nutritional status in participants with IBD compared to healthy controls .

A6. Total amount of Lottery funded applied for

$ 95,060

A7. Duration of the translational research project

Twelve months

SECTION B: TRANSLATIONAL RESEARCH PROJECT DETAILS

B1. Translational research proposal:

Translational research aims	• To assess nutritional status and bone turnover markers, in New Zealanders aged 8-30 years with Inflammatory Bowel Disease (IBD), compared to healthy controls. • To determine whether serum vitamin D levels correlate with disease activity and levels of inflammatory markers in participants with IBD. • To compare the efficacy of vitamin D supplementation in increasing serum vitamin D in participants with IBD and healthy controls. • To investigate whether vitamin D supplementation, as an adjunct therapy for 6 months, improves nutritional status and bone turnover markers in participants with IBD and healthy controls, and reduces disease activity and inflammation in participants with IBD.
Translational research need	Inflammatory Bowel Disease (IBD), an idiopathic disease of the digestive tract, has serious consequences for those affected including chronic pain, nutritional deficiencies, impaired bone health, and surgical intervention in approximately 65% of cases. The prevalence of IBD in New Zealand is one of the highest in the world, at approximately 308 per 100,000 people, and increasing in adults and children alike. Of particular interest is the role of vitamin D in the aetiology of IBD; lower vitamin D levels are observed in IBD compared to healthy controls; and vitamin D status is inversely associated with IBD risk, disease duration and disease activity. Vitamin D supplementation may reduce the severity of symptoms associated with IBD; animal studies support this hypothesis. There is an urgent need to investigate the potential of vitamin D in IBD treatment and its effects on nutritional status and bone health, especially in children and young adults who are still growing and accruing bone mass. Some medication, e.g. steroid treatment, known to be effective in older patients is not suitable for younger people. New Zealand is an ideal location for such a study based on its high IBD prevalence and also high prevalence of low vitamin D status.
Translational research design and methods	PARTICIPANTS: New Zealanders of any ethnicity, aged 8-30 with Inflammatory Bowel Disease (IBD), and healthy individuals without IBD, will be recruited for this randomised double-blind controlled trial. Confirmation of IBD diagnosis will be based on history of clinical, endoscopic, radiological and histological findings where available; alternatively confirmation of diagnosis will be based on evidence from an appropriately qualified medical professional eg a gastroenterologist. Exclusion criteria include renal dysfunction, serum vitamin D ($25(OH)D_3$) levels ≤ 12.5 nmol/L (indicative of severe deficiency in which case notification will be sent to the consenting participant's GP), and use of cholecalciferol supplementation ≥ 400 IU/day. Exclusion criteria for healthy controls are a family history of IBD or other conditions that affect the intestine. The study will be advertised at Gastroenterology practices, General Practitioner clinics, and Maori health providers. INTERVENTION: Participants who meet the inclusion criteria will be randomly assigned to either the high vitamin D treatment group (4,000 IU daily; Tishcon, New York), a dose that is known to be safe and well-tolerated; or the low vitamin D treatment group (200 IU daily; Tishcon, New York), the Adequate Intake ("usual intake at or above this level has a low probability of inadequacy")

	recommended by the Ministry of Health for males and females from birth to 50 years. The high dose of supplementary vitamin D is based on a dose shown to be effective in raising and maintaining serum 25(OH)D_3 at > 75 nmol/L, a level which is considered to be the minimum required for optimal health. Randomisation will be performed by an independent organisation and concealed from the researchers until after statistical analysis of the data. Vitamin D supplementation (in the form of capsules) will be mailed to participants monthly (to facilitate compliance) for a total period of 6 months. Symptom assessment tools (age matched IBD activity indices PCDAI, PUCAI, CDAI, or UCAI), a medication use questionnaire, and a request to confirm receipt and consumption of the vitamin D capsules (this will allow follow-up of non-responders) will also be sent out with the supplements. Participants will be encouraged to maintain their usual diet throughout the duration of the intervention and to advise the researchers if any significant dietary changes occur during this period. Participants based in the Manawatu region will visit the Massey Nutrition Research Unit at baseline and endpoint (6 months) when blood samples will be drawn for the measurement of vitamin D (serum 25(OH)D_3), indicators of nutritional status (calcium, vitamin B12, folate and iron), bone turnover markers (osteocalcin, alkaline phosphatase, parathyroid hormone, and phosphate), and inflammatory markers of disease (c-reactive protein, erythrocyte sedimentation rate, complete blood count, tumor necrosis factor alpha, and interleukin-6; in participants with IBD only). Participants outside the Manawatu region will be provided with the name and contact details of the phlebotomy clinic closest to them. STATISTICAL ANALYSIS: Statistical analysis will be carried out by intention to treat analysis (ITT) and reporting will be in line with the 2010 CONSORT guidelines. The multiple imputation approach will be used to deal with any missing data. An ITT analysis will principally be used but complier average causal effect (CACE) analysis will also be incorporated in lieu of the per-protocol analysis to estimate intervention effectiveness. CACE provides an unbiased estimate of intervention effects among participants who adhere to their randomly allocated intervention. As vitamin D levels are largely determined by skin exposure to ultraviolet-beta radiation in sunlight, vitamin D levels will be adjusted for season where appropriate. If high vitamin D supplementation proves to be beneficial, IBD participants allocated to the low vitamin D treatment group will be offered the equivalent treatment.
Potential impact of the translational research, including how the translational research meets the Lottery Health Research priorities, particularly community benefit?	The prevalence of Inflammatory Bowel Disease (IBD) in New Zealand (NZ) is one of the highest reported in the world and is increasing. Alongside an increasing prevalence, the age of IBD onset is also decreasing with around 20% of patients being diagnosed during childhood. International data demonstrates that individuals affected by IBD have significantly greater health risks including malnutrition, present in 40-85% of adults with IBD; compromised skeletal health, reduced bone mineral density; and nutrient deficiencies including iron, folate, vitamin B12, and vitamin D. These health risks are predicted to be higher in children, adolescents and young adults due to growth requirements and accrual of bone mass, however very few studies have assessed these risks. By undertaking a randomised, double-blind, controlled trial we will explore the extent of these health risks in a sub-group of New Zealanders and determine whether the risks are elevated by IBD.

	The minimum participant age of 8 years will ensure inclusion of growth phases that overlap with the lower age of IBD diagnosis, and the maximum participant age of 30 years will ensure inclusion of the peak bone mass attainment period. The trial will be carried out by Massey University which has a dedicated Vitamin D Research Centre. This project will complement other vitamin D research that has recently taken place, or is currently taking place in NZ including demonstration of an association between lower vitamin D status and increased risk of type 2 diabetes; increased vitamin D supplementation in aged care residents and a subsequent reduction in falls and fall-related fractures; and a potential association between vitamin D supplementation and cardiovascular disease, respiratory infections; psoriasis; and multiple sclerosis. Understanding both the risk of hypovitaminosis D and the possible effects of improving vitamin D status has the potential to improve the nutritional status of this group and aid in bone deposition before its peak in early adulthood. Identifying an elevated risk of inadequate vitamin D levels in IBD would highlight a risk factor for the condition that can screened for and rectified safely, easily, and at a low cost. Also, a vitamin D induced reduction in disease activity and inflammation could significantly influence the effectiveness of IBD treatment programmes without causing adverse side-effects associated with many medications currently available such as nausea, vomiting, headache, diarrhoea, fatigue, and reduced bone mineral density. Such benefits would equate to both immediate and long term reductions in health costs as a result of improved IBD treatment, a reduction in ailments triggered or exacerbated by impaired nutritional status, and enhanced bone health. The outcomes of this trial could also have broader implications for the management of other inflammatory conditions that present a significant health burden in NZ such as atherosclerosis, insulin resistance, rheumatoid arthritis, and especially asthma which tends to be most severe during childhood. Vitamin D is a safe, readily available and relatively inexpensive micronutrient, which if proven to be beneficial to health, makes it an ideal supplement to be incorporated into treatment programmes and health initiatives.
Key references	Bischoff-Ferrari, H.A., *Optimal serum 25-hydroxyvitamin D levels for multiple health outcomes*, in *Sunlight, vitamin D and skin cancer*. 2008, Springer. p.55-71. Gearry, R.B., et al., *High incidence of Crohn's disease in Canterbury, New Zealand: Results of an epidemiologic study*. Inflammatory Bowel Diseases, 2006. **12**(10): p.936-943. Ulitsky, A., et al., *Vitamin D Deficiency in Patients With Inflammatory Bowel Disease*. Journal of Parenteral and Enteral Nutrition, 2011. **35**(3): p.308-316. Vieth, R., P. Chan, and G. MacFarlane, *Efficacy and safety of vitamin D3 intake exceeding the lowest observed adverse effect level*. American Journal of Clinical Nutrition, 2001. **73**: p.288 - 294.

B2. Tell us about the translational research project team (please complete a Lottery Health Research CV template for each member)

First name	Family name	Position in team	Organisation (employer)
Jane	Coad	Leader	Massey University
Hannah	Morton	Researcher	Massey University
Genelle	Healey	Researcher	Massey University
Pamela	Von Hurst	Collaborator	Massey University
Andrew	Herbert	Clinical collaborator	MidCentral Health Palmerston North Hospital

(if you need to add any further people to this table, please attach them as a supplementary sheet)

B3. Does the translational research require Ethical Approval? (if so please provide written evidence from the relevant research ethical committee for your region and discipline).

☒ YES ☐ NO

B4. Does the translational research involve the development or importation of an organism modified through the use of recombinant DNA techniques, for example, a GMO?

☐ YES ☒ NO

B5. What support will the host institution provide for this proposal? (e.g. Accommodation, supervision, publication of results, maintenance of equipment, administration of a grant.)

The host institution will provide accommodation (offices and laboratories), supervision, IT and secretarial support, and administration of the grant.

B6. Please describe how findings from the translational research may be made publicly available beyond the normal scientific outlets. (Note: Findings from translational research projects funded by a Lottery grant must be made freely available. Please describe how you will do this, for example through publications or seminars for professionals or the general public.)

The results of the study will be published in distinguished peer-reviewed New Zealand and international journals, and presented as paper(s) at international conferences. This will allow the results to be disseminated to other researchers in the field. Depending on the results of the study it may be appropriate to hold workshops for key health personal working in areas where the results of the study could be valuable.
Many individuals with IBD are members of support groups managed by Crohn's & Colitis New Zealand (CCNZ). The research team is already involved with CCNZ and members of the research team have been invited to present physiology and nutrition seminars at CCNZ meetings. This would be an appropriate route of disseminating information.
The participants of the study will also be informed of the key findings of the study in a suitable presentation.
The results may also be disseminated via the media.

SECTION C: ABOUT THE FUNDS

(If the host institution is not registered for GST, all amounts must be GST inclusive.)

C1. What is the total cost of your translational research project?

$ 95,060

C2. What is the total amount of Lottery Health Research funding you are requesting? (this should equal C3 and C4.)

$ 95,060

C3. What salaries are you requesting funding for? **None**
Funding for salaries for a maximum of two years can be applied for. Please supply the actual salaries requested and show the salary scales and grades. Where funding for a partial salary is requested please indicate what percentage of a full time equivalent salary this will be.

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UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

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:	Hannah Morton
Name and title of main supervisor:	Professor Jane Coad
In which chapter is the manuscript/published work?	Three
What percentage of the manuscript/published work was contributed by the student?	70

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

Conceptualisation, investigation, ethical approval, methodology, data curation, data analysis, and writing.

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:

Morton H, Pedley KC, Stewart RJC, Coad J (2020). Inflammatory Bowel Disease: Are Symptoms and Diet Linked? Nutrients 12(10):2975

( ) **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:	Hannah Morton — Digitally signed by Hannah Morton Date: 2023.04.09 02:20:27 +12'00'	Main supervisor's signature:	Jane Coad — Digitally signed by Jane Coad Date: 2023.04.12 12:39:22 +12'00'

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

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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:	Hannah Morton
Name and title of main supervisor:	Professor Jane Coad
In which chapter is the manuscript/published work?	Seven
What percentage of the manuscript/published work was contributed by the student?	70

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

Conceptualisation, investigation, ethical approval, methodology, data curation, data analysis, and writing.

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:

  Morton H, Pedley KC, Stewart RJC, Coad J (2020). Vitamin D concentrations in New Zealanders with and without inflammatory bowel disease: do they differ? New Zealand Medical Journal 133:1511

- ( ) **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:	Hannah Morton — Digitally signed by Hannah Morton Date: 2023.04.11 20:50:41 +12'00'	Main supervisor's signature:	Jane Coad — Digitally signed by Jane Coad Date: 2023.04.12 12:40:33 +12'00'

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STATEMENT OF CONTRIBUTION
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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:	Hannah Morton
Name and title of main supervisor:	Professor Jane Coad
In which chapter is the manuscript/published work?	Eight
What percentage of the manuscript/published work was contributed by the student?	60

Describe the contribution that the student has made to the manuscript/published work:
Conceptualisation, investigation, ethical approval, methodology, data curation, data analysis, and writing.

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:
  Conceptualisation, investigation, ethical approval, aided in methodology, data curation, aided in data analysis, and writing.
- ◉ **The manuscript is currently under review for publication**
  Please provide the name of the journal:
  Digestive Disease and Sciences, status 'revisions being processed'.
- ○ **It is intended that the manuscript will be published, but it has not yet been submitted to a journal**

Student's signature:	Hannah Morton Digitally signed by Hannah Morton Date: 2023.04.11 20:55:19 +12'00'	Main supervisor's signature:	Jane Coad Digitally signed by Jane Coad Date: 2023.04.12 12:41:02 +12'00'

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