THE ASSOCIATION BETWEEN ENVIRONMENTAL EXPOSURES DURING CHILDHOOD AND THE SUBSEQUENT DEVELOPMENT OF CROHN'S DISEASE IN THE WESTERN CAPE

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KEYWORDS

Crohn’s disease

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Ethnicity

Race

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ABBREVIATIONS

$1,25(OH)_2D$:	1,25-Dihydroxyvitamin D
$1,25(OH)_2D3$:	1,25-Dihydroxyvitamin
D3 25(OH)D:	25-hydroxyvitamin D
AMP:	Antimicrobial peptide
APC:	Antigen-presenting cell
Anti-TNFα:	Anti-tumour necrosis factor alpha
BMI:	Body mass index
CAMP:	Cathelicidin antimicrobial peptide
CARD-15:	Caspase-activation recruitment domain
CD:	Crohn's disease
CDAI:	Crohn's Disease Activity Index
CI:	Confidence interval
CLSI:	Clinical and laboratory standards institute
CMV:	Cytomegalovirus
CRP:	C-reactive protein
DC:	Dendritic cells
dL:	Decilitre
DSS:	Dextran sulphate sodium
EC:	Epithelial cells
ECCO:	European Crohn's and Colitis Organization
GALT:	Gut-associated lymphoid tissue

ABBREVIATIONS

GNI:	Gross national income
GWAS:	Genome wide association studies
HBI:	Harvey Bradshaw Index
HLA:	Human leukocyte antigen
HSV:	Herpes simplex virus
IBD:	Inflammatory bowel disease
IgA:	Immunoglobulin A
IgE:	Immunoglobulin E
IgG:	Immunoglobulin G
IgG1:	Immunoglobulin G1
IgG2a:	Immunoglobulin G2a
IgG4:	Immunoglobulin G4
IL-1:	Interleukin-1
IL-Ra:	Interleukin-1 receptor antagonist
IL-Re:	Interleukin-1 receptor
IL-2:	Interleukin-2
IL-4:	Interleukin-4
IL-5:	Interleukin-5
IL-6:	Interleukin-6
IL-10:	Interleukin-10
IL-12:	Interleukin-12
IL-13:	Interleukin-13
IL-18:	Interleukin-18

ABBREVIATIONS

IL-21:	Interleukin-21
IL-23:	Interleukin-23
iLFs:	Intestinal lymph follicles
INF-γ:	Interferon gamma (also abbreviated IFN-γ)
IQR:	Interquartile range
IU:	International units
κ:	Kappa
Kg:	Kilogram
Kg/m²:	Kilograms per meter squared
KO:	Knock out
LCN-omega-3:	Long-chain omega-3 fatty acids
LRR:	Leucine-rich repeat proteins
LP:	Lamina propria
MDP:	Muramyl dipeptide
MHC:	Major histocompatibility complex
mL:	Millilitre
MLNs:	Mesenteric lymph nodes
mRNA:	Messenger ribonucleic acid
M.tb:	*Mycobacterium tuberculosis*
NF-AT:	Nuclear factor of activated T cells
NFκB:	Nuclear factor-κB
NHIS:	National health interview survey
NK:	Natural killer cell

ABBREVIATIONS

NKT:	Natural killer T cell
NLR:	NOD-like receptor
NOD-2:	Nucleotide oligomerization domain
NRAMP1:	Natural resistance-associated macrophage protein 1
OR:	Odds ratio
PP:	Peyer's patches
PAMP:	Pathogen associated molecular pattern
PCR:	Polymerase chain reaction
PGE_2:	Prostaglandin E_2
PID:	Participant information document
PR:	Prevalence ratio
PRR:	Pattern recognition receptors
PUFAs:	Polyunsaturated fatty acids
ROR-γt:	Retinoic-acid-receptor-related orphan receptor-γt
RXR:	Retinoid-X-receptor
SCL11A1:	Solute carrier family 11 member 1
SD:	Standard deviation
STAT6:	Signal transducer and activator of transcription 6
T-bet:	T-box protein expressed in T cells (also abbreviated TBX21)
TCGF:	T cell growth factor (also abbreviated TGFC)
TCR:	T cell receptor
TLR:	Toll like receptor
TGFβ:	Transforming growth factor beta

ABBREVIATIONS

Treg:	T-regulatory (cells)
TNF-α:	Tumour necrosis factor alpha
TSLP:	Thymic stromal lymphopoietin
UC:	Ulcerative colitis
UV:	Ultraviolet
UVI:	Ultraviolet index
VDR:	Vitamin D receptor
VDRE:	Vitamin D responsive element
VD3:	Vitamin D3
vs.:	Versus
WHO:	World Health Organization
WT:	Wild type

SELECTED TERMS USED INTERCHANGEABLY

Intestinal bacteria; intestinal microbiome; intestinal microflora; enteric flora.

LIST OF TERMS AND DEFINITIONS

Adaptive immune system: also known as the 'acquired immunity'. The components of the adaptive immune system include both humoral immunity and cell-mediated immunity. The cells of the adaptive immune system are T and B lymphocytes (i.e. T-cells and B-cells). Unlike the innate immune response (see definition), adaptive immunity is highly specific to pathogens. One of the main functions of vertebrate adaptive immunity is the recognition of specific 'non-self' antigens in the presence of 'self'.

Antigen: any substance (such as a toxin or enzyme) that stimulates an immune response in the body (especially the production of antibodies).

Anti-tumour necrosis inhibitors (anti-TNFα): a pharmaceutical drug that suppresses response to tumour necrosis factor, which is part of the inflammatory response.

Aphthoid (Aphthous) ulcer: a common cause of benign and non-contagious mouth ulcers.

Bioavailability: a subcategory of absorption and is the fraction of an administered does of unchanged drug that reaches the systemic circulation, one of the principal pharmacokinetic properties of drugs.

'Biologic' therapy: refers to the use of medication that is specifically tailored to target an immune or genetic mediator of disease.

C-reactive protein (CRP): a protein found in the blood, the concentrations of which rise in response to inflammation (i.e. C-reactive protein is an acute-phase protein).

Coloureds: people of a particular mixed-ancestry.

Commensal bacteria: a symbiotic relationship between two organisms of different species in which one derives some benefit while the other is unaffected; in this case the intestinal bacteria and the host.

Corticosteroids: a class of chemicals that includes steroid hormones naturally produced in the adrenal cortex of vertebrates, and analogues of these hormones that are synthesized in laboratories. Corticosteroids have a wide range of physiological processes, including stress response, immune response, and regulation of inflammation, carbohydrate metabolism, protein catabolism, blood electrolyte levels and behaviour.

Crohn's disease (CD): also known as 'regional enteritis' is an inflammatory disease of the intestines that may affect any part of the gastrointestinal tract from mouth to anus, causing a wide variety of symptoms. It primarily causes abdominal pain, diarrhoea, vomiting or weight loss, but may also cause complications outside of the gastrointestinal tract called extraintestinal manifestations (see definition).

Crohn's Disease Activity Index (CDAI): a research tool used to quantify the symptoms of patients with Crohn's disease.

Cytokines: a diverse group of soluble proteins, peptides, or glycoproteins which act as hormonal regulators or signalling molecules at nano- to-picomolar concentrations and help in cell signalling. The term 'cytokine' encompasses a large and diverse family of regulators produced throughout the body by cells of diverse embryological origin. The term 'cytokine' has been used to refer to the immunomodulating agents, such as interleukins and interferons. They are regulators of host responses to infection, immune responses, inflammation and trauma.

Enteric Flora: or 'gut flora' consists of a complex of microorganism species that live in the digestive tract of animals and is the largest reservoir of human flora. In this context 'gut' is synonymous with 'intestinal', and 'flora' with 'microbiota' and 'microflora'. The word 'microbiome' refers to the environment in which these bacteria live.

Enteropathy: refers to any pathology of the intestine.

Ethnicity or 'ethnic group': a socially-defined category of people who identify with each other

based on common ancestral, social, cultural or national experience.

Extra-intestinal manifestations (EIMs): occurrences situated outside the intestines. Extra-intestinal manifestations can involve any organ of the body, but musculoskeletal, dermatologic and ocular are the most commonly affected. Hepatobiliary and renal (nephrolithiasis) manifestations are especially seen with small bowel dysfunction.

Fissure: a tear in the lining (of the intestine or anus).

Fistula (fistulising): an abnormal connection or passageway between two epithelium-lined organs or vessels.

Genotype: the inherited instructions an organism carries within its genetic code: not all organisms with the same genotype look or act the same way because appearance and behaviour are modified by environmental and developmental conditions; similarly, not all organisms that look alike necessarily have the same genotype.

Harvey Bradshaw Index (HBI): a simplified version of the Crohn's Disease Activity Index (see definition).

Helminths: a polyphyletic group of eukaryotic parasites: worm-like organism living in and feeding on living hosts, receiving nourishment and protection while disrupting their hosts' nutrient absorption, causing weakness and disease: those that live inside the digestive tract are called intestinal parasites.

Hygiene hypothesis: a hypothesis that states that a lack of early childhood exposure to nfectious agents, symbiotic microorganisms (e.g., gut flora or probiotics), and parasites, increases susceptibility to allergic diseases by suppressing the natural development of the immune system.

Ileocolonoscopy: an endoscopic examination of the large bowel, where the last part of the small bowel (ileum) is also examined.

Industrialization: the period of social and economic change that transforms a human group from an agrarian society into an industrial one: part of a wider modernization process, where social change and economic development are closely related with technological innovation, particularly with the development of large-scale energy and metallurgy production; the extensive organization of an economy for the purpose of manufacturing.

Inflammatory bowel disease (IBD): a group of inflammatory conditions of the colon and small intestine; the major types of inflammatory bowel disease include Crohn's disease and ulcerative colitis.

Innate immune system: also known as the 'non-specific immune system': a subsystem of the overall immune system which defends the host from infection. One of the major functions of the vertebrate innate immune system is to recruit immune cells to the site of infection, through the production of chemical factors, including specialized chemical mediators called 'cytokines' (see definition for cytokine). Inflammation is one of the first responses of innate immunity to infection; it is initiated by macrophages, dendritic cells, histiocytes, Kupffer cells, and mastocytes. Unlike the adaptive immune system, the innate system recognizes and responds to pathogens in a generic way; it does not confer long-lasting or protective immunity to the host.

Intestinal lumen: in human anatomy, the intestine (i.e. bowel or gut) is the segment of the alimentary canal extending from the pyloric sphincter of the stomach to the anus; and, in humans and other mammals, it consists of two segments, the small intestine and the large intestine. In humans, the small intestine is further subdivided into the duodenum, jejunum and ileum, while the large intestine is subdivided into the cecum and colon.

***Intestinal microbiome/microflora*:** the aggregate of microorganisms: a microbiome that resides on the surface and in deep layers of the skin, in the saliva and oral mucosa, in the conjunctiva, and

in the gastrointestinal tract; they include bacteria, fungi, and archaea; the intestinal microbiome is also referred to as the enteric flora.

Immunoregulation: control of the immune response, as by manipulation of pathways involving suppressor and contra suppressor T-cells.

Immunosuppressant agents/treatments/drugs: drugs that inhibit or prevent activity of the immune system; used in immunosuppressive therapy.

Loci: in genetics, a locus (plural) is the specific location of a gene, DNA (deoxyribonucleic acid) sequence, or position on a chromosome.

Microbiome: the full collection of microbial genomes (bacterial, fungal, viral, etc.) that naturally exist within the human body; such as the community of microbes within the human gut.

Nucleotide oligomerization domain (NOD-2): also known as caspase recruitment domain-containing protein 15 (CARD15) or inflammatory bowel disease protein 1 (IBD1); a protein that in humans is encoded by the NOD-2 gene located on chromosome 16. NOD2 plays an important role in the immune system: it recognizes bacterial molecules (peptidoglycans) and stimulates an immune reaction.

Non-steroidal anti-inflammatory drug (NSAID): a class of drugs that provides analgesic and antipyretic (fever-reducing) effects, and in higher dosages, anti-inflammatory effects.

Pathogenesis: the 'pathogenesis of a disease' is the mechanism that causes the disease; the term can also describe the origin and development of the disease, and whether it is acute, chronic, or recurrent.

Penetrating (Crohn's disease): referring to 'fistulising' Crohn's disease. See definition for fistula.

Phenotype: any observable characteristic or trait of an organism, such as its morphology, development, biochemical or physiological properties, behaviour, and products of behaviour.

Phenotypes result from the expression of an organism's genes as well as the influence of environmental factors and the interactions between the two.

Polymorphism: a change in a nucleotide base in the DNA (deoxyribonucleic acid): a genetic polymorphism can also be interchangeable referred to as a 'mutation' or a 'variant'.

Race: a social concept used to categorize humans into large and distinct populations or groups by anatomical traits.

Stricture (stricturing): an abnormal narrowing of the intestine (or a duct or passage).

T-cells or 'T lymphocytes': a type of lymphocyte that plays a central role in cell-mediated immunity (adaptive immune system).

Th1 response: the pro-inflammatory response associated with the adaptive immune system (see definition for adaptive immune system). Activation of the Th1 response leads to cell-mediated immunity. The Th1 responses are more effective against intracellular pathogens such as viruses and pathogens.

Th2 response: an antibody-mediated response associated with the adaptive immune system (see definition for adaptive immune system). Activation of the Th2 response leads to humoral Immunity. The Th2 responses are more effective against extracellular pathogens such as bacteria and parasites.

Th17 response: a subset of T-helper cells. They create inflammation and tissue injury in 'autoimmune diseases' such as multiple sclerosis, rheumatoid arthritis, psoriasis, autoimmune uveitis, juvenile diabetes and Crohn's disease. Their normal role is to provide anti-microbial immunity at epithelial/mucosal barriers.

Transmural: existing or occurring across the entire wall of an organ or blood vessel.

Ulcerative colitis (UC): a form of colitis, a disease of the intestine, specifically the large intestine

or colon, which includes characteristic ulcers, or open sores, in the colon. The main symptom of active disease is usually constant diarrhoea mixed with blood, of gradual onset.

Westernization of lifestyle: a social classification of people influenced by the attitude, ethics and history of the American culture; this lifestyle affects this population sector's choice of recreation, clothing and consumption of goods.

ABSTRACT

Background: A subtype of inflammatory bowel disease, Crohn's disease is thought to represent a complex interaction between environmental factors, a defective immune system, the gastrointestinal microbiome and genetic susceptibility.

Aim: The focus of this study was to investigate the association between environmental exposures during childhood and the subsequent development of Crohn's disease, thus the two primary aims were to: 1) conduct a systematic review of the literature evaluating environmental risk factors during childhood, defined by studies either as, age intervals (e.g., 0-5, 6-10 and 11-18 years), or more 'broadly' as 0-18 years; and 2) investigate the association between childhood environmental exposures during three age intervals (0-5, 6-10 and 11-18 years), as well as frequency of childhood infections and the future development of Crohn's disease based on a score analysis, using a subset of previously collected data from a completed doctoral thesis involving a case control study design in study population, in the Western Cape, South Africa. The aim included a primary analysis of the latter dataset for childhood infections.

Design: For the first aim of the study, a systematic search was conducted during March 2015 in electronic databases, such as EMBASE, EBSCOhost (Medline), Ovid, Scopus and World Cat, PubMed and Biomed Central, to identify epidemiological studies that examined the association between childhood environmental exposures and the subsequent development of Crohn's disease. Studies evaluating childhood exposure either by age intervals, or more broadly, from birth until 18 years were included. The environmental exposures evaluated in the review were; farm animal contact, place of upbringing, sibship size, household pets, primary water source and hot water availability. Of the 181 identified articles, 16 were included in the final systematic review. The second aim of the study involved a post hoc analysis of a subset of findings from the

completed doctoral research by Abigail Basson with regard to the multiple logistic regression analysis evaluating environmental risk factor exposure during three age intervals; 0-5 years, 6-10 years and 11-18 years. In the present research, two different methodological approaches were undertaken. Briefly, exposure variables, of similar nature, were combined into subgroups and assigned weighting scores. The two 'subgroup models' were designated as: Group A and Group B. Based on these premises, a score analysis was performed, and the difference in scores, between case and control groups, was compared. In addition, multiple logistic regression models were conducted on a subset of original data from the aforementioned completed doctoral study to assess the association between the frequency of childhood infections between 0-20 years and risk of Crohn's disease development. Following this, a score analysis was again performed.

Results: Sixteen studies were included in the systematic review. Of the five studies that investigated the association between place of upbringing during the age interval 0-5 years and the subsequent development of Crohn's disease, three found no significant association; however of the three studies evaluating place of upbringing during the age intervals 6-10 and 11-18 years, only one study identified a significant association. Three studies investigated exposure to farm animals during the age interval 0-5 years, of which, two identified a significant association. Of the latter three studies, two investigated farm animal contact during the age intervals 6-10 and 11-18 years, but only one reported a significant association during these age intervals. Notably, this was the study which had failed to identify an association during the 0-5 year age interval. Both studies which broadly evaluated farm animal exposure during 'childhood' reported that not having contact with animals significantly increased the risk of developing Crohn's disease. Of the five studies that investigated exposure to pets during the age interval 0-5 years, only one identified a significant risk association, namely with exposure to cats. Of the three which investigated pet exposure during the age intervals 6-10 years and 11-18 years, one identified a significant

association, for both age intervals. Five studies investigated pet exposure during ‘childhood’; one found that having a pet significantly increased the risk of developing Crohn’s disease, two reported that not having a pet significantly increased risk in developing Crohn’s disease, whereas the remaining studies found no significant association. Only one study evaluated primary water source during the three age intervals; during the age interval 0-5 years and 11-18 years, having piped tap or bottled water was significantly associated with CD development. Of the four studies investigating primary water source during ‘childhood’, only one reported a significant association between primary water source and the development of Crohn’s disease. The availability of hot water during the age interval 0-5 years was significantly associated with Crohn’s disease development in one of the three relevant studies. Two studies investigated hot water availability during 6-10 and 11-18 years, however both failed to identify a significant association. When broadly evaluated, hot water availability during ‘childhood’ was significantly associated with Crohn’s disease risk, in two of the three relevant studies. None of the studies which investigated sibship size and the risk of future Crohn’s disease development during defined age intervals reported a significant association. Only two of the seven studies that evaluated sibship size during childhood reported a significant association.

Results of the score analysis revealed a significant difference during all three age intervals between the case and control groups with Group A and Group B, with cases having significantly lower exposure scores (approximately 30% and 40% lower, respectively), when compared with that of controls. On multiple logistic regression analysis, subjects who never had tooth decay/cavity (OR = 1.78; 95% CI, 1.05-3.04), periodontitis (OR = 1.95; 95% CI, 1.10, 3.48), diarrhoea (OR = 2.71; 95% CI, 1.62-4.62), gastritis (OR = 2.13; 95% CI, 1.30-3.35), or mouth ulcers (OR = 2.02; 95% CI, 1.12-3.70), at least once per year or more, were at an increased risk for later development of Crohn’s disease, when compared to those who were exposed to these

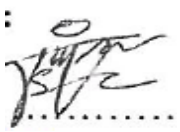
infections at least once per year or more. There was a significant difference in exposure scores between the case and control groups (OR = 0.88; 95% CI, 0.82-0.94), thus indicating that cases had 12% less exposure to childhood infections from birth until the age of 20 years, when compared to the controls.

Conclusion: The systematic review of the literature provides evidence in support of the hygiene hypothesis, in that delayed exposure to immunostimulatory microbes through the environmental exposures increases the risk for future CD development, in genetically susceptible individuals. In addition, the literature supports that the childhood environment plays an important role in the aetiology of Crohn's disease. However, the lack of consistent findings between studies, particularly those which have broadly defined 'childhood' implies that timing of exposure plays a crucial role in this ever evolving paradigm. Results from the score analysis provide insight into the 'compound' effects from multiple environmental exposures in the aetiology of Crohn's disease. While the present research was unable to provide any explanation for the underlying mechanism of disease pathogenesis, overall, the findings have important implications for future IBD-related studies as they demonstrate the importance of accounting for environment as a 'whole' when conducting epidemiological studies, as opposed to focusing on individual environmental factors, as well as that it is imperative to investigate environmental exposures within the context of defined age intervals.

DECLARATION

I declare that '**The association between environmental exposures during childhood and the subsequent development of Crohn's disease in the Western Cape'** is my own work that has never been published or submitted for any other degree or examination at any university and all sources used in this thesis have been cited and acknowledged.

Full Name: Mr Victor Tinashe Sabe **Date:** 30/06/2016

Signed: 

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Last but not least is my unending thanks to the One who sustains me, God Almighty to whom all praise and honour is due.

DEDICATION

To my father, the embodiment of perseverance

PREFACE

Crohn's disease (CD) and ulcerative colitis (UC), both subtypes of inflammatory bowel disease (IBD), are chronic, inflammatory disorders of the gastrointestinal tract that develop as a result of a deregulation of the T-cell mediated immune responses toward the intestinal bacteria. While there are similar overlapping features for both diseases, clinically, CD and UC are distinctly separate gastrointestinal disorders. The focus of this manuscript will thus be on the IBD subtype, CD in the adult patient.

This dissertation comprises of two aspects on environmental exposures of Crohn's disease based on the 'hygiene hypothesis'. Chapter 1 provides the background and rationale for the three main components of this dissertation, while Chapter 2 constitutes comprehensive description of the organizational framework of the study. In relation to the research theme, a broad literature review has been presented in Chapter 3. Chapter 4 includes the first component of the candidates' research, a systematic review of literature evaluating childhood environmental exposures of CD. The second component of the research is presented in Chapter 5. This includes a score analysis based on a subset of previously collected data from the completed PhD research (2011-2014) conducted by Dr Abigail Raffner Basson which focused on environmental exposures during childhood and the subsequent development of Crohn's disease in the Western Cape, South Africa. All tables and figures are located at the end of each chapter. The remaining components of the dissertation comprise the Bibliography and Appendices. The Bibliography includes all of the literature consulted throughout the dissertation.

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CHAPTER 1

INTRODUCTION

Inflammatory bowel disease (IBD) which is comprised of Crohn's disease (CD) and ulcerative colitis (UC) is a group of gastrointestinal conditions that result in chronic inflammation of gut (Frank, DN *et al.* 2007). So far, susceptibility to the CD has been linked to the highly complex interplay between genetic, immunologic and environmental risk factors (Knights D *et al.* 2013; Hanauer, SB *et al.* 2006; Danese S *et al.* 2004). Genome-wide association studies have identified more than 140 different susceptibility genes, many of which have been linked to the CARD15 (caspase-activation recruitment domain), also referred to as the NOD2 (nucleotide oligomerization domain) gene (Rioux, JD *et al.* 2007). Genetic predisposition has been linked to the dysregulation of the immune system of the gastrointestinal tract and dysbiosis of the intestinal microbiome Knights D *et al.* 2013; Manichanch C *et al.* 2012). However genetic factors fail to fully account for the aetiology of the disease (Shanahan F. 2002). Different susceptibility genes have been seen to influence the development of CD in different races and separate geographic populations (Shanahan F. 2002).

Despite the intensive research conducted over the years, no single microbial or infectious agent has been specifically linked to the pathogenesis of CD (Morgan XC *et al.* 2012). The exact role of intestinal microbiota in the aetiology of CD has not been clearly determined, although there is growing evidence which suggests that an inappropriate upregulation of the immune response may be triggered by the commensal microbiota in the gut (Round JL and Mazmanian SK 2009; Hooper

LV and Gordon JI 2001). The evidence also demonstrates that gut microbiota is pivotal in the initiation, maintenance, and determination of disease phenotype (Nagalingam NA and Lynch SV. 2012). The persistent antigenic stimulation ultimately results in the activation of specific mechanisms of the immune system that cause chronic intestinal inflammation and injury (Knights D *et al.* 2013; Baumgart DC and Carding SR. 2007). It has also been suggested that, exposure to pathogens in the gastrointestinal tract may be transitory to the onset of clinical disease (Eckburg PB and Relman DA 2007).

An accelerated increase in the incidence of CD in developing countries, such as Asia, has proposed the role of environmental factors in the aetiology of the disease (Molodecky NA and Kaplan GG 2010; Ng SC. 2014(a)). Traditionally, developing countries have reported low prevalence and incidence of CD, but as these countries have become increasingly industrialised and 'westernized', an accompanying noticeable rise in the prevalence of IBD has also been observed (Ng SC 2014(a); Sood A *et al.* 2003). Moreover, evidence from migrant studies has demonstrated that individuals migrating from regions of low IBD prevalence to regions with a high prevalence of IBD subsequently develop a higher risk of developing CD, comparable to that of the local population (Bernstein CN. 2008; Satsangi J *et al.* 1994). Other evidence supporting the role of environmental factors in CD pathogenesis is the lack of complete concordance in disease development between monozygotic twins (Thompson NP *et al*. 1996; Halfvarson J *et al*. 2003). Currently, three hypotheses have been postulated in a bid to understand the environmental factors underlying the pathophysiology of CD, these include; the 'cold chain hypothesis', the 'hygiene hypothesis' and the 'infectious hypotheses' (Koloski NA *et al.* 2008).

Smoking is one of the environmental risk factors that has been clearly implicated in CD pathogenesis (Loftus EV. 2004; Somerville KW *et al*. 1984; Bernstein CN *et al*. 2006). Other environmental factors, such as antibiotic use, breastfeeding, childhood hygiene, diet, oral contraceptive use and stress have been investigated, but findings proved inconsistent, therefore a further in-depth evaluation to substantiate these findings are required (Klement E *et al*. 2004; Allen S *et al*. 1999; Card T *et al.* 2004; Lashner BA *et al*. 1989).

Motivation for the Research

Environment is believed to play an integral role in regulating the biological functions carried out by CD susceptibility genes; however, studies have come to conflicting conclusions for the same or similar environmental exposure. While these discrepancies may in part, be explained by differences in study methodology, it is also possible that the impact of different environmental exposures, particularly those experienced during childhood, varies according to the timing, as well as the extent of exposure. This notion is supported by the recent findings by Basson A. 2014.

Primary Research Questions

1) What is the association between age at time of exposure to childhood environmental factors and the subsequent development of CD? (Chapter 4)

2) What is the association between multiple environmental exposures and the subsequent development of CD? (Chapter 5)

Aims of the Study

The first of two primary aims of the research was to conduct a systematic review of the literature evaluating a number of environmental factors, with an emphasis on timing of exposure; this is contained in Chapter 4. The second aim was to perform a score analysis on secondary data collected from a case control study design by means of the completed doctoral research by Abigail Basson (2011-2014) (Basson A. 2014). This is presented in Chapter 5.

CHAPTER 2

METHODOLOGY

2.1 Methodology Overview

The present research originally intended to investigate the association between serum 25-Hydroxyvitamin D (25[OH]D) concentration with 3 pro-inflammatory cytokines namely, tumour necrosis factor alpha (TNF-α), interleukin-2 (IL-2) and interferon gamma (IFN-γ) in light of their well-known interaction with the T-cell immune responses. In addition, the inflammatory marker, C-reactive protein (CRP) was to be evaluated. The study also aimed to evaluate whether vitamin D deficiency was independently associated with other known confounding factors such as disease duration, body mass index (BMI), oral contraceptive use, smoking, medication use (i.e. immunosuppressants, biologics, corticosteroids), surgical resection, season of study enrolment and ethnicity. The originally proposed research project is included as Appendix 1. However, due to unanticipated funding limitations, evaluation of the originally proposed inflammatory markers could not proceed. As a result of this, it was decided to conduct a systematic review of the literature on the association between environmental risk factors during childhood and the subsequent development of CD, as well as an evaluation of a subset of data collected from a completed doctoral research project performed by Abigail Basson (2011-2014) on a study population in Cape Town, South Africa.

Systematic Review: Methodology Overview

The systematic review evaluated the association between environmental risk exposures in childhood and the subsequent development of CD, with the main emphasis in the timing of the exposure in 3 age intervals; 0-5, 6-10 and 11-18 years. The theme of this investigation was based on the hygiene hypothesis, including the environmental factors associated thereof.

Secondary Data Analysis: Methodology Overview

Analysis of the subset of data was conducted in a bid to evaluate the interaction of environmental risk factor on the development of the CD and to provide insight into the impact in which multiple environmental exposures may shape the intestinal immune system during childhood, through the weighting of environmental exposures, followed by a score analysis approach. In this process, the timing of exposure during 0-5, 6-10 and 11-18 years was investigated.

Statistical consultation and analysis for the statistical methodology of this study was by the Bio-statistics Department of the Medical Research Council of South Africa through statistics consultants. The statistical distribution of continuous variables was appraised and expressed as means ± standard deviations (SD). Using an appropriate correlation structure, the predictors for CD were examined in a conditional logistic regression analysis. To measure the effect size, odds ratios and 95% confidence intervals (CI) were reported. Details of the statistical analysis performed have been described in the relevant chapters of the manuscript

Original Study Design (data obtained): Methodology Overview

From the original case control study conducted by Abigail Basson, 194 CD patients and 213 healthy controls were identified, of whom; 35 (18%) and 19 (9%) were White, 152 (78%) and

177 (83%) were Coloured, and 7(4%) and 17 (8%) were Black, respectively. Overall, 125 (31%) of the cohort were male and 281(69%) were female. There was a significant difference in the median age between the case and control group at study enrolment [47.0 (IQR 38.0-57.0) years and 32.0 (IQR 24.0-44.0) years, respectively, $P < 0.001$]. Ninety seven percent of case and control subjects were born in South Africa ($P = 0.02$). The majority (99%) received a monthly income below R10, 000 ($P = 0.39$). Only subjects residing in the Western Cape at the time of study enrolment were included. Clinical examination and an evaluation of medical and pharmacy records were conducted by a consulting gastroenterologist. Data on clinical and demographic variables were collected using an interviewer-administered questionnaire. A brief overview of the methodological approaches used for the original research design has been described under Point 2.3.1.

2.2 Systematic Review

A systematic review of literature was conducted on studies 16 studies focusing on childhood environmental risk factors of CD. Methodological and demographic data for these studies was compiled and evaluated. A detailed explanation of the methodology has been presented in Chapter 4

2.3 Secondary Data Analysis

This component of the research involved analysis of a subset of data obtained from a completed research project. Demographic data for the cases and controls is presented as frequencies (percentages) for the childhood infection and as medians, and as medians (interquartile range (IQR)) and mean (SD) for the environmental exposure variables (environmental risks factors). The score analysis was conducted for the environmental risk factors over the three age intervals

using logistics regression. The three age intervals were between 0 to 18 years for environmental risk factors. The logistics regression was also used to conduct the score analysis for the childhood infection between ages 0 to 20 years old. A detailed explanation of the methodology has been presented in Chapter 5.

The following is a brief description of original project from which the data for the present research was obtained:

2.3.1 Methodology of Original Project

The data used in this present research was obtained from a large case control study as part of the doctoral research of Abigail Basson. Recruitment of participants was performed in two ways; telephonically and physical approach of participants in both Groote Schuur Hospital (GSH) and Tygerberg Hospital (TBH) between September 2011 and January 2013. The recruitment process was standardized by means of verbal scripts. Data collection was conducted through an interviewer-administered questionnaire for both cases and controls. Clinical examination and a review of the CD patient medical and pharmacy records was part of the data collection process.

The same questionnaire was employed for cases and controls, with the exception of one section pertaining to CD onset, CD symptoms and smoking status at CD diagnosis for the cases.

In an attempt to minimize recall bias, the participants were allowed to complete the questionnaire at home if this would improve the accuracy of their responses. If it was necessary to consult relevant family members, patients were offered the use of a prepaid mobile phone, during the study interview. All inclusion and exclusion criteria were checked at the time of study enrolment,

then again during the interview, and finally, via patient medical records (cases).

The review of medical and pharmacy records of CD patients were performed by two independent clinical investigators using a standardized form. Results were compared and discrepancies were corrected by a re-examination of the relevant record by both clinical examiners, who discussed them until consensus was reached. The participant information documents (PID) (Appendix IV), hospital recruitment poster (Appendix V), participant consent forms (Appendix VI), questionnaire (Appendix VII), the medical review form (Appendix VIII) and the participant recruitment telephone script (Appendix X) are included in the appendix, sections.

Ethical permission for the amendment was granted by the Senate Research Ethics Committee of the University of the Western Cape (Reg No: 11/3/16), and the amended research proposal was approved by the Higher Degrees Committee of the University. The ethical approval letters have been included as Appendix III and IX.

2.3.1.1 Statistical Analysis

Registration of the research project was done with the Bio-statistics Department of the Medical Research Council of South Africa. Esme Jordaan provided statistical consultation for the statistical methodology of the study. Details of the statistical analysis performed for each manuscript are described in the relevant chapters. The statistical distribution of continuous variables was appraised and expressed as medians and interquartile ranges (IQR) or means ± standard deviations (SD) as deemed appropriate. Chi-square, Kruskall-Wallis or Student t-test were employed as appropriate for case and control comparison. Using an appropriate correlation structure, the predictors for CD were examined in a conditional logistic regression analysis. To measure the

effect size, odds ratios and 95% confidence intervals (CI) were reported. STATA 12.0; SAS 9.4; and R were the statistical programs used for the data analysis.

To determine the reliability of questionnaire data, an agreement analysis was performed for all the pertinent variables. The kappa statistic (ranging between 0 and 1, with 0 indicating no agreement and 1 indicating perfect agreement between the two occasions) was used to measure the agreement between repeated data for the questionnaire.

2.3.1.2 Population Description

At the time of study enrolment, the case and control groups comprised of South African Black, Coloured and White individuals aged between 18-70 years, residing in the Western Cape in South Africa.

2.3.1.3 Sample Size

A total of 537 study individuals consisting of 387 randomly selected CD cases and 150 controls (86 unrelated healthy and 64 non-IBD controls) was at first calculated. For childhood environmental exposure evaluation, a total of 200 cases and 200 non-matched healthy controls would be sufficient to provide over 90% strength to detect a risk factor with an odds ratio of 2.7 or greater and it would also have 80% strength to detect an odds ratio of 2.4 or greater. These figures were determined using data from the Western countries since no data is available for the South African population (Molodecky NA and Kaplan GG. 2010; Koloski NA *et al.* 2008). The final sample size achieved for the study was 194 cases and 213 controls, which was deemed sufficient to evaluate the final endpoints.

2.3.1.4 Sample Selection: Crohn's Disease Patients

Recruitment of Crohn's disease patients was conducted at Groote Schuur Hospital (GSH) and Tygerberg Hospital (TBH) gastrointestinal clinics, both of which are public teaching hospitals, and they offer treatment to the majority of IBD patients in the city's public health care sector. Approximately 900 CD follow-up patients are seen annually. These are large referral-based IBD centres in Sub-Saharan Africa. Of the 3.5 million persons who reside in the greater Cape Town area, approximately 90% rely on public-sector health services (Small K. 2008). Subjects with active disease, or inactive CD (i.e. 'remission') were enrolled in the study. Only patients with disease duration of more than five years, who had follow-up at the clinic within the past six months, were included for the study. Crohn's disease diagnosis was done according to the European Consensus Guidelines on the Diagnosis and Management of Crohn's Disease. This is the recommended standard of the European Crohn's and Colitis Organization (ECCO) (Van Assche G *et al.* 2010; Stange EF *et al*. 2006). The guideline includes typical history, radiographic and/or endoscopic appearance, compatible histological features if available, and the exclusion of infectious diseases, such as tuberculosis and amoebiasis, by histological and bacteriological examinations. Medical records were employed in performing a prior diagnosis of intestinal tuberculosis. After that a differential diagnosis of tuberculosis would be made according to an algorithm by Epstein D *et al* through interpretation of clinical, radiographical and endoscopic evaluations in CD patients attending the Groote Schuur Hospital and Tygerberg Hospital gastrointestinal clinics (Epstein D *et al*. 2007). It is also mandatory for all CD patients attending the Groote Schuur Hospital and Tygerberg Hospital gastrointestinal clinics to have a culture for *Mycobacterium tuberculosis* on endoscopic mucosal biopsy. In addition, prior to starting any immunosuppressant, anti-TNF-α or biologics treatment, all patients are given prophylactic

tuberculosis treatment.

2.3.1.5 Sample Selection: Control Group

In a bid to conduct an appropriate case-control study, careful selection of both cases and controls is crucial. There is a risk of selection bias on control selection if a dissimilar mechanism is employed as compared to the cases. Some studies are susceptible to misclassification bias because the selection of controls is through referrals from CD patients or patients with other non- IBD gastrointestinal diseases, such as irritable bowel syndrome and at times through administrative databases. Therefore, in light of this, the present research identified healthy controls unrelated to the CD cases in the following ways: 1) visiting family and friends of patients admitted to the spinal injury wards of Groote Schuur Hospital and Tygerberg Hospital; 2) outpatients from Groote Schuur Hospital and Tygerberg Hospital orthopaedic wards; and 3) porter and security personnel of Groote Schuur Hospital. Only one member from a family was included in the selection. The spinal injury rehabilitation units of Groote Schuur Hospital and Tygerberg Hospital are large referral-based centres for the Western Cape, which served as catchment for control selection. Orthopaedic outpatients, security and porter personnel at Groote Schuur Hospital were considered to be similar demographically to the CD cases, with comparable environmental exposures.

Exclusion of controls was done if any of the following were present: prior diagnosis of tuberculosis, IBD or any other gastrointestinal illness (e.g., irritable bowel syndrome); any immune-mediated (autoimmune) diseases; rheumatological disease (i.e. ankylosing spondylitis, enteropathy); or a family history of IBD. Exclusion criteria were determined at the time of participant recruitment and again at study enrolment.

2.3.1.6 Participant Recruitment: Crohn's Disease Patients

Identification of CD patients was done using the Groote Schuur Hospital and Tygerberg Hospital gastrointestinal clinic appointment lists for the day following the researcher's contact meeting. Eligible subjects were contacted telephonically on the day before their scheduled appointment with the gastrointestinal physician. A standardized verbal script was used and it was conducted in the first language of the participant. Upon engagement, details of the study were first explained to the prospective participant and their willingness to participate in the study was ascertained. At that time (and again at study enrolment), it was made clear to the patient that: 1) all information provided would be confidential; 2) they were not under any obligation to participate; and 3) participation (or non-participation) in the study would not affect their treatment at the clinic in any way. Subjects were informed that they were free to withdraw from the study at any time and, all data given would be destroyed. On the next scheduled appointment the principal investigator or the research assistant provided the patient with a review of the details of the study and provided with the PID (participant information document) to read before consenting to the study (the PID is contained as Appendix IV). A questionnaire was administered in a private room after an informed written consent was obtained by the interviewer.

The attending gastroenterologist captured the data during the patient's scheduled appointment (refer to Point 2.3.1.8). After participating in the study, a token of appreciation of R30 (in cash) was provided to participants. This however was only stated after completing the study so that it would not to be misinterpreted as an incentive or a form of coercion to participate in the study.

2.3.1.7 Participant Recruitment: Control Group

Individuals in the waiting areas of the spinal injury and outpatient orthopaedic wards were randomly approached to serve as the controls for the study. A4 size posters were also employed to advertise the study in three local languages namely Afrikaans, English, and Xhosa for recruitment of controls. These were posted within the hospital wards and on general notice boards (the poster is contained as Appendix V). Included in the posters were the details pertaining to the study, and the informed consent of the study subjects. The contact details of the study coordinator were available on the posters, allowing interested individuals the option of telephonic or mobile communication with the coordinator and stating their name and preferred language, in order to be contacted by the coordinator. There was also an option on the same posters whereby a secondary contact was provided indicating where the interested individuals could provide their contact details within the gastrointestinal clinics. A language appropriate standard verbal script was used when telephonically contacting the participants (the recruitment script is contained as Appendix X). Upon contact, the details of the study were carefully explained to the participant. The participant was then given the option of having the PID sent by mail to him/her before scheduling an appointment in order to participate in the research. An interview appointment was scheduled after obtaining a verbal consent. Medical exclusion criteria for controls were determined during initial telephone contact, and again while administering the interview questionnaire. Prior to study enrolment, the participant was provided with the PID to read before providing written consent to the study. A review of the PID was also conducted by the interviewer with the subject. Participants were again informed of their right to withdraw from the study at any time, and that all data collected would be destroyed upon request.

2.3.1.8 Structured Interview Process (Case and Control Groups)

For the sake of quality assurance, the principal investigator or a trained research assistant conducted the scheduled interview. The principal investigator trained the research assistant to ensure that structured and standardized interviews were conducted. All interview material was available in English, Afrikaans and Xhosa and interviews were conducted in the participant's language of preference. All participants however requested the interview to be conducted in English. The participant was verbally inquired as to whether he/she understood the nature of the study and what he/she was consenting to. Information pertaining to the study such as confidentiality, risks involved with blood collection, and withdrawal from the study were reviewed. The participants were however blinded to the hypothesis of the study. When the research interviewer was certain that the participant fully understood the study details, the participant was requested to sign the written consent form (contained as Appendix VI for both cases and controls).

On conducting each interview, the interviewer possessed two copies of the questionnaire: one was provided to the participant to read as the question was being posed by the interviewer, while the second questionnaire was used by the interviewer to read the questions aloud to the participant. The interviewer recorded the participant's responses on a predesigned instrument (contained as Appendix VII). In a bid to standardize the interviews, a document describing and defining terms had been generated by the principle investigator to clarify terminology to the participant if necessary. Explanation outside this document was strongly discouraged for the interviewers. At study enrolment, anthropometric measurements were performed. Body weight was measured to the nearest 0.1kg using a scale (A&D Personal Precision Scale, Tokyo, Japan) and height was measured using a portable stadiometer to the nearest millimetre. Measurements were performed

twice, using the same scale and stadiometer for all participants.

2.3.1.9 Response Rate: Crohn's Disease Patients

It was considered acceptable, having a participation rate of 80% out of the 255 cases originally calculated from those contacted, at the study centres (Groote Schuur Hospital and Tygerberg Hospital gastrointestinal clinics). Out of the total, only two patients declined participation in the research thus there was a 99% response rate. The CD patients expressed pleasure in contributing to the research project. The high response rate may assumedly be attributed to the following: 1) research involved no follow ups or additional blood sample collection and 2) the research was conducted within the already scheduled appointments with the subjects' gastrointestinal physicians and the within the waiting period before being seen by the physician.

2.3.1.10 Response Rate: Control Group

Response bias occurs when the 'exposure' number of control participants agreeing to participate in the study is different from those who refuse to do so. Many studies fail to account for non-responders, and in general, non-responders tend to have different socioeconomic or demographic profiles or unhealthy lifestyle behaviours, such as smoking (Molodecky NA *et al*. 2011; Van Loon ES *et al.* 2003; Korkeila K *et al.* 2001*).* The majority of IBD-studies evaluating environmental risk factors such as the 'hygiene hypothesis' have not accounted for non- responders; this is a concern since, a systematic difference may exist in exposure between cases and controls. For example, there is a significant correlation between a lower socioeconomic status and hygiene practices (Molodecky NA *et al.* 2011*).*

The principal investigator or research assistant recruited the majority of control participants, by physically approaching the subjects at Groote Schuur Hospital. In the spinal wards, family waiting areas as well as the orthopaedic outpatient waiting areas is where most recruitments were conducted. Porter and security personnel were also approached to serve as control subjects. In the participant recruitment processes, a standard verbal script was employed by both the principal investigator and the research assistant. Response bias was counteracted by the principal researcher or the research assistant by randomly selecting the subjects and approaching them appropriately while taking into cognisance the age, ethnicity and gender. This was done to reduce the possibility that response may be influenced by the subjects' perceptions of the recruiting researcher.

Out of the 159 subjects who were personally approached, only nine (6%) turned down the invitation to participate. The fairly high response rate may be attributed to the fact that those that were approached had sufficient time to participate in the study at that time, or were able to schedule a later study enrolment date, because they would be returning to the hospital within a short period. The remaining 63 control subjects were recruited via the advertising posters which were distributed throughout the hospital. None of the control participants who contacted the principal investigator about the study via this method later refused to participate.

2.3.1.11 Questionnaire

For data collection the principal investigator developed the structured questionnaire. It included seven demographic sections namely: General Information; Environmental Factors; Inflammatory Bowel Disease Medical History (cases only); Medical History; Family History; Smoking History; and General Health. The Environmental Factors section was structured to include three age intervals: 0-5 years; 6-10 years; and 11-18 years. Identical questions for each age interval were

included and race was self-reported. Six options were available for the participants: 'Asian', 'Black', 'Coloured', 'Indian', 'White' and 'Other'. However only Black, Coloured and White races were represented in the study. The questionnaire (English version only) is contained as Appendix VII for reference.

2.3.1.12 Medical Review Form

The principal investigator developed a medical review form which comprised of two sections. The first section would be completed during the appointment by the attending gastroenterologist with the CD patient, ensuring that all the required data was captured at the time of study enrolment. The other section would be used by the principal investigator and research assistant for gathering the patient's medical history from medical and pharmacy records. For the comprehensiveness of the information to be obtained, the forms were piloted by two separate gastroenterologists using CD patient charts. These gastroenterologists were not included in the research and their data was not included in the final research dataset. The CD patient medical review forms are contained as Appendix VIII.

It must be noted, that prior to the fairly recent initiation of computerized pharmacy records, data regarding medication use has been poorly captured in the past. As this was a retrospective study, details on the type of medical treatment used, duration of treatment, dosage and adherence could not always be determined.

2.3.1.13 Medical Review Form

Upon completion of data collection, 10% (n = 21) of the medical review forms were randomly

selected and completed by an independent investigator. This investigator was however not involved in the study, in a bid to ensure the reliability of the initially recorded data. There was minimal discrepancy between the results of the original records and the re-checked records.

2.3.1.14 Questionnaires

It was initially proposed in the study protocol that a Cronbach's alpha analysis would be performed, and a coefficient of 0.8 would be considered satisfactory in measuring the internal consistency of the questionnaire, and its freedom from random errors. However, after thorough discussion with the head of the Biostatistics Department of the Medical Research Council of South Africa, it was decided that due to the objective, 'factual' nature of the questions within the questionnaire, this analysis would not be relevant, and thus was not performed. Questionnaires were piloted and all Consent forms, questionnaires, PIDs and telephone scripts were translated from English to Afrikaans and Xhosa by four separate translators, who spoke native language. Four translators for each language comprised of both students and staff members in the Department of Dietetics and the Foreign Languages Department of the University of the Western Cape were recruited for the study. All participants, however, preferred the English consent form, PID and questionnaire. The Afrikaans and Xhosa consent forms, hospital recruitment posters (controls), PIDs and the questionnaires for the study participant groups. Only English versions have been included as reference.

2.3.1.15 Generalizability of Study

In many IBD case control studies, cases are identified from gastrointestinal clinics, owing to feasibility (i.e. participants are easier to identify). In this research study, identification of CD

patients was hospital-based, and not population-based. However, most subjects in state practice in the Western Cape attend one of the two hospitals from which patients were recruited; findings are therefore likely to be generalizable.

2.3.1.16 Ethical Clearance

Ethical clearance was accorded by the Ethics Research committee of the University of the Western Cape (UWC) (Reg No:11/3/16), the Human Research Ethics Committee of the University of Cape Town (UCT) (HREC REF: 122/2011), the Provincial Ethics Committee of the Department of Health as well as both the ethics committees of GSH and TGH. The research was performed in accordance to the Ethics on Medical Research General Principles, following the guidelines published by the Medical Research Council of South Africa (Benatar SR *et al*. 2004).

Informed Consent

Prior to data collection, written, informed consent was obtained from participants after full disclosure of study details. Participation was voluntary, and participants were informed of their right to withdraw from the study at any time, without any consequences, or loss of any benefits for which participants would otherwise qualify. Only participants, who could understand the consent form and the conditions of the study, were enrolled. There were two separate versions of the consent forms, one for the cases and one for the control groups. Informed written consent was obtained from all participants. If a signed consent was not provided, the subject was excluded from the study, and no personal data was collected.

Participant Anonymity

Anonymity and confidentiality of the participant information was maintained by removing identifiers from questionnaires. Medical information that was given by a participant or acquired from medical records was not shared with other participants such as, relatives or any other persons who were not co-investigators. Co-investigators only received information on a need-to- know basis. Questionnaires were coded using a unique number as an identifier, instead of participant names, or any other identifiable information. All identifiable information of the study participant (and participant code) was situated on the front page of the questionnaire. The rest of the questionnaire pages only contained the participant codes.

All of these materials were obtained for research purposes only, and data relating to participant identity was kept in strict confidence. Only the principle researcher had access to patient names and the identifier code key, which was at all times kept separate from the questionnaires, in a locked filing cabinet located in a locked office, and maintained with the strictest confidentiality precautions by the principal researcher. Coded data was entered into a password protected computer. All clinical or personal data acquired remained at all times anonymous, with the front page of the questionnaire removed.

2.4 Statistical Analysis

For cases and controls, the demographic data is presented as frequencies for categorical data an as medians and interquartile range (IQR) for the numerical data. Environmental risk factors and their impact for CD were evaluated through employing the multiple logistics regression models. Risk factors that were significant for ($P<0.05$) for a specific age interval were included in the final modes (0-5year, 6-10years, 11-18years); were all adjusted for age at study enrolment, gender and

ethnicity. Odds ratios and 95% confidence intervals were reported to measure the effect size. Risk factors with a cell frequency below 10 for any of the four cells in the cross tabulations of the risk factors with CD were not included in the models. Exact logistic regression was used for modelling with small cell sizes.

One year after study completion a total of 40 (10%) randomly selected participants completed the interviewer administered questionnaire for a second time in order to measure the agreement between repeated data for the questionnaire using a kappa statistic. Only data pertaining to the 3 age intervals was extracted in this process. Again, participants had the option of completing the questionnaire at home if they felt consulting family members may help with the accuracy of some responses. An agreement analysis was performed to determine the reliability of the questionnaire data for all the relevant variables. The kappa statistic (ranging between 0 and 1, with 0 indicating no agreement and 1 indicating perfect agreement between the two occasions) was used to measure the agreement between repeated data for the questionnaire. Standards by Landis and Koch were used to interpret the strength of the agreement (Landis JR and Koch GG. 1977) (Figure 1).

Data has been made publicly available via Figshare:

http://dx.doi.org/10.6084/m9.figshare.1159053 and http://dx.doi.org/10.6084/m9.figshare.1041586

Figures Referred to in Chapter 2

FIGURE 1: Interpretation of Landis and Koch's measure of agreement Kappa statistic (κ)

Agreement
Less than chance agreement
Slight agreement
Fair agreement
Moderate agreement
Substantial agreement
Almost perfect agreement

CHAPTER 3

LITERATURE REVIEW

3.1 Introduction

Crohn's disease (CD) is a chronic relapsing gastrointestinal condition that is characterized by a pronounced inflammation of the lining of the intestines. It can be explained as a chronic multifactorial disease that occurs in genetically susceptible individuals, involving environmental influences, intestinal dysbiosis, and altered immune responses (Foster A and Jacobson K. 2013). The condition forms one of two inflammatory bowel disease (IBD) subtypes, the second being ulcerative colitis (UC) and is classified under idiopathic IBD (Silverberg MS *et al.* 2005). While the actual aetiology of the disease remains unclear, it is believed that disease onset is due to an interaction between a broad range of susceptibility genes and the environment. Inflammation of the intestinal mucosa is the result of an abnormal activation of the gut immune system due to bacterial and dietary antigenic stimulation (Prideaux L *et al.* 2012). This involves the ability of dendritic cells (DCs), macrophages and neutrophils to distinguish between commensal or 'healthy' and pathogenic bacteria within the gut (Prideaux L *et al.* 2012). No infectious trigger has been singled out, however indirect evidence implicates mycobacteria as potential initiator of CD (Sechi LA *et al.* 2005).

Recent studies are suggesting that autophagy and host cell response to intracellular microorganisms are linked to the pathogenesis of the disease (Rioux JD *et al.* 2007). The development of CD is characterized by an increase in the production of potent pro-inflammatory cytokines namely, TNF-α, INF-γ and IL-2 (Podolski DK. 1991; Lemire JM. 1992; Cantorna MT

et al. 2004). Recent findings have also attributed the development of CD to a new effector helper T cell named Th17 which is responsible for the production of interleukin-17 (IL-17) (Bendix-Struve M *et al.* 2010).

3.1.1 Crohn's Disease Classification

In the bid to understand this clinically heterogeneous disease, attempts have been made to classify it into categories such as age of onset, disease location and disease behaviour. However the generally accepted standard is the Montreal classification system (modified from the revised Vienna classification) (Satsangi J *et al.* 2006), which takes into consideration the critical phenotypic characteristics of the disease (Table 1).

According to the Montreal classification system, the onset of the disease is mostly in the A2 (17-40years) category which is 78.6%. The onset in the A1 (≤16 years) and A3 (>40 years) categories are 16.8% and 6.6% respectively (Aljebreen AM *et al.* 2013). As for the disease location the most common location are the ileocolonic and the ileum which have an occurrence percentage of 48.8% and 43.55%, respectively. According to the system, colonic CD is not very common and has a 7.7% occurrence. Basing on the Montreal classification the behaviour of the disease is 41.8% non-stenotic or non-penetrating, 32.85 stenotic and 25.4% penetrating (Aljebreen AM *et al.* 2013). Crohn's disease usually appears between the ages of 15 and 30 years (Sands BE and Grabert S. 2009). According to a study involving 1015 CD patients, the anatomic distribution of the disease was reported to be as follows: 13.1% upper gastroduodenal, 27.2% right colon, 25.3% small intestines alone and 34.6% ileocolon (Freeman HJ. 2007). A study in Asia has revealed that the age of onset of the disease has an influence on the clinical features of the disease (Law ST and Li KK. 2013). Compared to adult-onset patients (≥29years old), young- onset patients (≤16 years

old) tend to exhibit more symptoms of fever, weight loss, isolated abdominal pain, lower body mass index (BMI), extra-intestinal manifestation (EIM) and perianal lesion (Law ST and Li KK. 2013). However in both groups inflammatory lesion was the dominant behaviour of the disease and ileocolonic manifestation was the most prevalent site of involvement.

3.1.2 Disease Activity

Crohn's disease is a lifelong disease that is characterized with periods of relapse and remission. Depending with the individual, periods of remission can span for years, even decades. Patients experience the disease in various ways. For instance, some patients exhibit a few symptoms and have long periods of remission, whereas others constantly exhibit severe symptoms. The severity of the disease or disease activity is measured in two formal grading systems namely; the Crohn's Disease Activity index (CDAI) (Table 2) and the Harvey-Bradshaw Index (HBI) (Table 3) (Ullman T. 2008).

The relapse-remitting nature of the disease is such that it demands expensive medications, such as corticosteroids, antibiotics, immunomodulators, biologics or even surgeries which often have many undesirable side effects and negatively impact the patients' quality of life (de Zoeten EF *et al.* 2013). Currently there is no known cure to CD but pro-inflammatory cytokine suppressor drugs (e.g., anti-TNF inhibitors) have shown to be beneficial in attaining and maintaining disease remission (Cosnes J *et al.* 2011). However there are risks of cancers and possible infection through the continued use of immune-suppressing drugs hence more research on less expensive and relatively safer therapeutic treatments is needed.

3.1.3 The Global Incidence and Prevalence of IBD

There is a generally observed high prevalence and incidence rate of CD in North America, Northern Europe, United Kingdom and Canada. In Northern America the incidence rate of the disease is 6.9 (Loftus EV *et al.* 2000) to 15.6 (Blanchard JF *et al.* 2001) per 10^5 population per year. The United Kingdom and Scandinavia have an incidence rate of 8.3 (Rubin GP *et al.* 2000) and 5.8 (Moum B *et al.* 1996 (a)), respectively. However there is a remarkably low incidence of CD in south and central Europe, Africa and Asia having incidence rates of 2.3 (Tragnone A *et al.* 1995), 1.8 (Wright JP *et al.* 1986) and 0.5 (Morita N *et al.* 1995) per 10^5 population, respectively. These statistics have been illustrated in Table 5.

The prevalence of IBD is highest in Europe and Canada, with countries such as Italy recording a rate of $322/10^5$ population and Canada having $319/10^5$ population (Molodecky NA *et al.* 2012; Bernstein CN *et al.* 2006). In the US, the prevalence rates reported for both UC and CD are 238 and $201/10^5$ population, respectively (Kappelman MD *et al.* 2007). According to age- standardised report from New Zealand, the incidence rates for UC and CD are 155 and $145/10^5$ population, respectively (Gearry RB *et al.* 2006). It is generally observed that the occurrence of IBD varies with geographical location. A general North-South gradient is observed in United States and Europe (Hovde and Moum B. 2012). However in other continents IBD is less prevalent a lower prevalence observed in Asia (Molodecky NA *et al.* 2012). This geographical difference has become less prominent over years and recent studies have shown the increased incidence and prevalence of IBD in Australia and New Zealand (Molodecky NA *et al.* 2012).

There is a traditional view that follows the occurrence of IBD that in low incidence areas, UC is

seen to emerge first, then followed by CD. Throughout the world in different regions and locations, there are variations in the ratios between the incidences and prevalence of UC and CD. In Australia, Canada and the United States, CD is more predominant as compared to UC (Ng SC. 2014(a)). Some studies reveal that UC to be more prevalent than CD in some North American states (Molodecky NA *et al.* 2012). Among the Nordic countries however, UC is shown to be more prevalent (Vind I *et al.* 2006; Lehtinen P *et al.* 2011). Studies from Asia have revealed a decrease in the ratio of UC and CD in the last two decades (Thia KT *et al.* 2008). In Korea the ratio of UC to CD has decreased from 6.8 to 2.3 and China reveals even a more drastic decrease that is from 41 to 15 (Wang WF 2010). The variations in the regions may be reflection of the differences in the environmental risk factors as well as the differences in genetic disposition of the individuals (Ng SC. 2014 (a)).

3.1.4 Westernization of Lifestyle

Inflammatory bowel disease tends to occur mainly in high socio-economic groups (Loftus EV and Sanborn WJ. 2003). There is a general trend of a high prevalence and incidence of CD and UC in heavily industrialized and developed nations such as United States, United Kingdom and Scandinavia. The industrialization tends to impact on lifestyle changes on individuals such as diet, sanitation, lifestyle, medications, microbial exposure, occupations pollution and exposure to industrial chemicals (Hanauer SB. 2006; Andres PG and Friedman LS. 1999; Molodecky NA and Kaplan GG. *et al.* 2010). However, IBD is emerging in nations that are becoming more westernized such as nations of Asia, Eastern Europe, French West Indies and North Africa (Ng SC. 2014 (a)). Such areas were previously reported as low incidence areas for IBD. It is also shown that IBD commonly occurs in urban areas as compared to rural areas therefore heavily

suggesting the role of environmental factors as strong determinants of IBD (Molodecky NA and Kaplan GG. 2010).

3.1.5 Emigration

Emigration studies have shown that individuals migrating from low IBD prevalent areas to high prevalent areas are at a greater risk of developing IBD especially the first generation children (Bernstein CN. 2008). Studies conducted in the United Kingdom's industrialized cities of Bradford (Findlay JM and Jayarantne SD. 1986), Leicester (Probert CSJ *et al.* 1992) and London (Chong SKF and Walker-Smith JA. 1986) among Asian immigrants and their children suggest that these immigrants have greater susceptibility to CD and UC when compared to those residing in Asia (Satsangi J *et al.* 1994). Among Jewish immigrants, a transition in the prevalence of IBD has also been observed. Compared to Sephardic Jews in the Middle East and North Africa, an elevated prevalence of CD and UC is observed in the Jews who migrated from Middle East and North Africa to Israel and the Ashkenazi Jews who migrated from Europe to Israel (Gilat T *et al.* 1986).

3.1.6 Ethnicity

Notably differences in the prevalence of CD and UC between various ethnic and racial groups have been reported (Satsangi J *et al.* 1994). In general, the prevalence of IBD is higher among the White race, when compared to non-Whites, with the highest prevalence and incidence observed among Ashkenazi Jews of Europe, United States and Cape Town (Gilat T *et al.* 1986). The prevalence and incidence is lower among the Black, Latin and Native Americans, the Maoris and the Asians (McConnell RB and Vadheim CM. 1992), although in recent years there has been an

observed rise in the occurrence of IBD in these previously low risk ethnic groups. Notably however, the majority of earlier studies have focused on populations of higher socioeconomic status, suggesting a potential bias.

In the United States accumulating evidence supports an increasing occurrence of IBD in Black and Hispanic populations (Calkins BM *et al.* 1984). A study by Malaty HM *et al.* 2010, on a population predominantly of low socioeconomic status in the United States revealed that Hispanics were more susceptible to UC than CD, and that there was greater prevalence of UC, when compared to their Black and Caucasian counterparts (Malaty HM *et al.* 2010). On the other hand, there was no statistically significant difference between the occurrence of CD and UC within the Afro-American and Caucasian population groups. However CD was more prominent in both groups (Malaty HM *et al.* 2010). The racial gap on the incidence and prevalence of the disease seems to be closing, and this again may be an indication that environmental factors have greater influence on the occurrence of the disease (Hanauer SB. 2006).

3.1.7 Asia

The epidemiology of Asia is of importance because more than half of the world population resides on this continent. For long it has been reported that the highest occurrence of IBD is in the Western countries. The occurrence of IBD is rising in areas which were traditionally known to have lower occurrence such as Eastern Europe (Lakatos L *et al.* 2004) and Asia (Sood A *et al.* 2003). The incidence and prevalence of IBD in Asia is however still significantly lower than in the West (Prideaux L *et al.* 2012). Nevertheless, Asia is experiencing a rise in the incidence and prevalence of IBD alongside an expeditious socio-economic development (Prideaux L *et al*. 2012). Japan (Ng SC. 2014 (a)) and Korea (Sood A and Midha V. 2006) are reportedly the only countries in Asia

which have reliable nationwide IBD registries. In the past decades, increase in the occurrence of IBD has been widely reported in these countries. A study conducted in Japan in the year 1991, revealed an incident rate of $1.95/10^5$ population and $0.52/10^5$ population for UC and CD, respectively (Morita N *et al*. 1995). The same study revealed a prevalence rate of $18.12/10^5$ population and $5.85/10^5$ population for UC and CD respectively in that same year. A cumulative number of IBD cases have been recorded to be three times greater in the year 2000 and it is estimated that a total of 10000 people are suffering from UC in Japan (Yao T *et al.* 2000).

It is also recorded in studies on IBD in Japan that between the years 1961 and 1991 the incidence rate of UC rose from 0.02 to $1.95/10^5$ population (Kitahora T *et al.* 1995; Morita N *et al.* 1995) and the incidence rate of CD rose from 0.60 in 1986 to$1.20/10^5$ population in 2008 (Yao T *et al*. 2000). According to two population-based studies conducted in Korea between 1986 and 2008, the incidence rate of CD rose from 0.05 to 5.1/ 10^5 population and the rate for UC rose from 0.34 to $5.4/10^5$ population (Yang SK *et al*. 2008; Shin DH *et al.* 2011). A cohort study in Hong Kong also revealed an increase in the incidence of CD and UC from 0.4 - 1.0 and 0.8 - $1.2/10^5$ population, respectively between the year 1990 and 2001 (Leong RW *et al.* 2004). There are variations in the incidence rates observed between different ethnic groups and geographic locations. It is however important to note that in Asia there is greater incidence of UC as compared to CD with the highest incidence rates reported in India, Japan and the Middle East (Ng SC. 2014 (a)).

Studies in Japan spanning from 1984 to 2005 have shown a drastic increase in the prevalence of UC from 7.85 - 63.6 / 10^5 population (Morita T *et al.* 1995; Asakura K *et al.* 2009) and that of CD rose from 2.9-$13.5/10^5$ population (Yao T *et al.* 2000). The figures from South Korea reveal an

alarming increase of UC from 7.6 - 30.9/10^5 population from 1997 to 2005 (Yang SK *et al*. 2008). A further increase was also seen in CD between the year 1986 and 1998 where a prevalence rates rose from 2.9 - 13.5/10^5 population (Yao T *et al*. 2008). Several studies from Asia have revealed a general increase in the prevalence of IBD. This strongly suggests that the occurrence of IBD is a dynamic process in which the incidence and prevalence of the disease gradually changes in different geographic locations due to alterations in environmental factors.

3.1.8 South Africa

The prevalence and incidence of CD in Africa in general has not been well documented. Data found in South Africa and mainly in the Cape Town area is the one used to represent the whole continent of Africa. According to a study conducted at Groote Schuur hospital from 1970-1974, Novis BH *et al.* reported that diagnosis CD had become more frequent over the years and that CD was becoming the major IBD among the Coloured and the White community (Novis BH *et al.* 1975). The reported incidences of CD during these years within the Coloured and White were 0.4 and 0.8 / 10^5 population. Wright BH conducted a retrospective study at the same hospital from 1975 to 1980. It was seen that the incidence rate of CD among the Coloured community had increased from 0.4/10^5 population (1970-1975) to 1.3/10^5 population (1975-1980) (Wright BH. 1992). Likewise among the White community, a subsequent increase was observed. There was an increase from 0.8/10^5 population (1970-1975) to 1.2/10^5 population among the Whites (Wright BH. 1992). It is however interesting to note that in both studies, there was only one Black patient therefore suggesting that CD is less common among the Black community. It was noted that within the Jewish community was the highest incidence rates of CD. Incidence rates of 5.0/10^5 population were noted from (1970-1975) and a rise in incidence to 7.2/10^5 population per year

(Wright BH. 1992).

3.2 Inflammation

Inflammation is the body's first line of defence against pathogens. It is a protective reaction of living tissue to infection, injury or irritation often characterized by swelling, pain, redness and heat. Inflammation is mainly mediated by intercellular inflammatory mediators known as cytokines. These are soluble cell-derived polypeptides which function as positive or negative regulator molecules of the immune system. Cytokines are the major orchestrators of cellular activation and serve as the determinants of the cellular infiltrates. In addition, cytokines serve as pleiotropic molecules for both paracrine and autocrine functions in the body. Inflammation can be subdivided into two main categories: acute inflammation and chronic inflammation (Abbas AB and Litchman AH. 2009).

Tumour necrosis factor alpha (TNF-α) is one of the key cytokines involved in the acute inflammation. This cytokine is produced chiefly by activated macrophages. However, it is also produced by mast cells, natural killer (NK) cells, T-cells and fibroblasts. Tumour necrosis factor alpha is an endogenous pyrogen, thus it is able to induce fever by either inducing the production of interleukin-1 (IL-1) or by stimulation of prostaglandin E_2 (PGE_2) synthesis. This cytokine has a major pro-inflammatory function through its association with other cytokines (Bradley JR. 2008). Tumour necrosis factor alpha and IL-1 stimulate interleukin-6 (IL-6) synthesis in a number of cells which in turn triggers a series of inflammatory reactions in association with other overlapping cytokines such as interleukin-11 (IL-11) and interleukin-8 (IL-8). Tumour necrosis factor is normally not detected in healthy individuals. However in infectious and inflammatory conditions exceedingly high levels are found in the blood serum and tissue samples (Robak T *et*

al. 1998). It is interesting to note that the levels of this cytokine are directly correlated to the level of infection (Waage A *et al.* 1987). Increased local production of TNF-α is the driving force behind effecting a chronic inflammatory response in tissue and serum (Maini RN *et al.* 1995). Tumour necrosis factor alpha can induce tissue injury in different pathways. It can have direct effects on the intestinal epithelial function and permeability (Gibson PR *et al.* 2004). An impaired epithelial barrier results in increased exposure to antigenic macromolecules, thereby triggering mucosal inflammation. This cytokine can also cause an upregulation of adhesion molecule expression on endothelium of the intestinal wall (Binion DG *et al.* 1997). Tumour necrosis factor alpha is pivotal in inducing the increased production of chemokines by various cell types of the immune system (MacDermott RP *et al.* 1998). The mucosal levels and the inflammatory cell production of TNF-α are elevated thus making it a cytokine of great significance in the pathogenesis of CD (Gibson PR *et al.* 2004).

Chronic inflammation is divided into two subunits: humoral inflammatory response and cellular inflammatory response. Interleukin-2 (IL-2) is a glycoprotein originally referred to as T-cell growth factor (TCGF). It is primarily involved in the cellular inflammatory response and it also plays a significant role in the humoral inflammatory response. Interleukin-2 is an α-helical cytokine which is chiefly produced by activated T-helper cells (Shaikh PZ. *et al* 2011). It is vital in the activation and regulation of white blood cells (both leukocytes and lymphocytes) in the immune system. Interleukin-2 effects the differentiation of CD4+ T-cells into Th1 and Th2 cells. In like manner 1,25D signalling functions to repress the transcription of the genes that encode for the Th1 immune response such as interferon gamma (IFN-γ) and interleukin-21 (IL-21) (Baeke F *et al.* 2010). CD4+ T-cells respond differently to antigenic stimulation, adopting specific phenotypes which are vital for varying immune functions and immune mediated responses (Herbst

RS *et al*. 2009). Activated T-cells also express the vitamin D receptor (VDR). Interleukin-2 is critical in the induction and maintenance of regulatory T-cells (Tregs). These are crucial in the maintenance of Fox P3 and the TGF-β effects (Davidson TS *et al.* 2007; Setoguchi R *et al.* 2005). Likewise the vitamin D3 (VD3) signalling enhances the tolerance of the dendritic cells which in turn promotes the production and function of Tregs which are the potent mediators of the immune system tolerance (Chung C *et al*. 2011). Interleukin-2 also functions to inhibit the differentiation of Th17 cells since the re-stimulation of the differentiated cells with IL-2 eliminates the production of interleukin 17 (IL-17) subsequently inducing the production of IFN-γ (Hoyer KK *et al.* 2008). Interleukin-2 functions as a growth factor thereby stimulates the production of NK cells and macrophages (Hoyer KK *et al.* 2008). It is crucial in the production of memory T-cells. It is also important to note that the production of IL-2 is inhibited through the binding of the VDR onto the nuclear factor of activated T-cells (NF-AT) binding site of the promoter for IL-2.

Interferon gamma falls in a category of cytokines that were originally identified and named for their antiviral properties. It is a type II interferon that is mainly produced by activated T-cells and natural killer (NK) cells. However some studies have shown that B- cells, natural killer T (NKT) cells and antigen-presenting cells (APCs) are also involved in its production. Interferon gamma is a homodimer, serving to stimulate the effector functions of mononuclear phagocytes, as well as to enhance the major histocompatibility complex (MHC) I and II expression on nucleated cells. Interferon gamma production is controlled by interleukin-12 (IL-12) and interleukin-18 (IL-18), by promoting the Th1 polarization. These two cytokines are produced mainly by APCs. The secretion of IFN-γ activates neutrophils and macrophages (Schroder K *et al.* 2004). Crohn's disease is characterized by a Th1 and a Th17 CD4+ immune response (Strober W and Ivan JF. 2011). Upon antigen stimulation, the CD4+ Th cells can be differentiated into Th1 and Th2 cells

depending on the cytokine profile. The secretion of IFN-γ in the Th1 response is induced by IL-12 derived from dendritic cells which is tangential to pathogen recognition receptor (PRR) signalling (Kaser A and Blumberg RS. 2010). The Th1 response subsets produce lymphotoxin (TNF-α) and IFN-γ which have been implicated in a number of immune mediated diseases (Boonstra A *et al.* 2001). A number of reports have shown IFN-γ to be produced in abundance by the colonic mucosal tissue and intestinal lamina propria mononuclear cells (Fais S *et al.* 1994). Studies on the wild type (WT) dextran sulphate sodium (DSS) treated mice have shown IFN-γ to be crucial in the induction and progression of CD-like symptoms. Interferon gamma subsequently provoked the infiltration of leukocytes into inflammatory lesions. The mice expressed a gigantic production of IFN-γ in the gut, body weight loss and a 60% mortality rate (Ito R *et al.* 2006).

C-reactive protein (CRP) is the classic acute phase response protein that functions as a marker of inflammation, tissue damage and infection. Pepys MB. 1995 describes it as an exquisitely objective sensitive biomarker. C-reactive protein is mainly produced by the hepatocytes in the liver (Vermeire S *et al.* 2004). It is a phylogenetically highly conserved plasma protein. C-reactive protein functions as a pattern recognition molecule that binds to specific molecular configurations on the surface of pathogens and infected host cells (Black S *et al.* 2004). It is rapidly synthesized after tissue injury or infection and the plasma concentration often increases up to a thousand fold during inflammation. This potent non-specific innate immune response molecule is produced by the cytokine TNF-α, IL-1 and IL-6 (Koenig W *et al.* 1999). C-reactive protein functions to promote phagocytosis by initiating the activation of the complement cascade and binding to high affinity Fc gamma receptors on phagocytes. It also promotes bacterial capsular swelling, agglutination and complement fixation (Faraj M and Salem N. 2012). C - reactive protein serves as a potent marker of an immune response. It is found in large quantities in the serum during the

acute phase and the chronic stages of inflammation (Vermeire S *et al.* 2004).

C- reactive protein has a half-life of 19 hours but however it remains stable and identical under all conditions therefore making it a reliable biomarker (Koenig W *et al.* 1999). This protein is up-regulated when an acute phase response is activated. There is a striking heterogeneity in CRP response among inflammatory diseases such as CD and rheumatoid arthritis (RA) (Vermeire S *et al.* 2004). In CD, CRP has a number of functions. It acts as a marker for diagnosis and differential diagnosis of IBD (Beattie RM *et al.* 1995) and it also serves as a marker for disease activity (Fagan EA *et al.* 1982). C-reactive protein is also important as a marker of outcome and risk assessment for surgery (Rutgeerts P *et al.* 2003) and a marker of treatment response (Louis E *et al.* 2002).

3.2.1 The Immune Response

Along with other cell types, T lymphocytes secrete cytokines, proteins that have autocrine and paracrine effects on T cell function. When first presented with a specific antigen, a naïve Th cell will produce IL-2 and begin to expand in number (Elliott DE *et al*. 2000). As the Th cells expand, members of the population produce other cytokines such as TNF-α, IFN-γ, IL-4, IL-5, IL-10 and IL-13. As antigen exposure is prolonged, the cytokine profile produced by the T cells will polarize to either the Th1 or the Th2 immune response (Romagnani, S. 1994). The Th1 response is characterized by the production of TNF-α, IFN-γ, LT and Th1 cytokines mediate delayed-type hypersensitivity reactions, macrophage activation, cellular cytotoxicity, and switch B cell immunoglobulin (Ig) production to subclasses that fix complement (murine IgG2a or human IgG1). On the contrary, the Th2 response produces IL-4, IL-5, IL-10, IL-13 and Th2 cytokines mediate eosinophilia, B cell proliferation, and switch B immunoglobulin production to IgA, IgE, and IgG subclasses that do not fix complement (murine IgG1 or human IgG4). The Th2 cytokines

IL-4, IL-13, and IL-10 inhibit delayed-type hypersensitivity reactions, macrophage activation, and cytotoxicity (Elliott DE *et al*. 2000).

The selection of the immune responses is mainly dependent upon the antigen dose, accessory cell function and costimulatory molecule display. However, the cytokine profile present during antigen stimulation is the controlling factor moulding the Th1 or Th2-type response (Paul WE and Seder RA. 1994). The presence of IL-12, IL-18, and IFN-γ promotes expansion of Th1 cells. Macrophages produce IL-12 and IL-18 which augment Th1 cell development and stimulates production of IFN-γ. Interferon-γ increases antigen presentation and IL-12 production by macrophages (Kubin M *et al.* 1994) and also increases Th1 cell high-affinity IL-12 receptor display (Gollob JA *et al.* 1997). Ultimately, IFN-γ inhibits the proliferation of Th2 cells, thus promoting the Th1 cells. The IL-12/IFN-γ positive feedback circuit augments Th1 while inhibiting Th2 cell development (Elliott DE *et al*. 2000).

The expansion of Th2 cells is promoted by the presence of IL-4 and IL-10. Interleukin-4 is an autocrine growth and differentiation factor for Th2 cells and it signals through the 'signal transducer and activator of transcription' 6 (STAT6) to augment its own production in a positive feedback circuit (Elliott DE *et al*. 2000). On the other hand, IL-4 inhibits release of IL-12 and other cytokines from macrophages (de Waal *et al*. 1993). Interleukin-13 and IL-10 also act in the same fashion to inhibit the release of IL-12 (Moore KW *et al*. 1993). Interleukin-10 inhibits macrophage accessory cell function required by differentiated Th1 cells, but not Th2 cells (Fiorentino KW *et al*. 1991). Thus, IL-4, IL-13, and IL-10 inhibit Th1 cell development while championing Th2 responses.

3.2.2 The Intestinal Microbiome

Commensal microbiota influences the development of the gut immune system by maintaining homeostasis between them and the pathogenic bacteria. The interaction between the mucosal innate immune system and the endogenous microflora ultimately functions to promote the development as well as maintain the intestinal ecosystem. The innate immune system has the ability to distinguish between pathogens and harmless antigens by means of pattern recognition receptors (PRRs) (Medzhitov R and Janeway C. 2000). Toll-like receptors (TLRs) which are present on immune cells such as macrophages, neutrophils, DCs and intestinal epithelial cells (ECs) enable the mammalian cells to recognize characteristic molecules present on microbes (Marshak-Rothstein A. 2006). The gut microbiome can regulate the intestinal immune system by modulating TLR expression on immune-sensory cells consequently triggering cytokine production through the activation of T cells (Purchiaroni F. 2013).

Microbial exposures are believed necessary in developing the gut's immune system. These exposures then optimize the acquired (adaptive) immunity and immunological memory, which will ultimately dictate balance between the Th1, Th2 and Th17 immune responses. It has been suggested that in those genetically susceptible, a limited exposure to microbes particularly early childhood may trigger a deregulated immune-mediated response to the commensal intestinal bacteria in the later stages of life. This theory is now referred to in the literature as the 'hygiene hypothesis' (Koloski NA *et al.* 2008).

According to metagenomic research, thousands of anaerobic bacterial species mainly from the phyla Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria reside in the human gut in an overall stratified orientation (Baumgart DC and Sandborn WJ. 2012). It is well established that

the number of anaerobic bacteria outnumbers the aerobic (facultative anaerobes) bacteria by a factor of 100-1000 (Simon GL and Gorbach SL. 1986). The predominant genera of anaerobes found in the GI tract of human beings are *Bifidobacterium, Clostridium, Bacteroides, Eubacterium, Peptococcus, Peptostreptococcus* and *Ruminococcus* (Simon GL and Gorbach SL. 1984; Salminen S *et al.* 1998). However there are other subdominant aerobes such as *Enterobacter, Klebsiella, Escherichia, Enterococcus, Lactobacillus* and *Proteus* (Simon GL and Gorbach SL. 1984; Moore WE and Moore LH. 1995). A healthy intestinal microbiome has an increased variation of microbiota, but the species diversity depends on dietary (Muegge BD *et al*. 2011), individual (Claesson MJ *et al.* 2011) drug induced (Dethlefsen L and Relman DA. 2011) and temporal (Costello EK *et al.* 2009) factors. In comparative studies on CD, a reduction in the diversity of the intestinal microbiota especially those belonging to the phyla Firmicutes and Bacteroidetes (Frank DN *et al.* 2007) has been noticed and also that the bacterial aggregations where in the form of clusters (Qin J *et al.* 2010).

Resident gut microflora has specific functions on the host's physiology and pathology (Falk PG *et al.* 1998). The main functions of intestinal microbial community are: metabolic, trophic and protective. The main metabolic function is the fermentation of the hard non-digestible dietary components and the endogenous mucus that is synthesized by the epithelia. A complex system of enzymatic and biochemical pathways is created due to a great diversity of microbes, resulting in the recovery of energy and absorbance of nutrients benefiting both the host and resident microbial proliferation (Guarner F and Malagelada JR. 2003). Within the trophic functions of gut microflora, growth and differentiation of epithelial cell is of paramount importance. Differentiation and growth of epithelial cell is affected by interaction with the gut microflora (Hooper LV and Gordon JI. 2001; Gordon JI *et al.* 1997). Studies on murine models have suggested that intraluminal

bacteria affect cell growth within the colon (Alam M *et al.* 1994). Crypt cell production is seen to be greatly reduced in the colon of rats that are bred in germ-free environments. It is also shown that the crypts in these rats contain fewer cells as compared to those rats which have resident microflora.

The gastrointestinal tract's immune system is an example of mucosa-associated lymphoid tissue and it is also known as the gut-associated lymphoid tissue (GALT). The intestinal mucosa therefore makes the link between the host immune system and the external environment. The interaction between the host and the resident microflora is crucial in the development of an effective immune system (Guarner F and Malagelada JR. 2003). The composition of the GALT is affected by diversity of the microbial colonies in the GI tract. Gnotobiotic animals tend to have low concentration of circulating immunoglobulin in the blood, fewer lymphoid cells in the gut mucosa and smaller specialized follicle structures (Tannock GW. 2001; Falk PG *et al.* 1998; Butler JE *et al.* 2000) but after exposure to the luminal microbiota, an intense expansion of all these is observed. Interactions between the intestinal microbiota, epithelium and the GALT serve in the fashioning of the memory mechanisms of the innate and adaptive immune system (Guarner F and Malagelada JR. 2003). It is also important to note that such an interaction needs to be moulded, more importantly in early life in order to develop complex and effective mucosal and systemic immunoregulatory circuits. In adults, the constant interaction between the host and the flora tends to shape the resultant immunity of the individual. Immune responses to microbes, depends on specific identifying components of the immune system such as immunoglobulins (Guarner F and Malagelada JR. 2003). Apart from macrophages and neutrophils, the innate immune response is also aided by the intestinal epithelial cells which coordinate the host's defence mechanisms through the synthesis of inflammatory mediators and signal transmission to the cells

in the mucosa (Kagnoff MF and Eckmann L. 1997). However there is need to discriminate between commensal bacteria and potential pathogens and this is achieved through the use of TLRs which recognize structural patterns on bacteria, which are not found on eukaryotic cells (Aderem A and Ulevitch RJ. 2000). The non-pathogenic bacteria are incubated within the inflamed intestinal mucosa thereby triggering a different cytokine response effecting a change in the phenotype of the lymphocytes found in the lamina propria (LP) (Borruel N *et al*. 2002).

Under the protective functions, the commensal bacteria act as an important line of resistance through the barrier effect. The presence of commensal bacteria limits the colonization of the gut ecological niche by exogenous microbes thus ensuring restricted growth of opportunistic bacteria. Equilibrium is created between the different resident bacterial species hence creating stability among the microbial population within the gut (Guarner F and Malagelada JR. 2003). However the use of antibiotics can shift the balance in favour of those bacteria which are potentially toxic to the host such as *Clostridium difficile* (Van der Waaij D. 1989). There are several different mechanisms which are employed to affect the barrier. Commensal bacteria aid in the competition for the adhesion sites situated in the brush borders of the intestinal epithelial cells, thus preventing the subsequent colonization by the pathogenic enteroinvasive bacteria (Bernet MF *et al*. 1994). There is also competition by bacteria for nutrients in the respective ecological niches. The host and the commensal bacteria have a symbiotic relationship in which the host provides the required amount of nutrient enrichment and the commensal bacteria indicates how much nutrient it requires and it consumes all the nutrients thereby maintain their habitat (Guarner F and Malagelada JR. 2003). The provision of nutrients is limited to the needs of the commensal bacteria so that invasive bacteria are deprived of nutrients to thrive on. The other way of inhibiting the growth and survival of invasive bacteria is through the production of bacteriocins (Brook I. 1999; Lievin V *et al.* 2000).

These are ribosomally synthesized proteinaceous toxins by bacteria to inhibit the growth of similar or closely related species. However the host is capable of controlling the production of these substances.

3.2.2.1 Commensal Bacteria and Crohn's Disease

It has been suggested that commensal bacteria influence the inflammatory processes of CD (Shanahan F. 2002). According to Pirzer U *et al.* 1991, there is hyperactivity of T lymphocytes against bacterial antigens and an elevated intestinal secretion of Immunoglobulin G (IgG) type antibodies in CD patients (Pirzer U *et al.* 1991, Macpherson A *et al*. 1996). Immunoglobulin G mediated immune responses tend to damage the intestinal mucosa through the activation of the complement and inflammatory mediators, contrary to an Immunoglobulin A (IgA) response (Brandtzaeg P *et al.* 1989). It is seen that patients with CD have greater amounts of bacteria, from different genera attached to their epithelial cell surfaces than do the healthy individuals (Swidsinski A *et al.* 2002) thus suggesting that there could be some uncontrolled activation of the gut immune system by certain elements of the commensal enteric bacteria.

3.2.2.2 The Role of Mucosal Dendritic Cells in the Gut

The gut mucosa is susceptible to antigenic stimulation through exposure to food antigens, foreign pathogens and resident microbiota (Cabezon R and Benitez-Ribas D. 2013). This stimulation results in controlled inflammatory responses that are carefully controlled by a number of nonimmune and immune mechanisms. As a result the gut immune system has the capability to distinguish between pathogens and harmless antigens. There are three main categories of the host's response to the intestinal microbiota namely: (1) the intestinal epithelium, which modulates

immune response through the secretion of inflammatory mediators, recruiting dendritic cells (DCs) and presenting antigens to T lymphocytes, (2) the innate immunity, which includes anatomical barriers, secretory molecules, and cellular components, that initiate the nonspecific immune response, and (3) the adaptive immunity, which is facilitated by B and T lymphocytes, responsible for antigen specific immune responses (Cabezon R and Benitez-Ribas D. 2013). This mechanized regulation serves to maintain intestinal homeostasis. Defects in this carefully regulated mucosal immune system as suggested by many studies to result in IBD, more specifically CD (Niess JH. 2008).

Dendritic cells are physiologically professional antigen-presenting cells linking the innate and adaptive immune system and they possess a pivotal role in the immunoregulation of the GALT (Radwan P *et al.* 2010; Cabezon R and Benitez-Ribas D. 2013). Dendritic cells originate from the bone marrow and are brought into the circulatory system as immature cells and then they are channelled to regions that are more susceptible to pathogens such as the intestinal mucosa (Radwan P *et al*. 2010). There, they monitor the intestinal lumen by processing the luminal antigens and via phagocytosis. Dendritic cells reside in mucosal tissues or recirculate in the blood and lymphoid tissues (Iwasaki A. 2007).

Intestinal immune system is mainly controlled by the activity of the local T-cells which are activated by DCs. They are programmed during the initial stage of activation to either initiate (innate and adaptive) immune responses or to control inflammation and maintain tolerance (Niess JH and Reinecker HC. 2006). The LP of the small and large intestines are the effector sites of mucosal tissues and here DCs are ideally situated to survey the constituents of the commensal microflora and monitor food antigens (Bjorck P. 2001; Niess JH. 2008). The inductive sites of DCs are Peyer's patches (PP), intestinal lymph follicles (iLFs), DC aggregates and mesenteric

lymph nodes (MLNs) (Niess JH. 2008). The phenotype of DCs is influenced by the local microenvironment, and can be divided into conventional DCs (CD8α+CD11b-, CD4+CD11b+, CD4-CD11b+) (Hochrein H *et al*. 2001) and plasmacytoid DCs (B220+CD11clow), characterized by a phenomenal plasticity between them (Kelsall BL and Rescigno M. 2004).

Dendritic cells differentiate into two forms which are distinct in their functions (i.e. immature and mature cells). The immature DCs mainly perform phagocytosis and are found mostly in peripheral tissues. Mature DCs are characterized by surface costimulatory molecules that are required for the activation of T-cells hence they are specialised APCs (Banchereau J and Steinman RM. 1998). The mature DCs are derivatives of the immature cells and the process of maturation is facilitated by inflammatory stimuli. This result in the migration of these DCs to draining lymph nodes and these cells are most likely to induce tolerance to self-antigens (Steinman RM and Banchereau J. 2007). CD4+ T-cells are divided into two groups namely T helper cells and Tregs. The T helper cells are subdivided into 3 classes: Th1, Th2 and Th17cells and they are distinct in function. Th1 cells are pro-inflammatory cells which are responsible for the immunity against intracellular pathogens through the release of IFN-γ. Th2 cells on the other hand are anti-inflammatory cells and through the activation of B cells stimulate the release of B cell growth factors such as IL-4. Th17 cells are mainly responsible for defence against bacteria and fungi, however under certain pathologic conditions, they are found to exacerbate the inflammatory response (Rescigno M and Di Sabatiano A. 2009). Regulatory T-cells serve to suppress the function of effector T-cells thereby counteracting inflammation (Powrie F and Maloy KJ. 2003). In the GI tract, subsets of DCs stimulate the development of Tregs which are the major drivers of immune tolerance (Coombes JL and Powrie F. 2008). The intestinal DCs also serve in conferring gut-homing properties to lymphocytes or aid to promote class switch recombination to IgA (Rescigno M and

Di Sabatiano A. 2009).

3.2.2.3 The Role of Mucosal Dendritic Cells in the Pathology of IBD

Dendritic cells play an important role in intestinal homeostasis. However the role of DCs in the aetiology of CD remains elusive but several murine and human models have suggested that DCs may be implicated in immune-mediated disease. The role of mucosal DCs is strictly regulated by local microenvironment which is comprised of luminal bacteria, immune cells and non-immune cells (Rescigno M and Di Sabatiano A. 2009). Deregulation at any of these levels may affect the function of DCs and may result in intestinal disease. The affected DCs may prime abnormal T-cell responses to the enteric microflora, stimulate T-cells and maintain them in an inflamed state and serve as effector cells through the release of pro- inflammatory cytokines. They may also cause an imbalance between Th17 inducing DCs and Treg-inducing DCs, in favour of the pro-inflammatory Th17 cell differentiation. In T-cell-independent models of colitis DCs have a pathogenic role. For example, DC activation via CD40 results in gut inflammation in the absence of B and T-cells through the release of inflammatory cytokines such as IL-23 and IL-17 (Uhlig HH *et al*. 2006).

Dextran sulphate sodium administered orally to mice induces an acute form of colitis. Diphtheria toxin-induced (DT-induced) denaturing of DCs in diphtheria toxin (DT) receptor-transgenic mice during DSS administration reverses the colitis (Abe K *et al*. 2007). Pre-treating mice with immunostimulatory DNA sequences before DSS administration, results in DCs having a protective function, partially because of the release of type I IFN. This cytokine serves to modulate the recruitment of neutrophils and monocytes and along with their inflammatory activities (Abe K *et al*. 2007). On the contrary, if DCs are ablated before DSS treatment, colitis is exacerbated

(Qualls JE *et al*. 2009), implying that DCs have a protective role in the early phases of colitis but have a pathogenic role in late phases of disease course. The engagement of DCs during the late phases of colitis development may be a result of abnormal activation of resident DCs, a recruitment of DCs that were not exposed to the local tolerogenic microenvironment and hence are immunogenic thereby causing an imbalance between tolerogenic and immunogenic DCs (Rescigno M and Di Sabatiano A. 2009).

Dendritic cell phenotype in murine colitis has shown an increase of mature DCs expressing higher levels of costimulatory molecules (CD40, CD80, and CD86) and elevated amounts of IL-12p40 and IL-23p19 upon CD40 ligation (Krajina T *et al*. 2003). Both, IL-12p40 and IL-23p19 form IL-23, a critical cytokine for the growth and stabilization of Th17 cells in mice and differentiation in humans (Korn T *et al*. 2009). NOD2-mutant DCs derived from CD patients failed activate effector Th17 cells upon stimulation with bacterial peptidoglycan or a combination of MDP and TLR ligands. Thus these data then imply a pathway for IL-1 and IL-23-dependent priming of effector Th17 cells through NOD2-mediated detection of intracellular MDP. This therefore reveals a connection between the 2 systems which are implicated in the pathogenesis of CD (Stetson DB and Ruslan M. 2007).

In IBD patients DCs accumulate at sites of inflammation (Vuckovic S *et al*. 2001), mainly as a result of upregulated mucosal expression of chemokines (Kaser A *et al*. 2004) or of addressins (Arihiro S *et al*. 2002). As a result of the overexpression of lymphoid chemokines, recruited mature myeloid DCs form clusters with proliferating T-cells in the inflamed intestinal tissue (Middel P *et al*. 2006). M-DC8+ cells, a subpopulation of DCs in human blood secreting TNF-α and expressing high levels of FcγRIII (CD16), are proliferated in the inflamed mucosa (de Baey A *et al*. 2003), thus demonstrating that DCs are a further source of TNF-α in CD. Activated DCs

may migrate from the mucosa to the MLNs in CD patients and there are at least 3 distinct myeloid DC populations are present in MLNs (i.e. immature DCSIGN+ DCs in the medullary cords), DCs expressing the myeloid marker BDCA3 (CD141) around the lymph follicles, and mature CD83+DCs expressing the S-100 protein in the T-cell areas (Verstege MI *et al.* 2008). Cross-talk between IECs and DCs may disrupt the intestinal immune homeostasis, thus promoting gut inflammation (Rescigno M and Di Sabatiano A. 2009). It was shown that IECs isolated from the majority of CD patients do not express Thymic stromal lymphopoietin (TSLP) and are not capable of controlling the DC-mediated pro-inflammatory response, thus resulting in an aberrant release of IL-12 by DCs, which drives Th1-type inflammatory responses (Rimoldi M *et al*. 2005). Available evidence reveals that NOD2 mutations may affect the response of CD monocyte derived DCs to bacteria and DCs derived from NOD2-deficient CD patients show an impaired capacity to induce IL-17 expression upon MDP triggering (Rescigno M and Di Sabatiano A. 2009).

3.3 Vitamin D and Immune System

3.3.1 Vitamin D Receptor (VDR)

Vitamin D interacts with many organs in the body. This is due to the ligand-dependent transcription factor vitamin D3 ($1,25(OH)_2D3$) receptors VDRs, the nuclear hormone receptor on many different cells which belong to the albumin family (Bikle D. 2014). After binding to vitamin D, the receptor associates with specific recognition sequences called vitamin D responsive elements (VDREs). These are present in the promoter region of target genes and involved in regulating their transcription (Haussler MR *et al.* 1998). The VDR modulates the expression of vitamin D- responsive genes. Vitamin D receptor typically functions as a heterodimer with the retinoid-X-receptor (RXR) (Basson A. 2014). This is basically achieved by positively regulating

the expression of some genes by binding to the VDREs in their promoter regions; by antagonizing specific transcription factors thereby inhibiting the expression of certain genes and by binding to negative VDREs thus negatively regulating the expression of the genes (Bikle D. 2014). Therefore all biological functions of either 1,25$(OH)_2$D or 1,25$(OH)_2$D3 are regulated by the VDR (Haussler MR *et al*. 1998).

All cells of the immune system including activated lymphocytes, APCs and DCs express the VDR and T-cell activation induces the expression of additional VDR (Cantorna MT *et al.* 2004). Exposure to 1,25$(OH)_2$D3 results in a dose-dependent inhibition of two lymphokines namely IL-2 and IFN-γ (Mathieu C and Adorini L. 2002). Interleukin-2 production is inhibited through the binding of the VDR onto the NF-AT binding site of the promoter for IL-2. Interferon gamma is inhibited in the same manner through the binding of VDR with a VDRE in the promoter region. These inhibitions suggest that suppression of Th1 activity is the major point by which active vitamin D metabolite can regulate the immune system and dictate the balance between the T-cell mediated immunity (Scalabrino G. 2009).

Studies have shown that 1,25-dihydroxy Vitamin D3, can have an effect on tissues and cells that are not directly involved in calcium regulation mechanism. It has been strongly proposed that 1,25-dihydroxyvitamin D3 can function as a regulator of immune cell differentiation and proliferation, thus vitamin D may have a similar function to that of immune-modulatory molecules such as cytokines (Hewison M. 2010). Vitamin D receptors, specific high-affinity intracellular receptors for 1,25-dihydroxyvitamin D3 are detectable in activated T cells, and activated macrophages are able to synthesize 1,25-dihydroxyvitamin D3 thus suggesting a possible paracrine and intracrine mechanisms of action (Hewison M. 2010). The explicit association of 1,25-dihydroxyvitamin D3 with cytokines is however not yet fully understood, but its ability to

modulate immune cells in vitro and its interaction with inflammatory diseases can be predicted.

3.3.2 The Effects of Vitamin D on the Innate Immunity

3.3.2.1 Vitamin D, Macrophages and Cathelicidin

The effects of vitamin D on macrophages have been pivotal to the observations which implicate vitamin D in the regulation of immune responses. Macrophages, NK cells, cytotoxic T-cells together with their monocyte precursors, function to initiate non-specific immune response to pathogens or tissue damage (cell-mediated immunity). Macrophages phagocytose pathogens and also interface with the adaptive immune system by utilizing phagocytic material for antigen presentation to T-cells. On macrophages, vitamin D stimulates differentiation of precursor monocytes to mature phagocytic macrophages (Abe E *et al*. 1983; Tanaka H *et al*. 1983). Observations showing differential expression of VDR and 1α-hydroxylase during the differentiation of human monocytes macrophages have supported the concept (Kreutz M *et al.* 1993). Studies have shown that human macrophages are capable of synthesizing 1,25$(OH)_2$D3 upon stimulation with IFN-γ (Koeffler HP *et al*. 1985).

In normal macrophages, localized activation of vitamin D, coupled with expression of endogenous VDR was heavily suggested to be an autocrine or intracrine system for vitamin D action. Seminal investigation on *Mycobacterium tuberculosis* (*M. tb*) revealed that the VDR and the gene for 1α-hydroxylase (CYP27B1) were induced by the toll-like receptor 2/1 (TLR2/1) (Liu PT *et al*. 2006). Further experiments confirmed that precursor 25(OH)D3 was able to induce intracrine VDR responses in monocytes that had been treated with a TLR2/1 activator. It was shown that the TLR2/25(OH)D3 combination stimulated expression of the antibacterial protein cathelicidin; in a

way that vitamin D was able to promote monocyte killing of *M. tb* (Liu PT *et al*. 2006). It is also shown that vitamin D supplementation in vivo can also enhance TLR2/1- induced cathelicidin expression (Adams JS *et al*. 2009). Cathelicidin was identified as a target for transcriptional regulation by 1,25$(OH)_2$D3-liganded VDR, in that its gene promoter contains a functional vitamin D response element (VDRE) (Gombart AF *et al*. 2005; Wang TT *et al*. 2004).

Other studies have however proposed that TLR2/1 induction of 1α-hydroxylase occurs indirectly as a result of TLR2/1 induced interleukin-15 (IL-15) which is a potent inducer of CYP27B1 and 1α-hydroxylase activity (Krutzik SR *et al*. 2008). In like manner, interleukin 17A (IL-17A) has been shown to enhance 1,25$(OH)_2$D3-mediated induction of cathelicidin (Peric M *et al*. 2008). It is also important to recognize that other ligands may interact with the VDR (Makishima M *et al*. 2002). Some compounds may act to disrupt normal 1,25$(OH)_2$D3-VDR-mediated immunity. For example; the polycyclic aromatic hydrocarbon benzo(A)pyrene, a product of cigarette smoking, is seen to attenuate vitamin D-mediated induction of macrophage cathelicidin in a VDR-dependent fashion by stimulating expression of 24-hydroxylase, and vitamin D catabolism (Matsunawa M *et al*. 2009).

The observations above show that vitamin D is an important stimulator of mechanisms associated with pathogen elimination. Vitamin D is associated with key feedback control pathways therefore it is crucial in innate immune regulation. Calcitriol has a catabolic enzyme in the form of 24-hydroxylase which attenuates responses to 1,25$(OH)_2$D3 and, in the case of the CYP24 splice variant, may also attenuate synthesis of this vitamin D metabolite (Ren S *et al*. 2005). Vitamin D (1,25$(OH)_2$D3) has been shown to downregulate expression of monocyte TLR2 and TLR4, thereby suppressing the associated inflammatory responses (Sadeghi K *et al*. 2006). By utilizing both CYP24 and TLR regulatory mechanisms, vitamin D may appropriately regulate innate

immune responses whilst preventing an over-expression of innate immune responses and tissue damage.

3.3.2.2 Vitamin D, Dendritic Cells and Antigen Presentation

The most highly recognized group of professional APCs is DCs. Studies using DCs isolated from Langerhans cells provided evidence that $1,25(OH)_2D3$ could act to attenuate antigen presentation (Dam TN *et al*. 1996) . *In vitro,* monocyte-derived DC models substantiate these effects of vitamin D metabolites on antigen presentation. It is shown in some studies that $1,25(OH)_2D3$ (Penna G and Adorini L. 2000) and its synthetic analogues (Griffin MD *et al*. 2000) inhibit the maturation of monocyte-derived DCs, thereby suppressing their capacity to present antigen to T-cells. It was therefore proposed that vitamin D could promote tolerance and this was supported by studies of pancreatic islet transplantation in which lower rejection rates were observed in $1,25(OH)_2D3$-treated mice (Gregory S *et al*. 2001). This response to $1,25(OH)_2D3$ appeared to be due to decreased DC maturation and concomitant enhancement of suppressor or Tregs (Gregory S *et al*. 2001). Further studies have emphasized the importance of Treg generation as part of the interaction between vitamin D and the immune system.

3.4 The Immunopathology of Crohn's disease

Despite the fact that the cause of IBD remains elusive, it is presumed to result from dysregulation of the intestinal mucosal immune system. Animal models of IBD show that normal intestinal flora induces intestinal inflammation in animals with a dysregulated immune system (Elliott DE *et al*. 2000). Inflammatory bowel disease is seen to likely result from uncontrolled immune responses to normal intestinal contents. Crohn's disease appears to be an overly vigorous Th1-type

inflammation that produces IFN-γ and TNF-α (Fuss IJ *et al.* 1996). The mucosal inflammation of these models generates excess amounts of IFN-γ and TNF-α thereby suggesting that excess production of Th1-type cytokines is one mechanism underlying the pathogenesis of disease. There are no actual animal models of human IBD; however there are several animal models of chronic intestinal inflammation. Murine models with genetically altered gene deletions develop chronic bowel inflammation similar to IBD such as mutant mice having targeted deletions for IL-2, IL-10, MHC class II, or T-cell receptor (TCR) genes (Elliott DE *et al*. 2000). Some of the models have shown that a dysregulated immune system itself mediates intestinal injury. Blocking Th1 circuitry has also been shown to prevent the inflammation (Berg DJ *et al*. 1996; Ehrhardt RO *et al.* 1997). Therefore these models strongly suggest that CD is dysregulated Th1 responses thus have direct implications regarding the immunopathology of this human disease process.

3.4.1 Th1/Th2 Paradigm in Crohn's Disease

Currently the view on CD is that inflammation is a result of an IL-12-driven Th1 response, which results in the production of the key inflammatory mediator, IFN-γ (Karczewski J *et al.* 2012). This view has been supported by multiple IBD mouse models of CD. CD4+ T cells in the intestinal lamina propria of CD patients produce large amount of IFN-γ and lower amount of IL-4 than that of healthy control (Fuss IJ *et al.* 1996). In CD patients, macrophages in the intestinal lamina propria produce large amounts of IL-12 (Monteleone G *et al*. 1997). Lymphocytes in the intestinal lamina propria of these patients also express elevated levels of IL-12R, and produce large amount of IFN-γ in response to IL-12 (Okazawa A *et al.* 2002). The peripheral blood of CD patients also exhibits the same pattern. Vast numbers of CD4+ T-cells that express T-box protein expressed in T-cells (T-bet) and STAT-4 have been found in the intestinal mucosa of CD patients (Karczewski

J *et al.* 2012). Among all the suggestions the most convincing evidence that CD is a Th1-cytokine mediated disease, is the efficacy of biologic therapies directed against TNF-α, IL-12 and IFN-γ (Rutgeerts P *et al*. 2009).

3.4.2 Th17 Cells

Recent research has given much attention to the possible involvement of Th17 cells in the pathogenesis of CD (Holtta V *et al*. 2008). The Th17 cells are a newly identified subset of T-helper cells distinct from Th1/Th2 which produces various pro-inflammatory cytokines, including IL-17A, IL-17F, IL-21, IL-22 and TNF-β. They play a pivotal role in the protective immunity against the extracellular pathogens such as bacteria and fungi (Weaver CT *et al*. 2007), as well as the protective immunity against intestinal pathogens (Maloy KJ and Kullberg MC. 2008). Th17 cells differentiate in the presence of IL-6 and transforming growth factor (TGF)-β, through the expression of retinoic-acid-receptor-related orphan receptor (ROR)-γt (Weaver CT *et al*. 2007). IL-21 is an important cytokine for the differentiation of Th17 cells. IL-6 promotes the production of IL-21 from Th17 cells independent of RORγt, and then IL-21 upregulates the expression of RORγt by activating the STAT3 pathway thus providing a positive feedback in the differentiation of Th17 cells, in a process called amplification. IL-21 is however not essential for the polarization of Th17 cells, but it is necessary for their amplification (Karczewski J *et al.* 2012).

Pivotal for stabilization and expansion of Th17 cells, is the cytokine IL-23, a member of IL-12 cytokine family. This cytokine is secreted by professional APCs during the inflammatory response to both pathogenic and non-pathogenic stimuli (Abraham C and Cho J. 2009). Intestinal microbiota is also essential for the development of Th17 cells (Atarashi K *et al.* 2008). A high

expression of IL-23 has been found in mucosa of human ileum, as well as an upregulation of Th17 cells in the human GALT (Maloy KJ and Kullberg MC. 2008).

3.4.2.1 The Role of Th17 Cells in Crohn's Disease

Interleukin-17A, generally referred to as IL-17, is a main pro-inflammatory cytokine produced by Th17 cells (Karczewski J *et al*. 2012). It has been reported that Th17 subset is involved in the pathogenesis of various diseases that were previously thought to be either Th1 or Th2 dominant. Interleukin-17A is upregulated in and has been associated with several immune-mediated diseases such as IBD, rheumatoid arthritis, psoriasis, psoriatic arthritis, ankylosing spondylitis and multiple sclerosis, among others (Witkowski J *et al*. 2004). Increasing evidence from both human studies and mice models supports the view that Th17 cells are involved in the pathogenesis of CD. Human studies have shown high expression of IL-17A mRNA in intestinal mucosa of CD patients (Brand S. 2009). The elevated faecal IL-17A levels were found in active CD, accompanied by high percentage of IL-17A and IL-23 producing cells in the LP of CD patients (Holtta V *et al*. 2008). Murine studies have identified IL-23 as a major driver of intestinal inflammation via inflammatory mediators including IL-17Aand IL-6 (Brand S. 2009; Elson CO *et al*. 2007). According to a genome-wide association study (GWAS), IL-23R and five additional genes involved in Th17 differentiation are associated with susceptibility to CD (Brand S. 2009). It is not clear how IL-23R polymorphism might predispose to CD however the identification of both disease-protective and risk-associated variants of the gene suggests that the IL-23 signalling may play a crucial role in maintaining immune homeostasis in the intestine (Duerr RH *et al*. 2006).

Clinical trials have reported that blockage of p40, the shared subunit of IL-12 and IL-23 is effective in CD treatment and has an identical therapeutic effect to that of anti-TNF-α therapy

(Mannon PJ *et al.* 2004; Sandborn WJ *et al.* 2008). The anti-p40 antibodies were thought to revoke IFN-γ mediated intestinal inflammation through blockage of IL-12. There is however another possible implication that inflammation is attributed to IL-12 is mediated by IL-23 and its downstream cytokines IL-17A and IL-22 (Karczewski J *et al*. 2012). These data have supported the notion that IL-17A plays a crucial role in CD pathogenesis and its inhibition might represent a potential approach for treating active course of the disease. The observations may suggest the importance of the IL-23/IL-17 axis in CD, however the exact role of IL-17 in the pathogenesis of disease remains unclear, since also its protective role in the intestine inflammation has been proposed based models of experimental colitis (O'Connor W *et al.* 2009 ; Ogawa A *et al.* 2004). Th17 cells induced in the presence of IL-1β, on the contrary, have a unique mRNA profile and increased capacity to induce inflammation (Ghoreschi K *et al*. 2010). In environments lacking TGF-β, IL-12 and IL-23 Th17 cells acquire the capacity to produce IFN-γ hence amplifying the pro-inflammatory effect (Karczewski J *et al*. 2012).

Crucial for immune homeostasis is a relationship of Th17 cells with regulatory T-cells through their shared use of TGF-β as a differentiation factor (Mangan PR *et al*. 2006; Maul J *et al*. 2005). Active CD is characterized by either numerical, functional or both defects of a regulatory T-cell subpopulation (Veltkamp C *et al*. 2011). Despite their presence in the inflamed gastrointestinal tract of CD patients, either their number, suppression function or both is probably inadequate to curb chronic inflammation (Maul J *et al*. 2005). Th17 cells constitute a heterogonous subpopulation, differing in properties and functions. There is however evidence that differentiation of Th17 cells in the absence of IL-23 leads to Th17 cells producing a potent anti-inflammatory cytokine, IL-10 (McGeachy MJ *et al*. 2007).

Immune regulation is maintained by a critical balance between the activities of the Th1, Th2 and Th17 cells. This is because the different cytokines produced by these cells tend have a counter (inhibitory) effect upon the different respective immune mechanism. In CD and many other immune-mediated diseases, we can therefore propose that the corresponding chronic inflammation is a result of a dysregulated immune response characterized by an imbalance between the Th1/Th2 immune response, the overproduction of Th1/Th17 associated cytokines or a combination of these immune mechanisms.

3.5. Genetic Factors

Crohn's disease is a complicated genetic disorder which is characterized by a recurring inflammation of the gut. More than 140 susceptibility gene loci have been discovered through research and these have in some way improved our understanding of the disease pathogenesis (Liu JZ and Carl AA. 2014). The onset of CD has been linked to a number of genetic factors. However the ones that reflect the strongest risk are the genetic susceptibility mutations in the Caspase-activation recruitment domain (CARD15)/Nucleotide-binding oligomerization domain (NOD2), ATG16L and IL23R genes. Individual gene mutations and also a combination of these mutations have also been demonstrated in CD patients (Csöngei V *et al.* 2010).

3.5.1 CARD15/NOD2

The most recognized high susceptibility gene influencing CD is the CARD15/NOD2 (Hugot JP *et al.* 2001). Its primary function is pattern recognition in the innate immune system (Economou M *et al.* 2004). The CARD15/NOD2 gene belongs to a family of nucleotide-binding domain and

leucine-rich-containing receptors (NLRs) which recognizes a modified peptidoglycan muramyl dipeptide (MDP) (a pathogen-associated molecular pattern (PAMP) from bacterial peptidoglycan breakdown) (Abraham C and Medzhitov R. 2011). The TLR family is involved in the recognition of pathogen-associated molecular patterns by the immune system. TLR4 recognises lipopolysaccharide using the extracellular LRR domain (Arnott IDR *et al.* 2004).

CARD15/NOD2 has also been shown to potentiate autophagy by acting as a mediator of the protein (ATG16L1) which forms an integral part of this process (Garg M *et al.* 2012). A deficiency of this protein has been shown to result in an overemphasized inflammatory response (Kaser A and Blumberg RS. 2011). CARD15/NOD2 contains a highly conserved domain which is linked to a nucleotide-binding domain, and they are thought to regulate apoptosis and to activate the nuclear factor-κB (NFκB) (Arnott IDR *et al.* 2004).

Upon several factors, mutations of CARD15/NOD2 are considered to be the contributing susceptibility mutation about 16% of CD patients (Van Montfrans C *et al.* 2002). Among these private mutations, only three occur more frequently. These alleles are the R702W, G908R and 1007fs (Hugot JP *et al.* 2007). These susceptibility alleles are located in the distal third part of the gene, of which two are missense point mutations and the other one is a frameshift mutation. The result is a number non-conservative variation of the protein (Lesage S *et al.* 2002).

3.5.2 IL-23R

IL-23R is a protein consisting of the heterodimeric receptor complex, an IL-12β1 and an IL-23R chain (Zhang Z *et al.* 2006). The expression of this complex regulates activities of IL-23 (Hradsyk O *et al.* 2010). This protein is expressed on the cell membrane of memory T-cells and other

immune cells which identify antigens, such as NK cells, monocytes, and DCs (Naser SA *et al.* 2012). IL-23R is mainly involved in the mediation of pro-inflammatory activities through the production of IL-17 via the activation of Th17 lymphocytes (Vermeire S *et al.* 2002). Th17 lymphocytes are a subset of T-helper cells, which are pivotal in the production of IL-17 and also produce IL-6 and TNF-α to a lesser extent (Wehkamp J *et al.* 2005). IL-23R interacts with IL-23, which is a cytokine that regulates the activity of immune cells and plays an important role in the inflammatory response against infection by bacteria and viruses (Naser SA *et al.* 2012).

It is suggested that IL-23 could be a key regulator in the differentiation of Th17 lymphocytes from memory T-cells and that IL-23 is vital in the maintenance of the already differentiated Th17 cells (Veldhoen M *et al.* 2006). The IL-23-IL-17 cytokine axis is a key pathogenic mechanism that mediates the development and progress of inflammation by Th17 cells (Naser SA *et al.* 2012). The mechanism of IL-23R is clearly important to understand because it is directly associated with CD.

3.5.3 ATG16L

A number of genetic factors predispose to CD, and one of the susceptibility allele is in the autophagy gene ATG16L1 (Xavier RJ and Podolsky DK. 2007; Hampe J *et al.* 2007). Autophagy is a catabolic process by which cellular components are degraded by delivery of double-membrane-bound vesicles containing cytoplasm and cytoplasmic organelles to the lysosome to form autophagosomes (Levine B and Kroemer G. 2008). Autophagy has an important role in cell and tissue homeostasis, and has been implicated in a range of human diseases (Cadwell K *et al.* 2008). During infection autophagy is important in getting rid of foreign antigens in the cells by breakdown of the pathogen. Mutation on the gene responsible for autophagy can result in a

shift in normal flora, and resulting in many gastrointestinal problems (Naser SA *et al.* 2012) Failure by the cells to regain nutrients or fight off foreign antigens within the gastrointestinal tract will cause them to undergo programmed cell death. This can however result in tissue damage (Naser SA *et al.* 2012). The ATG16L1 protein contains an amino- terminal domain that functions in autophagy as part of a complex with autophagy proteins ATG5 and ATG12 (Fujita N *et al.* 2008; Mizushima N *et al.* 2003). The ATG16L1 protein is expressed in many cell types such as in the colon, small intestine, intestinal epithelial cells, leukocytes, and spleen (Fujita N *et al.* 2008). ATG16 is responsible for the subcellular localization of the autophagy machinery (Fujita N *et al.* 2008; Rioux JD *et al.* 2007). Studies have shown that a mutation located on chromosome 2, on the ATG16L gene is linked to the onset of ileal CD, and therefore it is an important molecule in explaining the genetic aspects of the disease (Sventorairyte J *et al.* 2010).

3.5.4 IL-1

Interleukin-1α (IL-1α) and interleukin-1β (IL-1 β) have been implicated in the pathogenesis of a number of immune mediated diseases such as asthma, as well as IBD. These cytokines are strongly associated with the regulation of the inflammatory response and the immune response (Mwantembe O *et al.* 2001). Interleukin-1α and interleukin-1β are important in mucosal inflammation which is characterized by a massive infiltration of activated neutrophils and mononuclear cells. This in turn triggers the production of a cascade of other cytokines and inflammatory mediators (Dinarello CA and Wolff SM. 1993). The interleukin-1 receptor antagonist (IL-1Ra) is a modulating factor controlling the inflammatory process (Pillay V *et al.* 2000). It acts as an anti-inflammatory agent inhibiting the activities of IL-1α and IL-1β (McIntyre KW *et al.* 1991). The IL-1 gene cluster on chromosome 2 comprises of genes coding for IL-1α,

IL-1β, IL-1Ra and interleukin-1 receptor (IL-1Re). The IL-1Ra and IL-1β have a structural relationship and they compete for affinity to the IL-1Re (Duff GW. 1989). It is however important to note that IL-1Ra functions as a competitive inhibitor, but not as a trigger of signal transduction, therefore it serves as an endogenous inflammatory regulator (Tarlow JK *et al.* 1993). The balance between receptor expression; cytokine production and inhibitor concentration is pivotal in determining the resultant inflammatory response (Pillay V *et al.* 2000; Mwantembe O *et al*. 2001).

There is a well-known association between the 240-bp allele of the IL-1Ra and a number of immune-mediated diseases. A study conducted by Pillay V *et al.* 2000, showed that elevated levels of plasma IL-1Ra were corresponding to disease severity in asthma patients. A disparity between IL-1 and IL-1Ra in the intestinal mucosa and abdominal lavage has been shown in IBD patients (Andus T *et al.* 1997). A genetic association between IL-1Ra allele 2 and IBD has been demonstrated (Mansfield JC *et al.* 1994).

3.5.5 HLA

The human leucocyte antigen (HLA) genes have been implicated in many immune-mediated diseases. Such diseases include diabetes mellitus (Larsen CE and Alper CA. 2004), rheumatoid arthritis (Weyand CM *et al.* 2000), autoimmune hepatitis (De Silva S *et al.* 2006) and celiac disease (Green PH *et al.* 2003). Since the first reported HLA-IBD association many studies have been conducted investigate the link between HLA genes and he susceptibility and phenotype of IBD (Ahmad T *et al.* 2006). The MHC is a large genetic complex with multiple gene loci that is located on the short arm of chromosome 6. Genome-wide scans have revealed evidence point at the IBD3 (6p21.1-23), an area encompassing the HLA complex on the chromosome (6p21.3)

(Ahmad T *et al.* 2006). The MHC possesses highly polymorphic HLA genes (Tshabalala M *et al.* 2013). The polymorphic nature of HLA genes allows the presentation of a wide range of peptides to the immune system. HLA gene polymorphism is highly linked to the rate of disease progression (Just JJ. 1995). Gene loci within the MHC encode for two classes of classical glycoproteins, that is class I and class II HLA molecules (Tshabalala M *et al.* 2013). The HLA class I molecules bind to endogenous antigenic epitopes and present them to CD8+ T lymphocytes. Of particular importance to IBD are the HLA class II molecules which present antigenic peptides to CD4+ T lymphocytes. Their products play a pivotal role in the immune response (Stokkers PCF *et al.* 1999). Class II HLA genes encode cell-surface glycoproteins which are expressed on antigen presenting cells such as macrophages and dendritic cells (Ahmad T *et al.* 2006). The class II molecules are comprised of an α chain and a β chain that form the grove in which the antigenic peptide is presented to the T-cell receptor (Jardetzky TS *et al.* 1994). These function mainly to present peptides to T-cell receptors as a precursor to T-cell activation. HLA-DR, HLA-DQ and HLA-DP are the three types of HLA class II molecules that are expressed by a single cell (Ahmad T *et al.* 2006). However the mechanism by which HLA class II genes influence IBD remains unclear. Association studies have generally implied a role for HLA-DR alleles in susceptibility or resistance to IBD. In CD, an association with HLA-DR7, HLA-DRB3*0301 and HLA-DQ4 alleles have been found to have a positive association whereas HLA-DR2 and HLA-DR3 have a negative association (Stokkers PFC *et al.* 1999). However the total genetic contribution to disease susceptibility cannot be accounted for by the aetiological fractions associated with these phenotypes.

3.5.6 SLC11A1

The SLC11A1 (solute carrier 11A1) was formerly known as NRAMP1 (natural resistance-associated macrophage protein 1), however for the purpose of this section NRAMP1 has been used. In humans the NRAMP gene is located on the chromosome region 2q35 and it is made up of 15 exons covering 16kb of DNA. This gene encodes for an intrinsic membrane protein that is 550 amino acids long (Sechi LA *et al.* 2006). The protein is only expressed in the lysosomal compartments of monocytes and macrophages (Cannonne-Hergaux F *et al.* 2002). NRAMP1 gene polymorphisms have been associated with genetic susceptibility with *M. tb* and disease progression of tuberculosis in China (Zhang W *et al.* 2005). Reports have suggested a role of the NRAMP1 gene in immune-mediated diseases such as rheumatoid arthritis (Yang YS *et al.* 2000). Certain NRAMP1 variants have been shown to be specifically associated with CD (Hofmeister A *et al.* 1997). An association was shown between NOD2 polymorphisms and the pathogen *M.tb* in Sardinian CD patients (Sechi LA *et al.* 2005). An association was also shown between CARD15/NOD2 polymorphisms, at the 823 C/T locus and the 1729 +55del4 locus on the NRAMP1 gene in Sardinian patients (Sechi LA *et al.* 2006).

3.6 Environmental Factors

A vast number of epidemiological studies have indicated a rise in the incidence and prevalence of CD across varying geographic locations. The North-South gradient is one such indicator of the influence of the environment in the development of CD. However, this hypothesis has not been consistently substantiated and the factors not yet proved as causal. Inflammatory bowel disease tends to occur in high socio-economic groups (Loftus EV and Sanborn WJ. 2003). There is a

generally noticeable trend of a high prevalence and incidence of CD in heavily industrialized and modernized nations. Industrialization tends to modify the lifestyle of individuals causing changes in diet, exercise regiments, sanitation, and increasing exposure to occupations pollution and industrial chemicals (Hanauer SB. 2006; Molodecky NA and Kaplan GG. 2010).

Crohn's disease is emerging in countries that are having a transition from developing to developed, thus becoming more westernized of which such areas were previously reported as low incidence areas for CD (Ng SC. 2014 (a)). When developing countries become industrialized, improved sanitary conditions likewise follow, resulting in changes in the patterns of childhood hygiene. It is further shown that CD commonly occurs in urban areas as compared to rural areas therefore heavily suggesting the role of environmental factors as strong determinants of IBD (Molodecky NA and Kaplan GG. 2010). Emigration studies have also brought compelling evidence suggesting a change in the epidemiology of CD alongside changes in the environmental factors and lifestyle. As compared to those remaining, migrants from the low-risk countries tend to exhibit an elevated risk of CD when they migrate to developed nations thus suggesting a role of the modifiable external environment. Their children also tend to take on the risk factors of the new environment, thus furthermore suggesting that environmental influence during childhood is crucial.

3.6.1 Vitamin D Deficiency

Accumulating evidence now suggests that there is a strong association between low vitamin D (as an environmental constituent) and immune-mediated diseases such as rheumatoid arthritis, multiple sclerosis, insulin dependent diabetes mellitus and IBD (Kriegel MA *et al.* 2012). To

explain this, the northern hemisphere generally receives less sunlight as compared to the southern hemisphere and the least exposure occurs in winter months. In Canada and the northern parts of the United States and Europe, where these diseases are typically more prevalent, it was observed that the severity of multiple sclerosis fluctuates seasonally and that the disease exacerbations occur mostly in spring when there is very little sunlight exposure (Moum B *et al.* 1996 (b)). This is attributed to the observation that circulating concentrations of serum 25(OH)D are lower in winter and higher in summer, corresponding with the relevant amount of seasonal sunlight exposure (Maxwell JD. 1994). Vitamin D is manufactured in the skin directly from the ultraviolet (UV) rays of sunlight and researchers suggest that low levels of vitamin D may be an important environmental factor that increases the risk of CD in populations specifically in the northern latitudes (Butcher RO and Limdi JK. 2012).

3.6.2 The Hygiene Hypothesis

Hygiene is generally defined as conditions favourable to maintaining good health and preventing ailment, notably through cleanliness (Soanes C and Angus S. 2008). In the context of IBD, the hygiene hypothesis' emanates the rising disease incidence, corresponding with improvements in hygiene over the last few decades. Microbial exposure in childhood is believed to program the enteric immune system thus moderating the future response to the specific antigen (Koloski NA *et al.* 2008). At delayed exposure to the entero-pathogenic microbial agents, an inappropriate immune response may be triggered. This can also be referred to as the 'sheltered child hypothesis' (Rachmilewitz D. 1986). The hygiene hypothesis predicates that individuals raised in sanitary environments tend to be more susceptible to develop CD as a result of limited exposure to environmental or immunostimulatory agents thus resulting in dysbiosis or rather a shift in the

balance between the commensal and the entero-pathogenic agents. It also infers that this chronic inflammatory reaction in genetically susceptible hosts can be as a result of the loss of saprophytic microbes, subsequently leading to the development of a defective maturation of regulatory T-cells and antigen-presenting cells.

Environmental influence in the industrialized society encompasses a wide array of factors which are potential causative agents of CD. In the last three decades, many studies have examined possible associations of CD with interrelated factors as food substances, chemicals, harmful micro-organisms, noxious fumes, sanitation, household amenities etc. It is easy to point out that environmental factors may have an influence on the development of CD; however it has proved unyielding to isolate the specific cause. No hygienic factor also has been singled out to demonstrate a consistent association with CD. This can be attributed to the effect of timing of exposures, overlapping factors and confounding factors, which probably occurs during susceptible periods of development and may precede onset of the disease by lengthy periods of time (Foster A and Jacobson K. 2013). Table 5 shows the categories and examples of the possible childhood exogenous risk factors involved with CD.

Environmental factors are undoubtedly important in the pathogenesis of CD. These factors act as potential triggers for the onset or development, and also affect the course and the response (to medication) of the disease. There are several factors that have been identified to have an association with CD in various ways. Some of these include hygiene and sanitation (Gent AE *et al.* 1994), air pollution (Kaplan GG *et al.* 2010), diet (Cohen AB *et al.* 2013), stress (Mawdsley JE and Rampton DS. 2005), exercise (Bernstein CN *et al.* 2001), industrialization (Aamodt G *et al.* 2008), breastfeeding (Barclay AR *et al*. 2009) and smoking (Seksik P *et al*. 2009). However the latter has been the best studied and shown to have the greatest association with the disease.

3.6.2.1 Injury to the Puerile Gut

It has long been suspected that the modifiable environmental factors can create a susceptibility to IBD by influencing intestinal permeability, disrupting the gastrointestinal microbiota and modifying the enteric mucosal immune system (Loftus EV Jr. 2004). There is accumulating evidence that events early in life may have long term effects on health and disease. Several epidemiological studies have suggested a role for perinatal or childhood events in the aetiology of IBD (Loftus EV Jr. 2004; Marshall JK and Hilsden 2003).

A study by Koletsko S *et al*. 1989 showed that lack of breast feeding and periods of diarrhoeal disease during infancy were two factors independently associated with the subsequent development of CD. It was shown in the same study that if breast feeding was combined with formula feeding in infancy, a resultant decrease in the significance of breast feeding in being protective against CD was observed. According to the findings of the meta-analysis by Klement E *et al.* 2004, breastfeeding has been shown to be associated with lower risks of CD. In support of this Goldman AS and Smith CW. 1973 posit that breast milk contains factors such as immunoglobulins that may be protective against gastrointestinal infections. The meta-analysis however revealed that many studies had methodological flaws and small sample sizes therefore putting a compromise to the validity of the observations.

A case-control study on 51 UC patients and 57 CD patients conducted in the United Kingdom by Whorwell PJ *et al.* 1979 found bottle feeding within the first two weeks of life predisposing some individuals to the development of ulcerative colitis later in life but could not significantly implicate it to the development of CD. This study suggests that bottle feeding may injure the gut

by causing sensitization to milk formula or cow's milk in the early stages of life causing increased permeability to macromolecules or by altering bacterial flora by introducing bacterial antigens at the time of sensitization. However the study revealed that independent of bottle feeding the CD patients had a significantly increased occurrence of gastroenteritis within the first six months of birth. Rotavirus infection may be the cause of the gastroenteritis which may have a prolonged effect resulting in an abnormal immune response and the future development of CD.

3.6.2.2 Nutritional Factors

Gastrointestinal inflammation can be triggered by the diet through immunological and biochemical mechanisms such as alteration of the gut microflora, offsetting prostaglandin balance and antigen presentation (De Filippo C *et al.* 2010, Sharon P *et al.* 1978). IBD incidence is rising in nations that are becoming more westernized such as nations of Asia, Eastern Europe, French West Indies and North Africa (Ng SC. 2014 (a)). Highly implicated to the rise is the "Western" diet which is characterized by a high content of saturated fats and protein (Amre DK *et al.* 2007). Many studies on the association between diet and IBD had certain methodological flaws which led to their findings being questioned. However modern studies have addressed the issues and have reported that refined sugar (Rief S *et al.* 1997; Matsui T *et al.* 1990), fat (Rief S *et al.* 1997) and fast foods (Persson PG *et al.* 1992) increase the risk of developing CD. On the other hand it was revealed that fruits (Persson PG *et al.* 1992; Russel MG *et al*. 1998), vegetables (Persson PG *et al*. 1992; Russel MG *et al*. 1998) lower the risk of developing CD.

A Japanese epidemiological study (Shoda R *et al*. 1996) revealed that increased intake of animal fat, animal protein, milk protein, n-6 polyunsaturated fatty acids (PUFAs), and the increased ratio

of n-6 to n-3 fatty acid was strongly associated with the increased incidence of CD. The strongest independent risk factor from this study was animal protein which therefore suggests that an increased intake of animal protein can greatly increase the risk of developing CD. A study by Amre DK *et al.* 2007 also reported that consumption of long-chain omega-3 fatty acids (LCN-omega-3) was significantly associated with lower risk of developing CD (OR = 0.44, 95% CI 0.19-1.00).

3.6.2.3 Chemical Factors

3.6.2.3.1Smoking

Smoking is one of the crucial environmental factors that have been implicated through several studies to have an influence on the onset and course of the disease (Loftus EV. 2004). Smokers are at a greater risk of developing CD as compared to non-smokers (Somerville KW *et al.* 1984). Second-hand cigarette smoke exposure during childhood has been associated with an increased risk of CD development; however these findings have been inconsistent (Bernstein CN *et al.* 2006; Mahid SS *et al.* 2007; Gerry RB *et al.* 2010; Russel RK *et al.* 2005). It is also noted that CD patients who quit smoking had less recurrence of the disease, compared to those who continued with smoking (Bernstein CN *et al.* 2006; Cosnes J *et al.* 2001; Russel MG *et al.* 1996). The cessation of smoking after diagnosis of CD in an intervention study strongly suggests that smokers have worse disease symptoms than non-smokers (Johnson CJ *et al.* 2005). Ryan WR *et al.* 2003 posit that smoking also tends to cause an increase in the severity and risk of CD. Smoking leads to a more aggressive disease course and an increased possibility of failure to respond to medical therapy (van der Heider F *et al.* 2009). Other studies revealed that smokers have a lower response rate and shorter response time to medication than non-smokers and also higher relapse rate of the

disease (Arnott IDR *et al.* 2003; Parsi MA *et al.* 2002; Rudolph SJ *et al.* 2008). It is also interesting to note that smokers have a higher chance of undergoing surgery due to CD as compared to non-smokers (Cosnes J *et al.* 1996).

In CD patients, cigarette smoking has been associated with lower serum 25(OH)D concentrations as compared to non-smoking patients (Gilman J *et al.* 2006; Siffledeen JS *et al.* 2003). In addition, when vitamin D supplemented CD patients were compared with non-supplemented CD patients, Jorgensen SP *et al.* 2013 found that the smokers had significantly lower serum 25(OH)D concentrations. This evidence suggests that there is an independently direct association between low serum 25(OH)D concentrations and smoking and disease activity.

3.6.3.3.2 Oral Contraceptive Use

The association between oral contraceptive use and CD has been extensively investigated through several case-control studies. Findings from these studies generally reveal that individuals taking oral contraceptives are at a greater risk of developing CD. A study conducted by Godet et al revealed that use of these drugs results in individuals being twice as susceptible to the risk of developing the disease as compared to the healthy controls (Godet PG *et al.* 1995). A study by Corrao G *et al.* 1998 also reveals that oral contraceptive users had a greater risk of CD. A case-control study performed by Persson P *et al.* 1993 on the risk indicators of CD re-emphasises that oral contraceptive use is associated with CD by showing that there was a 70% increase in the risk among users when compared with the controls. Certain studies however suggest that there is only a dose-dependent association between oral contraceptive use and CD as a result, long time users of oral contraceptives poses greater risk (Boyko EJ *et al.* 1994). On the other hand certain studies

find no significant association between oral contraceptive and CD (Halfvarson J *et al.* 2006). The actual mechanism on how oral contraceptive use affects the gut immune response still remains elusive, however it has been suggested that the thrombogenic characteristics of the contraceptives may be associated.

3.6.3.3.3 Antibiotics and Medication Use

The gut microflora plays a pivotal role in the regulation of the gut immune system and it is considered to be an initiator as well as a mediator of the long term inflammatory mechanism in IBD (Hviid A *et al.* 2011). Early life exposure to antibiotics can potentially harm the intestinal microbiota thus altering the balance between mutualistic microbes and opportunistic microbes (Sullivan A *et al.* 2001). The extent of the harm is dependent on various factors such as; the type of the antibiotic, the chemical properties of the antibiotic, and the dose and duration of use. According to a nation-wide register-based Finnish case-control study conducted by Virta L *et al.* 2012, the commonly used antibiotics were broad-spectrum penicillins, macrolides and cephalosporins. Antibiotics such as phenoxymethylpenicillin and combinations of sulphonamides and trimethoprim were less frequently used. The study revealed that exposure to cephalosporins was significantly associated with the risk of developing CD. It was also shown that if one was continued exposure to phenoxymethylpenicillin from birth to the age of 1 year was significantly associated with an elevated risk of CD development. No association was identified for any of the other antibiotics studied.

3.6.3.3.4 Vaccinations

Vaccinations in early childhood have been implicated in CD and are thought to be capable of altering the development of the intestinal immune system (Thompson NP *et al.* 1995). Common vaccinations include vaccinations against diphtheria, measles, pertussis, polio, rubella, tetanus and tuberculosis. Data from a case-control study based on a Danish inception cohort showed that vaccination against pertussis and polio led to an increase in the risk of developing CD (Hansen TS. 2011). Baron S *et al.* 2005 showed that there was a positive association between poliomyelitis, smallpox and BCG vaccination and an increased risk of CD.

The live attenuated vaccine for measles has been implicated in the aetiology of CD (Thompson NP *et al.* 1995). It has as well been suggested that there was an increased risk of CD among individuals with a persistent measles infection and those born during a measles epidemic (Ekbom A *et al.* 1994). However the study by Baron S *et al.* 2005 failed to confirm these findings, thus their results showed that measles mumps rubella (MMR) vaccine had a negative association with the risk of developing CD. The findings from a case-control study of measles vaccination and inflammatory bowel disease by Feeney M *et al*. 2002 also do not support the notion that measles vaccination predisposes an individual to subsequently develop CD. In CD patients, the matched odds ratios for measles vaccination was 1.08 (95% CI, 0.62-1.88).

3.6.4 Infections

It has been highlighted in many studies that infections in early childhood play an important role in as risk factor for IBD. It is generally unclear whether frequent childhood infections confer protection against CD or if they actually increase the risk of subsequently developing the disease.

Some paediatric studies however suggest that increased frequency of gastroenteritis (Whorwell PJ *et al.* 1979) and diarrhoea (Koletsko S *et al.* 1989) increase the susceptibility to CD. The results from a case control study conducted by Baron S *et al*. 2005 also suggests that frequent exposure to infections in childhood results in an increased risk of developing CD. The study by Gradel KO *et al.* 2009 confirmed that gastrointestinal infections were associated with CD development and the implicated microbes were *Salmonella* and *Campylobacter*. It is however important to consider that different infectious agents interact differently with the immune system so it is worthwhile to consider the interactions as separate entities.

3.6.4.1Viral Infections

Frequent exposure to infections in early childhood is thought to increase the risk of developing CD and to augur the early onset of the disease. Wurzelmann JI *et al.* 1994 investigated the association between childhood viral infections and the IBD. The viral infections which were investigated are colds, measles, mumps, rubella and varicella. Findings from the study only implicated cold to the increased risk of developing CD. Contrary to the common hypothesis, the study found a weak association between measles infection and the risk of CD.

3.6.4.1.1Measles Virus

Viruses have been implicated in the aetiology of IBD. The possibility that the causative agent for CD is a transmissible pathogen such as a virus is another plausible hypothesis (Thayer WR. 1990). The paramyxovirus in particular has caused great speculation in the pathogenesis of the disease. This is commonly known as the measles virus. Fragments of this virus have been observed in the mesenteric microvascular endothelium and if they persist, they result in chronic granulomatous

vasculitis. The measles hypothesis suggests an increased risk of CD in individuals vaccinated with the live attenuated measles vaccine in childhood (Thompson NP *et al.* 1995). The results from a study performed by Feeney M *et al.* 1997 in Dorset, UK however reveal no evidence linking live attenuated measles vaccination in childhood and the subsequent risk of developing CD. The study by Fisher NC *et al.* 1997 using serological techniques examined the association between measles virus infection in early childhood and predisposition to CD. Serum analyses for adenovirus, cytomegalovirus (CMV), herpes simplex virus (HSV) and measles virus were performed. Results from this study also confirm that the measles virus is not involved in the causation of CD, which seemed contrary to the accepted disease pathogenesis models of the time (Fisher NC *et al.* 1997).

3.6.4.2 Bacterial Infections

A great number of bacteria have been investigated as possible causative agents of CD. Among them are bacteria of the following species: *Klebsiella spp, Chlamydia spp, Eubacterium spp, Peptostreptococcus spp, Bacteroides fragilis, Enterococcus faecalis, Escherichia coli, Campylobacter jejuni, Campylobacter faecalis, Listeria monocytogenes, Brucella abortus, Yersinia pseudotuberculosis, Yersinia enterocolitica and Mycobacteria spp* (Thompson DE. 1994). Among the different species investigated, one *Mycobacterium avium paratuberculosis* (MAP) has received greater attention. Certain studies have linked drinking water to IBD, and the implicated microorganisms is *Mycobacterium paratuberculosis* (Abubakar I *et al.* 2008).

Found in ruminants, paratuberculosis is a chronic digestive disorder which is characterised by granulomatous enteritis that subsequently results in excessive weight loss and ultimately death. MAP is an extremely slow growing bacterium and this makes it difficult to isolate and grow on

conventional media. It can only be grown in conventional culture in 5% of cases and then only after months or years of incubation (Ryan P *et al.* 2002). Naser SA *et al.* 2004 found MAP in 46% of CD cases using the polymerase chain reaction (PCR) technique. There is still speculation on the association of MAP and CD, whether MAP's presence in individuals results in the subsequent development of CD or that MAP colonises the gut as a result of CD. It is however important to note that the association between MAP and CD is still unclear.

3.6.4.2.1 Helicobacter pylori

Helicobacter pylori (H.pylori) is a bacterial infection that is often acquired in the early years of childhood. It has been identified that *H. pylori* is associated with overcrowding, poor sanitary conditions and large sibship size (Feeney MA *et al.* 2002). It has been observed that CD seems more prevalent in regions less commonly colonized with *H. pylori* infection (Molodecky NA and Kaplan GG. 2010). According to Luther J et *al.* 2010, the mechanism in which *H. pylori* infection may confer protection against CD is by increase in gene expression of Treg cell function.

3.6.4.3Parasites Exposure

Helminths are complex multicellular worms that play an important role in the aetiology of CD. Helminths which are commonly known as flatworms are more specifically termed platyhelminths. They parasitically inhabit the human gut and possess intricate life cycles and development (Mansfield LS *et al*. 2004). Helminths are more prevalent in warm climates and common in populations subject to overcrowding and poor sanitation. It is interesting to realise that IBD is less prevalent in helminth infested places. The common host for helminths are children who normally acquire them through contact with food, water or soil contaminated with the infective form of the

parasite (Elliott DE *et al*. 2000). Many helminths survive for years within the gastrointestinal tract, thus beginning in childhood these worms release antigenic molecules that are bound to activate the Th2-type cytokines which in turn inhibit the Th1 response to antigens therefore causing a dysregulated Th1 response (Else KJ *et al.* 1994). A dysregulated Th1 mucosal immune response over a long period of time promotes greater likelihood of developing CD. People in affluent communities live in increasingly hygienic environments thus making them less likely to acquire helminths. It is possible that the failure to acquire helminths and to experience mucosal Th2 conditioning predisposes to CD (Urban JFJ *et al*. 1992). The decreasing frequency of helminthic colonization appears to correlate with the increasing prevalence of CD (Elliot DE *et al*. 2000).

3.7 Domestic and Socio-economic Factors

3.7.1 Environmental Upbringing

Different environments, diverse in their unique potential exposures each based on the socio-economic attitudes and activities that take place in such areas, will influence the gut microbiota and in turn, immune development. Among those genetically susceptible, such environmental differences are likely to play a role in which certain immune-diseases are expressed, and thus in the incidence and prevalence of disease. Many studies have identified dissimilar occurrence of CD between urban and rural inhabitants (Bernstein CN *et al.* 2006; Gearry RB *et al.* 2006; López-Serrano P *et al.* 2010; Wurzelmann JI *et al.* 1994). Greater occurrence was found to be in the urban settlement. This can possibly be attributed to the different environmental exposures, diet, contact with animals, levels of sanitation and pollutants within these environments. Some studies found no association between the place of upbringing and CD (Basson A. 2014; Castiglione F *et al.* 2012; Feeney MA *et al.* 2002; Malekzadeh F *et al.* 2009). Certain observational studies tend

to infer that there is increased incidence of CD in highly populated settlements (Klement E *et al.* 2008; Radon K *et al.* 2007; Wulzermann JI *et al.* 1994). These differences in observations warrant for a better understanding of how the place of upbringing interacts with CD.

3.7.2 The Sharing of Bathrooms and Living Spaces

Early childhood is crucial in programming the immune system and exposure to microbial antigens helps to sensitize and regulate the immune response. It is therefore follows that lack of exposure to infections in early childhood may potentially increase the risk of subsequently developing CD. Social interactions are an important consequence of access to the natural environment and they serve to increase the diversity of micro-organisms to which an individual is exposed (Kuo FE *et al.* 1998). It has been observed that cohabiting individuals tend to share microbiota (Song SJ *et al.* 2013). Sharing basic household amenities facilitates the transmission of microbes which are responsible for the triggering of the immune responses. A study on the environmental risk factors of IBD conducted by López-Serrano P *et al.* 2010, the authors reported a significant association between bedroom sharing and IBD. Data from this study indicated that bedroom sharing was associated with a lower risk of developing CD. Conversely, two studies (Basson A. 2014; Malekzadeh F *et al.* 2009) could not confirm this finding. Observations from these studies reveal no significant association between bedroom sharing and the subsequent development of CD.

3.7.3 Water Source and Hot Water Availability

Aamodt G *et al.* 2008 used data from a population-based cohort recruited in South-eastern Norway in 1990-1993, investigated the association between the quality of drinking water and the risk of developing CD. The main sources of drinking water in the region are lakes, reservoirs and rivers.

Water quality data extracted from the Norwegian waterworks included measurements of pH, colour, turbidity, coliform bacterial count and metal content of iron and aluminium. The risk of developing CD was significantly increased by 21% and this was attributed to the high iron content of the water. From these findings the authors suggested that the high iron content serve as a catalyst for oxidative stress, thus resulting in inflammation or an increased rate of cell mutations. Another possible explanation was that the high iron content triggers the rapid growth of bacteria, which in turn stimulate an inappropriate immune response in genetically predisposed individuals.

Several observational studies (Basson A. 2014, Bernstein CN *et al.* 2006, Duggan AE *et al.* 1998 and Gent AE *et al.* 1994) have investigated the association between drinking water and the risk of developing CD. Findings from these studies revealed that there was increased risk of CD in individuals who had access to tap water. By contrast, similar studies conducted by Baron S *et al.* 2005 and Gilat T *et al.* 1987 could not confirm the findings. An epidemiological study conducted in the United Kingdom by Duggan AE *et al.* 1998 revealed that before the age of 11 years, the a fixed supply of hot water supply was significantly associated with CD development. Likewise, a population-based study on the Asian population also found that availability of hot tap water increased susceptibility to develop CD (Ng SC *et al.* 2014 (a)). However other studies found no significant association between heated water and the subsequent development of CD (Basson A. 2014; Hampe J *et al.* 2003).

3.7.4 Number of Siblings, Birth Order and Family Size

It has been suggested that an increased number of household residents consequently results in an accrued likelihood of infections. This implies that individuals raised in households with fewer residents, as well as those with fewer siblings, have a reduced microbial exposures, including a

reduced chance of acquiring gastrointestinal or other such infections, believed to form crucial elements in the programming of intestinal immunity (Baron S *et al.* 2005). Nevertheless, the majority of studies have yielded inconsistent findings in support of this. For instance, findings from a case-control study conducted by Bernstein CN *et al.* 2006 reported that CD patients tended to have a smaller family size and fewer siblings, whereas another study reported the tendency for CD patients to be raised in families with a greater number of older siblings, when compared with controls (Baron S *et al.* 2005). Gearry RB *et al.* 2010 investigated the association between the age and number of siblings in a family with the risk of developing CD yet found no significant association. In like manner Han DY *et al*, also found no significant association between of number of siblings and birth order and CD risk (Han DY *et al.* 2010).

3.7.5 Exposure to Animals

The hygiene hypothesis infers that individuals who are raised in extremely hygienic conditions tend to have greater propensity to develop disease compared to those who are exposed to the various immunostimulatory microbial agents such as viruses, bacteria, and parasites (Koloski NA *et al*. 2008). The most significant phase of immune system development is the initial year of life and it is important for an individual to be exposed to certain microbes which are necessary in the programming of an effective immune system. Soon after birth, the human body requires microbial exposures to provide the molecular inputs of the surrounding environment (Rook GA. 2013). There are three main reasons why the body needs to be exposed. Firstly, exposure enables the body to build up immunological memory of various molecular structures of the microbial antigens in its surrounding which in turn aid to subsequently accelerate recognition of potentially dangerous organisms (Naik S *et al.* 2012). Secondly, the immune system has to develop regulatory

pathways and Tregs that are of paramount importance in preventing inappropriate immune responses on self, gut contents and harmless allergens (Rook GA. 2013). Thirdly, microbial antigens taken up in the gastrointestinal tract are vital in maintaining an optimum level of activation of the gut innate immune system (Clarke TB *et al.* 2010).

Data observed from the study by Fujimura and colleagues revealed that microbiota found in dust from households which have dogs is more diverse than that present in households without them (Fujimura KE *et al.* 2010). In line with these findings, it was demonstrated in the study by Aichbhaumik N *et al* that exposure to dogs in early childhood, confers protection against allergic sensitization and disorders (Aichbhaumik N *et al.* 2008). A study by Bernstein CN *et al* indicated that before the age of 5 years, having pet cat was protective against CD and also displayed that CD patients were less likely to have had a household pet than controls (Bernstein CN *et al.* 2006). Radon K *et al* highlighted that contact with regular farm animals such as cattle, goats, pigs and sheep during the first year of childhood tends to decrease the risk of developing juvenile onset of disease CD (Radon K *et al.* 2007). This study also investigated the association between exposure to household pets, specifically cats and dogs and the immune system.

On the contrary, Han DY *et al* determined that having a pet caused significant increased risk of subsequently developing CD and that even regularly feeding pets was not sufficient to confer protection against the risk (Han DY *et al.* 2010). In support of this notion Hlavaty T *et al* confirmed that having a cat in the home posed significant increased risk of developing CD (Hlavaty T *et al.* 2013). These findings therefore warrant more research into the hygiene hypothesis which might lead to the understanding the intricate aetiology underlying CD.

3.7.6 Appendectomy and Tonsillectomy

Both the appendix and the tonsils are crucial elements of the gut-associated lymphoid system. Surgically removing these organs can possibly alter the balance between immunologic helper and suppressor functions therefore resulting in changes in the immune response. That makes these organs important in the pathogenesis CD. In a case control study performed in Crete by Koutroubakis IE *et al.* 1999, an association between CD cases and appendectomy was observed. However, the association did not reach statistical significance. A positive association between appendectomy and CD was confirmed in studies conducted by Gilat T *et al.* 1987 and Gent AE *et al.* 1994.

In the same aforementioned study by Koutroubakis IE *et al*, tonsillectomy was shown to have a significant positive association with the prevalence of CD. Data from the study showed that 25% of the CD cases and 9.2% of their matched controls had undergone tonsillectomy. The results revealed that an individual who had undergone tonsillectomy had an 83.7% percent risk of developing CD as compared to one who had not undergone the operation. On multivariate logistic analysis, tonsillectomy was identified as a significant independent risk factor for developing CD (Koutroubakis IE *et al.* 1999).

3.8 Summary

Vast research has been devoted in understanding the exact aetiology of Crohn's disease; however the actual cause still remains unknown. Nonetheless, it has been established that susceptibility to the disease is a result of a combination of genetic and environmental risk factors. The assumed basis of environmental factors is the disruption of the gastrointestinal immune function towards

commensal microflora. Under environmental factors, three hypotheses have been postulated: cold chain; hygiene and infection hypothesis. To date the only environmental risk factor that has been clearly established for CD is cigarette smoking. Other environmental factors have been widely investigated, only to yield conflicting findings. With such different findings, the suggestion is that there is need to refine research on these interactions and to come up with plausible conclusions. Generally evaluation of environmental factors has not achieved much in identifying the cause and effect of the disease. More research in this area needs to be done in a bid to ascertain its pathophysiology. This study seeks to investigate the element of the timing of exposure and the possible interplay between the environmental risk factors in the subsequent development of CD.

Tables Referred to in Chapter 3:

The following tables from this chapter are represented:

Table 1: Montreal Classification System

Table 2: Crohn's Disease Activity index CDAI)

Table 3: Harvey-Bradshaw Index (HBI)

Table 4: The Global Incidence of Crohn's disease

Table 5: Potential Risk factors of Crohn's Disease

TABLE 1: Montreal classification scheme

Age at diagnosis (years)		
	A1	≤16
	A2	17- 40
	A3	> 40
Disease location		
	L1	Isolated to the terminal ileum
	L2	Isolated to the colon
	L3	Ileum and colonic involvement
	L4*	Upper gastrointestinal tract
Disease behaviour		
	B1†	Inflammatory; non-stricturing, non-penetrating
	B2	Stricturing
	B3	Penetrating disease, with or without stricturing, excludes perianal penetrating disease
	p‡	Perianal disease modifier

*Upper gastrointestinal (GI) modifier (L4) can be added to L1-L3 when concomitant upper GI disease present.

† B1 category should be considered 'interim' until a pre-specified time has elapsed from time of diagnosis. Suggested time period is between 5-10 years.

‡ 'p' is added to B1-B3 when concomitant perianal disease is present.

TABLE 2: Crohn's Disease Activity Index (CDAI)

Score	Definition
CDAI <150	In-active or quiescent disease (i.e. 'remission')
CDAI 150-220	Mild disease characterized by <10% weight loss, potentially increased CRP levels above the upper limit of normal, with no presentation of dehydration, fever, abdominal mass, tenderness or obstruction.
CDAI 220-450	Moderate disease characterized by weight loss >10%, presence of tender mass (no overt obstruction), or unsuccessful response to treatment and elevated CRP.
CDAI >450	Severe disease characterized by intestinal obstruction, abscess, elevated CRP, cachexia (BMI <18 kg/m^2), in treated yet persistently symptomatic patients.

CRP, C-reactive protein; BMI, Body mass index.

Developed by WR Best and colleagues in 1976, The CDAI consists of eight factors, each summed after adjustment with a weighting factor.* Often used in research purposes, the score is then used to classify patient disease activity.

*For full details, refer to Best WR et al., 1976 (Best WR., et al. 1976).

TABLE 3: Harvey Bradshaw Index (HBI), a simple index of Crohn's disease activity

	Response/ Variable	Possible score	Received score
1.General Well-being (yesterday)			
	Very well	0	
	Slightly below par	1	
	Poor	2	
	Very poor	3	
	Terrible	4	
2.Abdominal Pain (yesterday)			
	None	0	
	Mild	1	
	Moderate	2	
	Severe	3	
3.Number of liquid stools per day (yesterday)			**_____stools**
4.Abdominal mass			
	None	0	
	Dubious	1	
	Definite	2	
	Definite and tender	3	
5.Complications (*check any that apply below; score one per item except for first box*)			
	None	0	
	Arthralgia	1	
	Uveitis	1	
	Erythema Nodosum	1	
	Aphthous ulcers	1	
	Pyoderma gangrenosum	1	
	Anal fissure	1	
	New fistula	1	
	Abscess	1	
Harvey Bradshaw index score = (add scores of questions 1-5)		**TOTAL:**	
HBI Scoring Key:			
Remission		**<5**	
Mild disease		**5-7**	
Moderate disease		**8-16**	
Severe disease		**>16**	

Table 4: The Global Incidence of Crohn's disease

Place	Time period	CD incidence Per 100000
North America		
Loftus EV *et al*	1984-1993	6.9
Blanchard JF *et al*	1987-1996	15.6
Scandinavia		
Moum B *et al*	1990-1993	5.8
United Kingdom		
Rubin GP et *al*	1985-1994	8.3
Southern/Central Europe		
Tragnone A *et al*	1989-1992	2.3
Asia		
Morita N *et al*	1991	0.5
Africa		
Wright JP *et al*	1980-1984	1.8

*Adopted from Viazis N. 2006 (incidence cases per 100000)

Table 5: Potential Risk factors of Crohn's Disease

Injury to the puerile gut	Nutritional Factors	Chemical Factors	Infectious Factors	Domestic / Socio-economic Factors
Bottle feeding Gastroenteritis Diarrhoea	Food Additives GMOs Sugar Cholesterol Modified fats	Antibiotics Immunization Medications Smoking Pollution	Viral infections Bacterial infections Parasites exposure Pet exposure Farm animal exposure	Bathroom sharing Bedroom sharing Swimming Family size Running water Hot water supply Sibship Birth order Surgery Rural/urban environment Central heating

CHAPTER 4: THE ASSOCIATION BETWEEN ENVIRONMENTAL EXPOSURES DURING CHILDHOOD AND THE SUBSEQUENT DEVELOPMENT OF CROHN'S DISEASE: A SYSTEMATIC REVIEW

4.1 Background

Environmental risk factors in childhood are believed to play a role in the subsequent development of CD. Numerous studies have evaluated the different environmental exposures during childhood, albeit findings for many have been inconsistent. These inconsistencies may be attributed to differences in both the timing and the extent of the various environmental exposures during childhood, as well as the heterogeneity in CD susceptibility mutations both between and within individual population groups. Alternatively, these findings may be a result of methodological issues such as study sample size, participant characteristics (i.e. demographics, socioeconomic factors), identification of inappropriate control subjects, or the failure to account for potential confounding environmental variables. Therefore, in a bid to explore the available evidence surrounding childhood environmental risk factors and the association with future development of CD, a systematic review of the literature was conducted, with a specific emphasis on timing of exposure.

4.2 Aim

The aim of this systematic review was to investigate the association between childhood environmental risk factors, timing of exposure and the subsequent development of CD. Comparisons aimed to evaluate exposures during three specific age intervals; 0-5years, 6- 10years and 11-18years. However due to the limited number of studies identified in the initial literature search, in which these age interval definitions had been used, the original aim was amended to

also include studies which had broadly defined ‘childhood’ (0-18 years); in the literature this was found to be defined either as 0-12 years, or as 1-18 years; to evaluate the association between childhood environmental exposures and future CD development.

4.3 Objective

The objective of the systematic review was to:

- Determine the environmental factors associated with CD development.

Under the original objective; the environmental risk factors during childhood included:

Antibiotic use

Availability of heated water

Bathing in a swimming pool

Bathroom sharing

Bedroom sharing

Breastfeeding

Childhood infections

Exposure to pets

Farm animal contact

Feeding pets

H. pylori status

Having a sand pit at home

Having a vegetable garden at home

Place of upbringing

Preschool/Day-care attendance

Primary water source

Proportion of carpet in home

Sibship size

Type of toilet facility

4.3.1 Amended Objective

The final number of possible variables included in the systematic review was limited as a result of the inconsistencies in environmental variables investigated between the identified studies (under the amended aim).

The objective of the systematic review was to:

- Determine the environmental factors associated with CD development.

Under the amended objective; the environmental risk factors during childhood included:

Availability of running tap water

Availability of hot tap water

Exposure to household pets

Farm animal contact

Place of upbringing

Sibship size

4.4 Materials and Methods

A systematic review of the literature including all original case-control, cross-sectional, cohort studies and controlled trials was conducted.

4.4.1Search strategy

A systematic search of the literature was conducted using a research protocol developed by the candidate. During March 2015, a comprehensive search was conducted in electronic databases, such as: EMBASE, EBSCOhost (Medline), Ovid, Scopus and World Cat, PubMed and BioMed Central. Additional search terms were constructed after a review of the relevant literature. A manual search of reference lists of retrieved articles was also performed. In addition, a systematic search of the grey literature was performed, although no articles were identified using this method.

4.4.2 Methodology

A systematic search for literature using a controlled vocabulary of Medical Subject Headings (MeSH) was undertaken to identify articles published after 1 January 1950 and up to 25 June 2015. Identification of eligible articles was performed by two reviewers (V. Sabe and A. Basson) and screening was conducted by reading of abstracts and titles. Only original studies were considered and selection encompassed cross-sectional, cohort and case-control studies, as well as controlled trials. The search was limited to human studies and both retrospective and prospective evaluations were considered. Elimination of articles was performed on the basis of the following; diagnostic accuracy, publications reporting on experimental animal research or articles that were not based on original research, including all systematic reviews and meta-analysis reports (Appendix XIII). Duplicated studies were also excluded from the selection.

Set of search entry terms

Three sets of entry terms were applied separately and combined in the database search. The list of the sets of search entry terms is shown in Table 6.

4.4.3Variables of Interest and Definitions

Availability of running tap water: having access to the water that comes out of the taps that are connected to the main supply of the local water system as the primary water source.

Availability of hot tap water: having access to heated water that comes out of taps that are connected to the main supply of the local water system.

Exposure to household pets: having direct or indirect contact with any household pets within the household.

Farm animal contact: having a farm animal on the property.

Place of upbringing: the place where an individual was raised during childhood.

Sibship size: the number of individuals born of the same parents living in the same household.

4.4.4 Selection Criteria

Inclusion criteria

- All retrospective and prospective: case control, cohort and cross-sectional studies, as well as controlled trials

- Studies evaluating the association between one or more childhood environmental exposures and the subsequent development of CD.

- Studies which evaluated childhood exposure defined as age intervals (e.g. 0-5, 6-10 and 11-18 years).

- Studies which evaluated 'childhood exposure' broadly defined as 0-18 years.

- Studies published from 1 January 1950 (inclusive) and until the date of the literature search (March 2015).

Exclusion criteria

- All non-English language literature.

- Unpublished articles without an available abstract or full text.

- All systematic reviews, meta-analyses and studies of diagnostic accuracy.

- Articles reporting on paediatric onset (diagnosed under the age of 16).

- Articles investigating CD remission, as the primary outcome.

- Articles investigating dietary habits, as the primary outcome.

Search findings

Of an initial 181 articles, 153 were considered after the removal of duplicates, and 16 were selected for the data extraction and quality appraisal process in this review. A flow diagram of how the literature was identified is shown in Table 7. This process has been described under Point 4.5.5.1 (Literature Search Discussion). All included literature has been and listed within the bibliography (listed at the end of the present dissertation).

Analysis of identified literature

The researchers carefully screened and examined all titles and abstracts of the articles identified from the search. Articles which did not meet the inclusion criteria were automatically excluded from the batch. Full text copies were obtained of all articles meeting the initial inclusion criteria based on the title and abstract. After a thorough review of the preliminary articles by both reviewers and a consensus was reached, the data extraction process was initiated. The data extraction tool was performed developed according to PRISMA (preferred reporting items for systematic reviews and meta-analyses) guidelines (Appendix XVIII) and the PICOS (participants, interventions, comparisons, outcomes, study design) (Appendix XIV). A template of the data extraction tool is presented in Table 8. After performing the data extraction, a quality appraisal was carried out by the investigators prior to data collection (Table 10 and 11). One investigator performed data extraction and quality appraisal while the other analysed the results. The authors of the articles were contacted via email and specific questions pertaining to their study were asked.

Data extraction

A standardized form, developed by the candidate was employed for the process of data extraction (Table 8).

The following specific data points were collected for each study:

- Study setting
- Methodological approaches for data collection (i.e. questionnaires, medical records)
- Data sampling frame
- Number of IBD case and control participants
- Method of participant recruitment
- Demographic information of participants
- Environmental exposures investigated
- Age interval(s) and/or age range(s) used for environmental variable investigated
- Study findings and data analysis methods

After the data extraction process was completed, authors of the identified articles were then contacted via email based on the contact details provided within their respective publication. Emails included an attachment of a formal letter requesting the following (specific to each author); 1) missing statistical results, methodological details pertaining to the study design which were not mentioned in the publication, or other variables the authors may have investigated during their study, but did not include in the original publication, and 2) where needed, any clarification from the author on various aspects of the study design (e.g., participant demographics, age at time of study enrolment) which was not mentioned in the original publication. Of the 16 included publications, 6 had missing statistical results. In an attempt to source all statistical data, authors

were contacted. From a total of 16 letters sent to the respective authors, 7 responded to the call. Two among the seven authors responded by providing a copy of their doctoral thesis. However, of the latter 2 authors that supplied a copy of their doctoral thesis, only one was written in the English language and translation of the second proved difficult. Four of the authors responded by saying that their study had been conducted so long ago that they no longer had a copy of the information, beyond what was available in the original publication. The letter template is as shown in Appendix II.

Data Extraction Outcome

The data extraction outcome is presented in Table 9. Based on the data extraction outcome of the included articles, the environmental exposures that were considered as environmental exposure variables for this systematic review of the literature were; exposure to household pets, farm animal contact (including having a donkey/horse/sheep or cow living permanently on the property), place of upbringing, sibship size, primary source of water (i.e. availability of tap water) and the availability of hot water. The childhood environmental exposure variables considered in the present review are in line with the hygiene hypothesis and specifically relate to the immediate household and the surrounding environment to act as potential avenues for both microbial exposure and antigen immunostimulation.

Although environmental risk factors such as, breastfeeding (Klement E *et al.* 2004), passive cigarette smoke exposure (Mahid SS *et al.* 2006) and antibiotic use (Khan KJ *et al.* 2011) scored relatively high according to the data extraction analysis of variables, these variables were excluded from the present systematic review in light of the fact that systematic reviews, focusing

specifically on these exposures, have recently been published. After reviewing the available literature, it was also decided that surgical history and childhood infections and antibiotic use would be excluded from review. The other reason for this was because the great majority of studies were limited by recall bias and data had not been confirmed by medical records. Dietary factors (in general) were not investigated in the systematic review for three reasons, namely; 1) the focus of the review was intended on the hygiene hypothesis 2) methodological issues associated with this variable, including the strong recall bias which often entails retrospective dietary intake reports, and 3) there has been a fairly recent systematic review on this variable (Hou JK *et al.* 2011). The interaction between dietary compounds and the gut microbiome cannot be discounted, particularly given the dynamic differences in diet between population groups; however this was outside the scope of the present systematic review.

4.4.5 Data Synthesis

Data Collection

The majority of studies (n=12) used either interviewer administered or self-administered questionnaires as the main method of data collection. Four studies also included patients' medical records as a means of data collection (Appendix XI and XII).

Article Coverage

The observational studies that were under investigation seem to representative of the world, since all the continents are covered (Appendix XI and XII).

4.4.6 Literature Search Discussion

After careful review, 20 full text articles remained eligible for inclusion into the systematic

review; however another 4 were later excluded based on a consensus reached by both reviewers (Appendix XI; XII and XIII). Of the 16 included publications, 6 had missing statistical results. In an attempt to source all statistical data, authors were contacted. From a total of 16 letters sent to the respective authors, 7 responded to the call. Two among the seven authors responded by providing a copy of their doctoral thesis. However, of the latter 2 authors that supplied a copy of their doctoral thesis, only one was written in the English language and translation of the second proved difficult. Four of the authors responded by saying that their study had been conducted so long ago that they no longer had a copy of the information, beyond what was available in the original publication.

The study by Guo AY *et al* was eliminated because it examined the association of early life exposures and the modification of risk of IBD (Guo AY *et al.* 2014). The main focus of the study was how early environmental risk factors were associated with the risk of surgery in CD patients, which was not the focal point of the present systematic review. The case-control study performed in Sweden by Hildebrand J *et al* examined the early-life exposures associated with antibiotic use and the risk of subsequent development CD (Hildebrand H *et al.* 2008). The study included cases that had an early diagnosis of CD, before the age of 16 years. Given that early onset CD is associated with a more severe form of the disease compared to that of later onset, as well as that different classification models are used for adults when compared with paediatric CD patients, to distinguish disease phenotype, the latter using the Paris classification model, the study was excluded from the present systematic review.

Corrao G *et al* conducted a nationwide case-control study in Italy (Corrao G *et al.* 1998). The study examined the risk of IBD attributable to smoking (Mahid SS *et al.* 2006), oral contraceptive use (Cornish JA *et al*. 2008) and breastfeeding (Klement E *et al.* 2004), aspects which have

been considered in earlier systematic reviews. In addition, oral contraceptive use is not considered to be a childhood environmental risk factor.

The Swedish-Danish population based co-twin control study conducted by Halfvarson J *et al* was excluded as the study evaluated variables which were not the focus of this systematic review, namely; diet, smoking, oral contraceptive use and appendectomy (Halfvarson J *et al.* 2006). In addition, it was felt that recall bias was a strong limitation to the findings surrounding childhood infections and childhood hospitalization as this information was collected via questionnaire and not confirmed via medical records of patients.

Overview

The summary overview of the literature under examination has been presented in Appendices XI-XVII. Among the 16 observational studies that were under examination, 2 of them were cohort studies and the rest were either population-based or hospital-based case-control studies. It is important to note that only 4 articles performed investigated environmental risk factors based on predefined age intervals; either as 0-5, 6-11 and 12-18 years, or as; 0-5, 6-10 and 11-18 years (Basson A. 2014; Gearry RB *et al.* 2010; Han DY *et al.* 2010; Wurzelmann JI *et al.* 1994). The remaining studies evaluated exposures in the 'broad childhood' category which articles defined as; 0-12 years, 0-16 or 1-18 years.

4.5 Results

Place of Upbringing

0-5 years

Of the 5 studies that investigated the association between the place of upbringing during the age interval 0-5 years and the subsequent development of CD, 3 found no significant association. In a population based case-control study conducted in Cape Town, South Africa by Basson A including 194 CD patients and 213 controls, there was no significant difference in the risk of CD development between individuals who lived in a suburban or urban type of community and those who lived in a rural, farm or informal settlement [(OR = 1.21; 95% CI, 0.71-2.10); (κ = 0.86; 95% CI, 0.79-0.92)] (Basson A. 2014). Notably, one year after study completion a total of 40 (10%) randomly selected participants completed the interviewer administered questionnaire for a second time in order to measure the agreement between repeated data for the questionnaire using a kappa statistic. The κ statistic for the majority of findings ranged between 0.70-0.99, strongly supporting the reliability of their data. In a matched case-control pair study conducted at a district general hospital in the United Kingdom including 139 CD patients and outpatient controls, Feeney MA *et al* also found no association of the urban environment with the risk of developing CD (OR = 0.59; 95% CI, 0.28-1.24) (Feeney MA *et al.* 2002). Similarly, a case-control study performed in Canterbury, New Zealand by Gearry RB *et al* involving 638 CD patients and 600 controls, revealed that there was no difference in association between city dwellers (OR = 1), town dwellers (OR = 0.88; 95% CI, 0.67-1.16) and country dwellers (OR = 0.86; 95% CI, 0.65-1.14), with the future development of CD (Gearry RB *et al* 2010). In this study, each established predictor for CD was adjusted for age, sex, ethnicity and social class.

By contrast, in a large population-based cohort study conducted in Northern Europe by Timm S *et al,* the authors showed that being born, and raised on a livestock farm before the age of 5 years was protective against developing CD [(OR = 0.54; 95% CI, 0.31-0.94); (HR = 0.55; 95% CI, 0.31-0.98)] (Timm S *et al.* 2014). In the same study however, village life [(OR = 0.71; 95% CI, 0.49-1.03); (HR = 0.75; 95% CI, 0.52-1.10)] and city life (OR = 1; HR = 1) conferred no protective effect against future CD development. The study was performed in three phases of follow-up, and study population identification. The first, was conducted during 1989-1992, with a population of 21 802 responders. This was followed by the second phase conducted during 1999-2001 and included 16 202 responders. The last phase included 15 167 responders, and was conducted during 2010-2012. After considering all the variables of interest, the final study population included 10 864 participants. Statistical analysis included both multiple logistic regression and multiple Cox regression models. Sub analysis of the multiple logistic regression model of place of upbringing and CD was further stratified by the year of birth and also adjusted for BMI, sex and smoking. It was revealed that individuals born after the year 1952 and living on a livestock farm (OR = 0.25; 95% CI, 0.11-0.61), as well as those living in a village (OR = 0.52; 95% CI, 0.34-0.79), were less likely to develop CD, when compared to their city-dwelling counterparts. However there was no significant difference in the association with developing CD between dwelling on a livestock farm (OR = 1.64; 95% CI, 0.64-4.20), living in a village (OR = 1.60; 95% CI, 0.71-3.60) and city dweller [(OR = 1; (95% CI, data not available)] for the individuals born during, or prior to 1952 (Timm S *et al.* 2014). In line with these findings, a study performed by Wurzelmann JI *et al* in North Carolina, United States found that city dwelling was associated with the future development of CD (Wurzelmann JI *et al.* 1994). The authors performed a case-control study, including 322 CD patients obtained from the membership rolls of the North Carolina chapters of the Crohn's

and Colitis Foundation of America and 403 controls who were the patient's closest neighbour. It was established that the risk of developing CD significantly increased as the environment became more 'urbanised'; rural (OR = 1.32; 95% CI, 0.76-2.29), small town (OR = 1.34; 95% CI, 0.82-2.18), suburb (OR =1.43; 95% CI, 0.77-2.67) and city (OR = 1.81; 95% CI, 1.07-3.03).

6-11 years

Three studies investigated place of upbringing during the age interval 6-11 years; Basson A maintained a lack of association between place of upbringing and the risk of developing CD [(OR = 1.58; 95% CI, 0.91, 2.76); (κ = 0.68; 95% CI, 0.53-0.75)] (Basson A. 2014). However, during this age interval Gearry RB *et al* identified town and country dwelling to be protective against CD development when compared to city dwelling. Interestingly, the positive association between town dwelling and CD development was only significant after adjusting for age, sex, ethnicity and social class [(OR = 0.79; 95% CI, 0.60-1.04) vs (OR = 0.69; 95% CI, 0.51-0.94), respectively], whereas a significant positive association was identified for country dwellers both before and after adjustment [(OR = 0.70; 95% CI, 0.53-0.92) and (OR = 0.64; 95% CI, 0.46-0.89), respectively] (Gearry RB *et al.* 2010). Wulzermann JI *et al* also found that, compared to individuals living in rural (OR = 1.34; 95% CI, 0.77-2.35), small town (OR = 1.04; 95% CI, 0.62-1.7), and city (OR = 1.57; 95% CI, 0.92-2.67) areas, 'suburbanites' living was significantly associated with CD development (OR = 2.05; 95% CI, 1.12.-3.77). Notably, there remained a general trend of the risk increasing with the urban environ (Wurzelmann JI *et al.* 1994).

11-18 years

Three studies investigated place of upbringing during the age interval 11-18 years; Basson A.

continued to find no significant association between the place of upbringing and CD risk [(OR = 0.97 95% CI 0.55-1.70); (κ = 0.74; 95% CI, 0.35-0.67)] (Basson A. 2014). As with the previous age interval, Gearry RB *et al* the protective association for town dwelling and CD risk was only significant after adjustment [(OR = 0.82; 95% CI, 0.62-1.07) vs (OR = 0.69; 95% CI, 0.51-0.94), respectively]. Country living still remained consistently protective against CD, both prior to (OR = 0.67; 95% CI, 0.50-0.90) and after (OR = 0.64; 95% CI, 0.46-0.89) adjustment (Gearry RB *et al.* 2010). Wulzermann JI *et al* also continued to show that city dwelling was significantly associated with CD development (OR = 1.73; 95% CI, 1.01-2.97) (Wurzelmann JI *et al.* 1994).

Childhood (0-18 years)

Of the 4 reports which broadly evaluated exposure during 'childhood', Bernstein CN *et al* demonstrated that living on a farm (OR = 0.62; 95% CI. 0.46-0.85) to be protective against CD development. The authors performed a case-control study in Canada through questionnaire using data from the Manitoba Health Registry (Bernstein CN *et al.* 2006). Childhood was defined as the age younger than 12 years. The study included 364 CD patients and 433 controls matched by age, gender and geographic residence. In a 2010 Spanish hospital-based case-control study by López-Serrano P *et al* urban dwelling was significantly associated with risk of CD (OR = 4.58 95% CI 2.17-10.0), when compared to rural dwelling (López-Serrano P *et al.* 2010). The study included 124 CD patients and 235 age and sex matched controls. Data was collected via both self-administered questionnaires and medical records. By contrast, Castiglione F *et al* found no significant association between urban upbringing and the subsequent development of CD (OR = 0.73; 95% CI, 0.52-1.02) in a multi-centre case-control study conducted in Southern Italy including 468 CD patients and 562 controls, the latter consisting of physicians, nurses and health

support service professionals (Castiglione F *et al.* 2012). Similarly, an Iranian case-control study by Malekzadeh F *et al*, including 199 CD cases and 207 age-matched controls, the authors failed to find a significant association between place of upbringing and the risk of future CD development (Malekzadeh F *et al.* 2009). Overall, 89% of cases and 85% of controls reported an urban rather than rural upbringing.

Farm Animal Contact

0-5 years

During the age interval 0-5 years, Timm S *et al* revealed that farm animal contact, by means of living on a livestock farm, was protective against the subsequent development of CD (OR = 0.54; 95% CI, 0.31-0.94) (Timm S *et al* 2014). Basson A. found no association for never having a cow, donkey, horse or sheep living permanently and the risk of developing CD [(OR = 1.67; 95% CI, 0.83-3.45); (κ = 0.64; 95% CI, 0.19-1.00)] (Basson A. 2014), whereas Gearry RB *et al* identified a weak association between CD and contact with farm animals (data not available) (Gearry RB *et al.* 2010).

6-10 years

Two studies investigated the risk association between farm animal contact during the age interval 6-10 years and CD development. On multiple logistic regression analysis Basson A. identified a significant-risk association for never having a cow, donkey, horse or sheep living permanently on the property and future CD development [(OR = 3.10; 95% CI, 1.42-7.21); (κ = 0.84; 95% CI, 0.12-1.00)]. In addition, an independent risk-association was identified for never having a donkey,

horse, sheep or cow on the property (OR = 2.48; 95% CI, 1.09-5.98) during the age interval 6-10 years (Basson A. 2014). On the other hand, Gearry RB *et al* reported no significant association between farm animal contact and future risk of CD development (Gearry RB *et al.* 2010).

11-18 years

During the age interval 11-18 years, the aforementioned observations remained consistent for both studies; [Basson A: (OR = 4.31; 95% CI, 1.36-16.14); (κ = 1.00; 95% CI, 1.00-1.00)], although the publication by Gearry RB *et al* did report data on the non-significant association between farm animal contact and future risk of CD development (Basson A. 2014; Gearry RB *et al.* 2010).

Childhood (0-18 years)

Of the 2 studies evaluating the association between farm animal exposure during 'childhood' and CD risk, Hlavaty T *et al* found that not having contact with animals significantly increased the risk of developing CD (Hlavaty T *et al.* 2013). The 2013 case-control study was conducted at Bratislava University hospital through self-administered questionnaires to 190 CD patients, age and sex matched with 355 healthy volunteers. Statistical analysis examined the frequency of the individual's contact with either dogs, cats, poultry, cattle, horses, pigs or small rodents, and revealed that having little or no contact with cattle (p = 0.05), cats (p = 0.03) and dogs (p = 0.02) during childhood (0-20 years) significantly exposed one to the risk of developing CD. Bernstein CN *et al* investigated living on a farm, as well as whether primary activity was either cattle or pig or poultry husbandry. The authors found that CD patients were significantly less likely to have contact with farm animals (OR = 0.62; 95% CI, 0.46-0.85) compared to the controls. However for

the individuals who lived on a farm, there was no significant difference between either living on a cattle farm or pig farm or poultry farm and the controls (data not available) (Bernstein CN *et al.* 2006).

Exposure to pets

0-5 years

Five studies investigated the association between exposure to pets during the age interval and CD risk, of which only that of Bernstein CN *et al* identified a significant association. The authors revealed that before the age of 5 years CD patients were less likely to have had a pet as compared to the controls (65% vs 71.9%, respectively), and that exposure was protective (OR = 0.73; 95% CI, 0.53-1.0) against CD risk. Further analysis also revealed that prior to the age of 5 years, fewer CD patients had pet cats within the household, when compared to controls (33.8% vs 44.1%, respectively), and that exposure was protective against CD risk (OR = 0.68; 95% CI, 0.50-0.92)]. Comparison among only those who had pets at home the study found that CD patients (50.8%) were less likely to have cats than controls (61.3%), however these findings were not significant (OR = 0.72; 95% CI, 0.51-1.03). It must be noted that Bernstein CN *et al* evaluated only select variables during the age interval 0-5 years, while evaluating others under a more broad definition of 'childhood' (0-12 years) (Bernstein CN *et al.* 2006).

Feeney MA *et al* investigated the association between various household pets, namely cat, dog, bird and rodent and CD risk; however no significant risk association was identified for any of the variables [(Cat in home: OR = 0.81; 95% CI, 0.41-1.63), (Dog: OR = 0.65; 95% CI, 0.34-1.24), (Bird: OR = 1.76; 95% CI, 0.75-4.14), and (Rodent: OR = 0.89; 95% CI, 0.43-1.81)] (Feeney MA *et al.* 2002). Results of a questionnaire-based, case-control cohort of 315 CD patients

and 536 controls, conducted by Han DY *et al* in North Island, New Zealand, revealed no significant association between exposure to pets prior to the age of 5 years (OR = 1.26; 95% CI, 0.92-1.73) and the risk of developing CD (Han DY *et al.* 2010). None of the other studies identified a significant association between pet exposure and the development of CD (Basson A. 2014; Gearry RB *et al.* 2010). The statistical data was presented as in the studies as: Basson A. [(OR = 1.47; 95% CI, 0.92-2.34); (κ = 0.69; 95% CI, 0.46-0.91)] and Gearry RB *et al* (no data given).

6-10 years

For the age interval 6-10 years, only the study by Han DY *et al* reported a significant association between childhood exposure to pets and the development of CD (OR = 1.98 95% CI 1.28-3.06) (Han DY *et al.* 2010). The remaining two studies found no significant association [Basson (OR = 1.13; 95% CI, 0.71-1.79); (κ = 0.72; 95% CI, 0.51-0.94)] and Gearry RB *et al* (no data shown)] (Basson A. 2014; Gearry RB *et al.* 2010).

11-18 years

During the age interval 11-18 years, Han et al remained the only study to identify a significant association between childhood pet exposure and CD development (OR = 1.61 95% CI 1.07-2.42) (Han DY *et al.* 2010), while the other two studies [Basson A (OR = 1.01; 95% CI, 0.64- 1.59); (κ = 0.74; 95% CI, 0.53-0.94), and Gearry RB *et al* (no data available)] continued to report no significant association between exposure to pets and CD risk (Basson A. 2014; Gearry RB *et al.* 2010).

Childhood (0-18 years)

Among the 5 studies investigating childhood exposure to pets during 'childhood', López-Serrano P *et al* found that having a pet significantly increased the risk of developing CD (OR = 0.5; 95% CI, 0.3-0.9) (López-Serrano P *et al.* 2010). By contrast, Hlavaty T *et al* found that not having a cat significantly increased risk in developing CD (OR = 0.6; 95% CI, 0.4-0.9 (Hlavaty T *et al.* 2013). In a recent multi-centre, case-control study including 186 CD patients and 940 controls conducted by Ng SC *et al,* the authors reported that having a pet dog significantly decreased the risk of developing CD before the age of 15 years (OR = 0.54; 95% CI, 0.43-0.91). This Asia-Pacific study included Australia, China, Hong Kong, Indonesia, Macau, Malaysia, Singapore, Sri Lanka and Thailand. Statistical analysis was adjusted for sex, age and country income based on gross national income (GNI). Findings for Asia only were significant both prior to (OR = 0.50; 95% CI, 0.33-0.76), and after adjustment, supporting the notion that dogs were protective against CD (OR = 0.54; 95% CI, 0.35-0.83). In addition, a significant association was observed after adjustment for both Asia, and Asia and Australia (OR = 0.64; 95% CI, 0.44-0.94), whereas a weak association was revealed for cats, rodents, birds and aquarium fish with the development of CD (Ng SC *et al.* 2014 (b)). On the other hand, both Castiglione F *et al* (OR = 0.96; 95% CI, 0.75-1.24) and Malekzadeh F *et al* (data not available) found no association (Castiglione F *et al.* 2012; Malekzadeh F *et al.* 2009).

Water Source

0-5, 6-10, 11-18 years

Only one study evaluated the association between source of water during the 3 age intervals (0-5,

6-10 and 11-18 years) and CD risk. Basson A defined primary water source as either piped or bottled water, or that of an outside tap, borehole, well water, or a river or a dam. During the age interval 0-5 years and 11-18 years, having piped tap or bottled water was significantly associated with CD development [(OR = 2.10; 95% CI, 1.20-4.00); (κ = 0.63; 95% CI, 0.37-0.89) and (OR = 1.92; 95% CI, 0.80-4.60); (κ = 0.89; 95% CI, 0.69-1.00), respectively]. No significant association was identified for the age interval 6-10 years (OR = 2.05; 95% CI, 1.10-4.10); (κ = 0.65; 95% CI, 0.30-0.74) (Basson A. 2014).

Childhood (0-18 years)

Of the 4 studies investigating primary water source during 'childhood', only Bernstein CN *et al* identified a significant association between primary water source and the development of CD. The study revealed that in childhood, CD subjects were more likely to have had primary source of water being a tap rather than being a well, lake or other non-tap source as compared to the controls (data not available) (Bernstein CN *et al.* 2006). However, Hampe J *et al* reported that there was no significant association between tap water as a primary water source and developing CD (OR = 0.94; 95% CI, 0.53-1.66) (Hampe J *et al.* 2003). In agreement with these findings Hansen TS *et al* failed to identify a significant association when defined as having access to running water at home (OR = 0.50; 95% CI, 0.05-5.51) (Hansen TS *et al.* 2011). Ng SC *et al* also reported no significant association between in-house tap water and CD development in Asia [(unadjusted: OR = 1.21; 95% CI, 0.80-1.82); (adjusted: OR = 0.76; 95% CI, 0.48-1.22)]. The results failed to reach significance when analysed for both Asia and Australia (OR = 0.85; 95% CI, 0.53-1.34) (Ng SC *et al.* 2014 (b)).

Heated Tap Water

0-5 years

In a 2 year double blinded case-control study including 1468 CD patients and 3364 unaffected family members as controls in Germany, Hampe J e*t al* demonstrated that there is no significant association between heated tap water and the development of CD before the age of 5 years (OR = 1.25; 95% CI, 0.78-2.00), although a general trend towards a high risk of developing CD and the availability of hot tap water was observed by the authors (Hampe J e*t al.* 2003). Similar findings were reported by Basson A who found no association between heated tap water and CD [(OR = 1.18; 95% CI, 0.71-2.00); (κ = 0.81; 95% CI, 0.73-0.89)] (Basson A. 2014).

Feeney MA *et al* however found the availability of hot tap water to be significantly associated with developing CD, and this was reported by over 95% of all subjects (data not available) (Feeney MA *et al.* 2002).

6-10 years

In the age interval 6-10 years only one study (Basson A. 2014) examined the association although found no significant association [(OR = 1.52; 95% CI, 0.92-2.54); (κ = 0.86; 95% CI, 0.89-0.90)] between heated tap water and developing CD.

11-18 years

Basson A again found no significant association between the availability of hot tap water and the

subsequent development of CD in this category [(OR= 1.52; 95%CI, 0.93, 2.51); (κ = 0.70; 95% CI, 0.69-0.76)] (Basson A. 2014). A case-control study performed by Duggan AE *et al* at Nottingham University hospital, United Kingdom, including 110 CD patients and 337 controls (surgical inpatients having elective surgery with no history of IBD) also did not identify a significant association between heated tap water and CD development [Unadjusted: (OR = 0.84 95% CI, 0.3-1.4); and Adjusted: (OR = 1.74 95% CI, 0.8-3.8)](Duggan AE *et al.* 1998).

Childhood (0-18 years)

Of the 3 studies which investigated the association between the availability of hot tap water and CD, 2 identified a positive association. Duggan AE *et al* analysed childhood exposure between 0-11 years and showed a significant, independent association (at 5% level) between the availability of hot tap water and CD risk, after adjustment (OR = 0.56; 95% CI, 0.3-0.9), after adjustment for age and sex (Duggan AE *et al.* 1998). In the Asia-Pacific study, Ng SC *et al* identified a positive significant association in the Asian population (OR = 1.48; 95% CI, 1.01-2.13), however no association was observed in the Australian population; after adjusting for sex, age and country income (Ng SC *et al.* 2014 (b)). Conflicting findings were also demonstrated by the study conducted by Malekzadeh F *et al* which found no significant association between heated water and the development of CD (data not available) (Malekzadeh F *et al.* 2009).

Sibship Size

0-5 years

Feeney MA *et al* focused on the association of family characteristics and the development of CD

before the age of 5 years. No significant difference in association was observed between being a single child (OR = 0.71; 95% CI, 0.18-2.77) in the family and having been two or more siblings (OR = 0.48; 95% CI, 0.1-2.01) with CD development. In addition, no significant association was seen for individuals not having older siblings (OR = 1.38; 95% CI, 0.37-5.20), for individuals with one older sibling (OR = 0.96; 95% CI, 0.25-3.65), or those who had two or more older siblings (OR = 1.71; 95% CI, 0.48-6.16) (Feeney MA *et al.* 2002).

Gearry RB *et al* investigated the association between sibling number and older sibling number with CD risk, finding no significant association (data not available) (Gearry RB *et al.* 2010). Han DY *et al* reported a weak effect of number of siblings and birth order on the risk of developing the disease (data not available) (Han DY *et al.* 2010).

6-10 years

During this age interval, both Gearry RB *et al* and Han DY *et al* investigated the association between number of siblings in the family and birth order or the older sibling number with risk of CD development, however both authors reported finding no significant association (data not available) (Gearry RB *et al.* 2010; Han DY *et al.* 2010).

11-18 years

During the age interval 11-18 years, both Gearry RB *et al* and Han DY *et al* continued to report no significant association between number of siblings in the family and birth order, or the older sibling number with risk of CD development (data not available) (Gearry RB *et al.* 2010; Han DY

et al. 2010).

Childhood (0-18 years)

Castiglione F *et al* found no significant association between having one, or less brothers or sisters and having 2 or more brothers or sisters (OR = 1.02; 95% CI, 0.76-1.37) with risk of developing CD (Castiglione F *et al.* 2012). Similarly, Hampe J *et al* reported that having 2 or more siblings had no significant association with the risk for CD ($\chi^2 = 0.48$, 1df, $p > 0.20$) (Hampe J *et al.* 2003), while López-Serrano P *et al* found no significant association between number of siblings and CD. The latter study displayed that there was no difference between having none or one sibling versus having two or more siblings, hence no significant association with the disease (OR = 0.6; 95% CI, 0.2-1.7) (López-Serrano P *et al.* 2010). These findings were supported by the study performed by Malekzadeh F *et al* in which sibship size was analysed as co-factor for exposure to domestic refrigeration in childhood. No association with CD identified (data not available) (Malekzadeh F *et al.* 2009). In a Swedish study, Persson PG *et al* also demonstrated no association between either having, or not having siblings (OR = 1.8; 95% CI, 1.0-3.4) with the subsequent development of CD (Persson PG *et al.* 1993). In contrast to these findings, 2 studies confirmed a positive association between sibship size and the risk of developing CD. Bernstein CN *et al* analysed the association based on the sex of the siblings, taking into separate account the number of brothers and the number of sisters the individual. Results of the study showed that, compared to controls, CD patients were less likely to have brothers [(mean: 2.12 vs 1.67, respectively); (OR = 0.78; 95% CI, 0.67-0.9)] or sisters [(mean: 2.11 vs 1.77, respectively); (OR = 0.82; 95% CI, 0.7-0.96)] (Bernstein CN *et al.* 2006). Hlavaty T *et al* also established that CD patients had significantly less number of siblings compared to controls (1.6+/-1.3 vs 1.9+/-1.3, respectively) (statistical results not available) (Hlavaty T *et al.* 2013).

4.6 Discussion

Overall, results from the studies examined in this systematic review show congruence with the hygiene hypothesis, in that delayed exposure to immunostimulatory microbes through the environmental exposures associated with improved sanitation, is associated with increased risk for future CD development, in genetically susceptible individuals. In addition, the literature supports that the childhood environment plays an important role in the aetiology of CD. However, the lack of consistent findings between studies, particularly those which have broadly defined 'childhood' implies that timing of exposure plays a crucial role in this ever evolving paradigm.

The human gut offers an optimum environment for growth and proliferation of mutualistic microbes known as the gut microbiota (Ley RE *et al.* 2006; Whitman WB *et al.* 1998), which are critical in maintaining the well-being of the host (O'Hara AM and Shanahan F. 2006), by regulating the host's immunologic, metabolic and trophic functions (Hooper LV and Macpherson AJ. 2010; Stecher B and Hardt WD. 2011; Renz H *et al.* 2012). There is complex interaction between the host and gut microbiota which is vital for the maintenance of the gut homeostasis that is the development and regulation of the gut immune system (Chow J *et al*. 2010; Chung H and Kasper DL 2010; Hill DA and Artis D. 2009). Studies carried out on germ-free (GF) animals establish that the gut microbiota is requisite for the development of the gut mucosal immunity. It has been shown that microbiota-driven immune response can counteract the development of inappropriate inflammation. This therefore promotes the survival of the microbiota in the absence of unnecessary inflammation. Nevertheless, dysbiosis of the gut microbiota can lead to the development of immune-related disorders (Min YW and Rhee PL. 2015).

Place of Upbringing and Farm Animal Contact

The results of the present systematic review revealed notable findings on how the place of upbringing influences CD development in relation to contact with farm animals. There is an indication that there is greater risk of developing CD in people who have a more urban upbringing as compared to those with a rural background where there is livestock. Urban areas, in general, are places typified with high "hygiene" standards and less contact with farm animals. Exposure to farm animals would increase exposure to immunostimulatory antigens thereby programming the gastrointestinal (GI) immune system. As a result of that, we see consonance between city dwelling and limited exposure to farm animals in childhood as cogent causative agents of future development of CD. For instance, Hlavaty T *et al* revealed that being raised on a cattle farm specifically offers protection against the development of the disease (Hlavaty T *et al.* 2013). It is also important to note from the present results that, exposure to farm animals, regardless of the child's developmental stage from birth up to adolescence, appears to confer protection against the development of CD, as demonstrated in the study by Wulzermann JI *et al* (0-18 years) (Wurzelmann JI *et al.* 1994). On the other hand, the timing of this exposure is unclear, and it is possible that the protective effect reflects exposure either early in childhood, or late in childhood, or a combination of both. Therefore, it is interesting to observe the parallels between the study by Basson A and Gearry RB *et al*. Both studies examined the association within the 3 childhood age interval and both studies found no significant association for never having farm animal contact and place of upbringing below the age of 5 years (Basson A. 2014; Gearry RB *et al.* 2010). From the findings of the study performed by Hlavaty T *et al* that cattle specifically confer protection, it may be argued that Basson A. broadly grouped the farm animals which could have watered down the association in this age category (Hlavaty T *et al.* 2013). Had the study examined

the influence of the farm animals as individual species, the findings could have come out differently. It is important also to consider that below the age of 5 years, an individual is less mobile within the surroundings making both urban and farm dwellers to have almost the same blanket of protection from farm animal contact therefore making the influence of animal contact to be limited thus the weak association at this age. Although during the age interval 6-18 years, the study by Basson A showed no significant association between place of upbringing and CD development, the study confirmed an association between farm animal contact and the development of CD (Basson A. 2014). Specifically, during the age intervals 6-10 and 11-18 years, Basson A identified a significant association for never having a cow, horse, donkey or sheep living permanently on the property, including the identification of an independent risk association for the latter exposure during the interval 6-10 years. The latter may be explained by the fact that in Cape Town, South Africa (Western Cape), it is not unusual to find farm animals roaming 'freely' within urban areas, implying that the exposures to farm animals in both urban and rural areas, is somewhat uniform, thus limiting differences between the association with the variable.

Vice versa, while the study by Gearry RB *et al* showed a significant association between place of upbringing and CD development, it confirmed no association between farm animal contact and the eventual development of the disease (Gearry RB *et al.* 2010). It is of paramount importance to also note that Gearry RB *et al* defined contact with animals as 'more than 4 times per week' and this measurement of contact may have influenced the association (Gearry RB *et al.* 2010). Interaction with farm animals when defined as 'four times or more' versus 'four times or less' , for instance, could impact on these associations with regard to an individual and the protective effect conferred by the exposure.

Basson A also identified an independent risk association for never having consumed unpasteurised milk; it is likely that unpasteurized milk would increase antigen exposure, again supporting the hypothesis (Basson A. 2014). It is plausible that a significant association was not identified by Gearry RB *et al* because of the different livestock farming activity carried out in the place under study (Gearry RB *et al.* 2010). New Zealand is well known for sheep husbandry (Boutonnet JP. 1999) and the microbial interaction may be different from those exposed to cattle. It is also possible that the microbial component of unpasteurised sheep milk differs to that of cow milk. Notably, there has not been a single study showing exposure to farm animals increasing the risk of developing CD.

The method of categorization, along with the context in which an area is considered to be 'urban' or 'rural' may influence findings from studies that have identified an association between the place of upbringing and risk of CD (Basson A. 2014; Castiglione F *et al.* 2012; Malekzadeh F *et al.* 2009). For instance, in the South African context, the definition of city, suburb, town and village may differ compared to that in the European context. In addition, areas such as 'informal settlements' exist only in South Africa and this definition is not necessarily applicable in Europe and North America. Furthermore, some studies have categorised the settlements based on socioeconomic status, while others have considered only the size of the population. The other discrepancy is that some studies have been conducted in different regions of one nation, so although the categorization of a may be similar, there may be regional differences in the socioeconomic level between population groups. The other confounding factor worth mentioning is that the type of livestock varies between regions, including those present on individual farm. While some studies did examine the interaction on farms by clearly identifying the primary type of livestock farming activity conducted (Basson A. 2014; Bernstein CN *et al.* 2006; Timm S *et al.*

2014), others simply investigated the area of study as 'farm' (Hlavaty T *et al.* 2013). If the farm were to be a crop farm, this may potentially bias the outcome and contribute to inconsistent findings. Overall, despite the fact that some studies have found no significant association, there remains a general consensus that contact with farm animals is protective of the subsequent development of CD.

The study conducted by Timm S *et al.* 2014 also brings to light a different perspective with regard to urban dwelling. Smoking has been is a well-recognized risk factor for CD (Loftus EV. 2004; Somerville KW *et al.* 1984). The study revealed that, farm dwellers were predominantly non-smoking females, whereas city dwellers were predominantly males who smoked. It is worthy to note that city dwelling as a child has been associated with limiting the microbial exposure contact that may help stimulate an appropriate immune response, while at the same time increasing the risk of exposure to potent chemical agents such as cigarette smoke and other environmental pollutants. The subsequent development of CD would possibly due to a complex interaction of different risk factors, some being caused by exposure to them while others, being caused by lack of exposure to them.

Pets

Contact with pets has been generally accepted to be protective against developing CD. However findings from the studies on the association between exposure to pets and the subsequent development of CD have been largely inconsistent. There is evidence suggesting that exposure to pets, namely cats appears to exert a greater impact during early childhood (0-5 years). Bernstein CN *et al* reported exposure to cats prior to the age of 5 years, as protective against CD, although other studies have reported conflicting findings. For example, López-Serrano P *et al* found having

a pet significantly increasing the risk of developing CD and Castiglione F *et al* and Malekzadeh F *et al* found that exposure to pets was not significantly associated with CD, whereas Ng SC *et al* found having dogs decreased the risk and Hlavaty T *et al* found that not having cats and dogs was associated with an increased risk (Castiglione F *et al.* 2012; Malekzadeh F *et al.* 2009; Ng SC *et al.* 2014 (b); Hlavaty T *et al.* 2013; López-Serrano P *et al.* 2010; Bernstein CN *et al.* 2006). Notably, Bernstein CN *et al* also investigated the relationship between farm animal contact and found that CD patients had a lower likelihood of living on a farm, while not having farm animals was associated with an increased risk of CD. Taken together these findings may indicate that while exposure to cats is a contributing factor, it is not an independent risk factor. Thus the positive association with cats may be a reflection of a concomitant exposure to other farm animals, thus greatly affecting the outcome (Bernstein CN *et al.* 2006).

Intriguingly, the study by Han DY *et al* found that pet exposure significantly increased the risk for CD development, as this is in opposition to the majority of evidence and the hygiene hypothesis. Notably, Han DY *et al* only investigated CD risk and pet exposure and did not take into account other environmental factors; this may offer a potential explanation to their findings (Han DY *et al.* 2010). It is possible that having pet reflects a multifactorial interaction between various other environmental variables which were not investigated by the authors. Upon assessing the risk posed by exposure to pets, it is also imperative to quantify the amount of physical exposure associated with pets, or other animals on the property. It may be argued that the generalization of the pet exposure might have a great effect on the outcome; this also holds true for all exposure variables under investigation. In general, certain household pets, such as dogs and cats tend to have greater contact with humans, when compared with others, such as birds, rodents

and fish, which predominantly stay enclosed in cages. Such discrepancies may affect the outcome of the variable in question because of the pooling effect of the statistical analyses.

Water Source and Hot Water Availability

In line with the hygiene hypothesis, the availability of tap water was shown to be associated with the development of CD, particularly when exposure occurred below the age of 5 years. In addition, based on the studies included in the present review there appears to be a relationship between place of upbringing (rural, country and farm vs urban, suburban and city) and the availability of tap water. Furthermore, the notion that the programming of the immune system is associated with age-related intervals, including specific environmental exposures and antigens was supported in the report by Basson A. 2014 who identified a significant association between the availability of tap and the development of CD during the age intervals 0-5 and 6-10 years, but not during the age interval 11-18 years. According to Zinkernagel RM *et al.* 2001, the time soon before and after birth is crucial in understanding immunologic memory therefore exposure to environmental antigens helps creates this memory. It can therefore be inferred that immune system differentiation occurs during specific time periods, or 'developmental windows' during childhood, and that various microbial antigens may influence this intricate process. Similarly, the association between the availability of hot water and CD development was not consistently observed for all 3 of the age intervals. It is convincing to point that the availability of clean tap water entails an improved sanitary environment and this tends to dominate the influence of heated tap water. While studies that examined exposure in the broad 'childhood' sense have often revealed a significant association between availability of hot tap water and CD, it can be argued that the outcome is not representative of the actual time period when the risk exposure occurred and that it is a reflection

of a cumulative effect from multiple other environmental risk factors. In addition, availability of hot water may not necessarily equate to daily use of hot water. This may be particularly relevant if 'availability' has not been delineated in terms of whether hot water source originates from boiled water, or from a geyser, as well as frequency of use for the washing machines. Although this delineation was made in the study by Basson A, further studies are required to investigate this association.

Sibship Size

Contrary to common consensus, results of the present systematic review suggest that there is no association between sibship size and the risk of future CD development. All of the included studies failed to identify a significant association, including those which defined childhood by predefined age intervals. Notably however, it is likely that sibship size, as a single variable, does not influence CD development, and that it must be investigated in the context of multiple factors. For instance, many studies fail to examine sibship size together with other important household characteristics that may influence the 'microbial impact' of sibship size, such as total family size, number of bedrooms in the home, and number of bathrooms in the home. In addition, other important aspects, such as the type of toilet facility and bedroom sharing may have a direct impact on the absolute microbial contact of the individual. It is entirely plausible that many family members reside in one home; however each individual makes use of his or her own bedroom, as well as bathroom facilities. The latter household should not be compared to one, in which, fewer family members reside, yet where two or more individuals share bedrooms and multiple people may share a single bathroom facility. Thus, sibship size should not be considered in isolation, but rather in context to various household-related environmental factors. There are a number of possible other

confounding factors which are capable of greatly influencing the findings, such as preschool attendance, the regular use of swimming pools and other outdoor activities, surgical procedures (e.g., appendectomy, tonsillectomy), antibiotics use, vaccinations and childhood infections. While recall bias is often a strong limitation to the majority of the latter variables, overall, it appears that most studies fail to take into account these factors.

Epidemiological Study Design

Retrospective studies are typically engendered by a number of limitations when compared to prospective studies (Hess DR. 2004). Retrospective evaluation however allow easier evaluation of conditions which exhibit a long latency between exposure and disease, thus lend themselves for the generation of hypotheses, which can be evaluated in more timely prospective research. Of the studies included in this present systematic review, 90% were retrospective in nature. However, CD is typically characterised by extended periods of latency between the exposure and the disease (Virta L *et al.* 2012) making retrospective evaluation one of the best suited methods for examining possible aetiological factors involved in the development of CD.

Some of the disadvantages in retrospective evaluation include recall bias, unavailable 'missing' data, as well as the difficulties surrounding both the identification and control of potentially confounding factors (Hess DR. 2004). These factors are some of the limitations encountered by the present systematic review. Notably, any study that depends on a subject to recall information about a specific exposure is subject to recall bias. For example, studies which use a questionnaire to recall information such as dietary habits, childhood environmental exposures and medication use, are subject to recall bias. Retrospective studies predominantly rely on the recall of individuals, or the accuracy of written record, thus exposing them to potential recall bias (Hess DR. 2004).

However, it is possible that the recall bias pertaining to some information is lower than that of other information. For instance, when asking a 60 year old subject to recall information about his/her dietary habits at the age of 15, the recall bias is likely to be very high; but the same subject may easily and accurately recall information regarding the number of bedrooms in his/her home at age of 15, the number of people living in the home, types of pets, or number of people sharing a bathroom; for this information, the recall bias is likely to be lower. Notably, the same 60 year old subject may even be able to provide the latter type of information for his/her infant years, not because of a 'cognitive' memory during that time period, but because the subject has been made aware by family members, for example, of where he/she grew up, the type of home and how many siblings, relatives or other individuals were in the home; this may also be the case for information related to breastfeeding. Even if the subject does not personally recall this more 'factual' information, it may often be fairly accurately obtained, with the help of other family members. Nevertheless, recall bias is among the challenges encountered by many retrospective assessments with regards to environmental risk factors of CD. Such inconsistencies in methodology and other limitations surrounding retrospective studies however make it difficult to determine whether or not these inconsistencies actually represent the complex pathogenesis of CD.

Limitations

The primary limitation encountered during the critical appraisal process for the present systematic review was missing statistical data, including information pertaining to methodological approaches. For instance, some publications failed to include standard methodological information, such as; the age range of the subjects **(**Wurzelmann JI *et al.* 1994; Hampe J *et al.* 2003; Hansen TS *et al.* 2011; Malekzadeh F *et al.* 2009; Guo AY *et al.* 2014; Halfvarson J *et al.*

2006**),** the number of control subjects (Bernstein CN *et al.* 2006; Corrao G *et al.* 1998; Guo AY *et al.* 2014; Feeney MA *et al.* 2002); control selection (Guo AY *et al.* 2014), age categories pertaining to the relevant subjects under evaluation (Castiglione F *et al.* 2012; Hlavaty T *et al.* 2013; López-Serrano P *et al.* 2010; Malekzadeh F *et al.* 2009; Ng SC *et al.* 2014 (b); Corrao G *et al.* 1998; Guo AY *et al.* 2014; Hildebrand H *et al.* 2008**)** and the specific study centre (Gearry RB *et al.* 2010; Han DY *et al.* 2010; Hampe J *et al.* 2003; Persson PG *et al.* 1993). The second limitation was the number of variables included in the final systematic review in light of the staggering inconsistency between all identified studies with regard to the environmental variables investigated.

Conclusion:

It is critical to expose the body to early microbial sensitisation, thus stimulating it to develop an appropriate immune response (Francino MP. 2014). Modern "hygienic" lifestyles at birth and throughout early childhood tend to limit the exposure of the individual to microorganisms necessary for the programming and maturation of a healthy immune system therefore an individual becomes susceptible to subsequently develop disease (Stanwell-Smith R *et al.* 2012). It is of paramount importance to conduct investigation of the association of environmental risk factors with the development of CD, with a specific focus on defined age intervals during childhood. Assessing exposure at the different child developmental stage assists in rooting out diluting of the weight of the exposure variable under study and also aids in determining true interactions.

Tables Referred to in Chapter 4:

The following tables from this chapter are represented:

Table 6: Set of search entry terms

Table 7: Flow Diagram Results

Table 8: Data Extraction Tool

Table 9: Data Extraction Outcome

Table 10: Critical Appraisal Tool; Cohort Studies

Table 11: Critical Appraisal Tool; Case-control Studies

Table 6: Set of search entry terms

SET 1		SET 2		SET3
Inflammatory bowel disease Inflammatory bowel syndrome Regional ileitis Terminal ileitis Regional enteritis Granulomatous enteritis Crohn's disease Crohn's colitis Crohn's enteritis Crohn's ileitis Ulcerative colitis Colitis Gravis Proctocolitis	AND	Environment Environmental exposure Environmental factors Environmental Risk factors Childhood exposure Childhood risk factors Early exposure Early childhood Risk indicators	AND	Hygiene hypothesis

Table 7: Flow Diagram Results

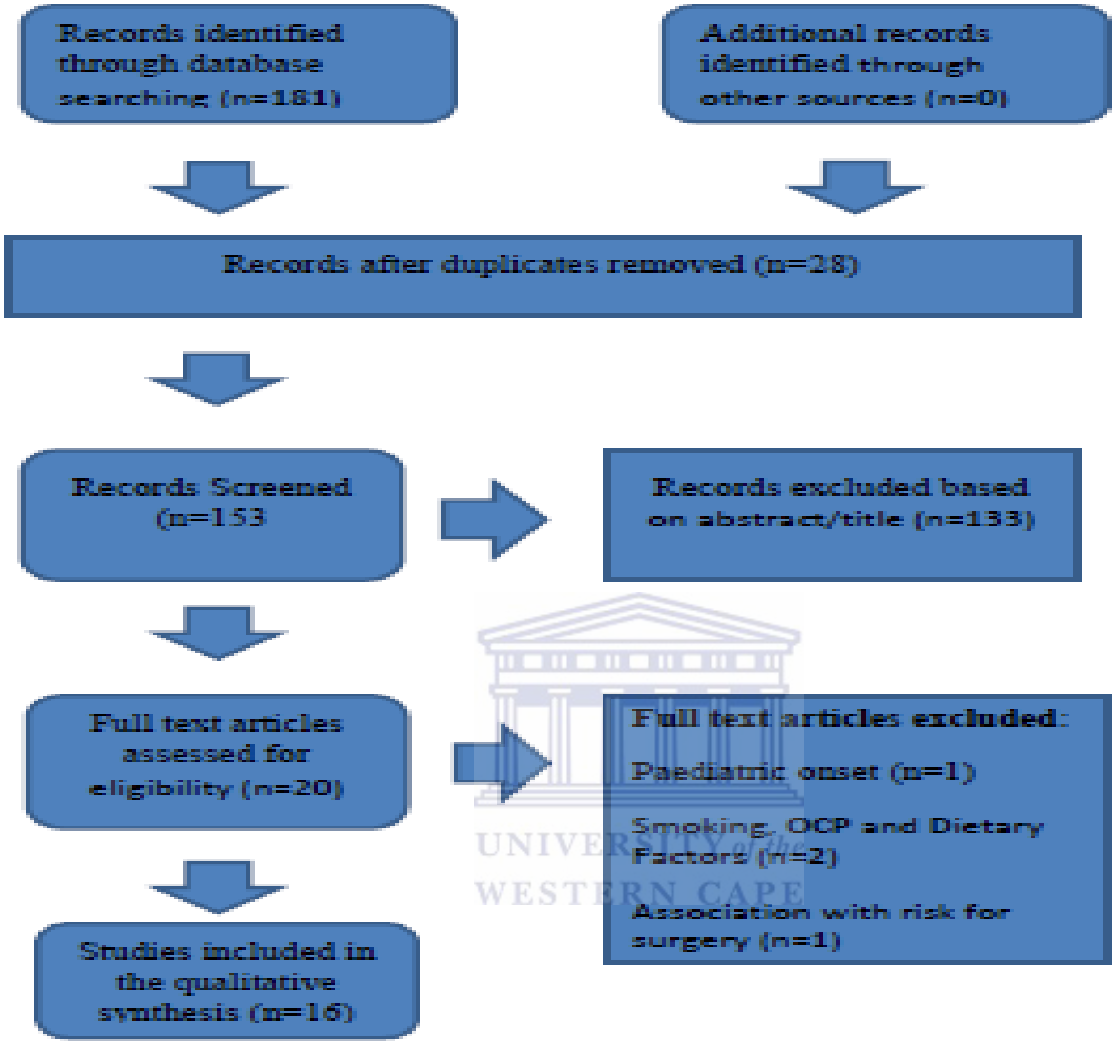

Table 8: Data Extraction Tool

Author	Study Centre	Study Design	Data Collection Methods	Methodological approach	Age intervals or range	Subjects (n=?)	Participant Selection	Environmental Variables	Results and analysis method	Score
A										
B										
C										
D										

Table 9: Data Extraction Outcome

Variables	Feeney	Timm	Wurzelmann	Gearry	Han	Bernstein	Hampe	Corrao	Hildebrand	Hlavaty	Hansen	Basson	Duggan	Malekzadeh	Persson	Guo	Lopez	Halfvarson	Castillogne	Ng	TOTAL
Breastfeeding	X	X	Y	Y	Y	Y	X	Y	X	Y	Y	Y	X	X	Y	Y	Y	X	Y	Y	13
Exposure to pets	Y	X	X	Y	Y	Y	X	X	X	Y	X	Y	X	Y	Y	Y	Y	Y	Y	Y	13
Farm animal contact	X	Y	X	Y	X	Y	X	Y	X	Y	X	Y	X	X	X	Y	X	X	X	X	7
General Childhood Infections	Y	X	Y	Y	X	Y	X	Y	Y	Y	Y	Y	X	X	Y	X	Y	Y	Y	Y	14
Helicobacter Pylori status	Y	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	X	X	X	X	2
Antibiotic use/regular medication	X	X	Y	Y	Y	Y	X	X	Y	X	X	X	Y	X	X	Y	X	Y	Y	Y	10
Surgical history	Y	X	Y	Y	X	Y	X	Y	X	Y	Y	X	Y	X	X	X	Y	Y	X	Y	11
Vaccination/Immunisation	X	X	X	Y	Y	Y	X	X	X	X	Y	X	X	X	X	X	X	Y	Y	Y	7
Childhood Hospitalisation	X	X	Y	X	X	X	X	X	X	X	X	X	X	X	X	Y	Y	X	X	X	3
Place of upbringing (Urban/Rural/Farm)	Y	Y	Y	Y	X	Y	Y	Y	X	Y	X	Y	X	Y	X	X	Y	X	Y	X	12
Family size/household residence	X	X	X	Y	X	Y	X	X	X	Y	X	Y	Y	X	X	X	X	X	Y	X	6
Sibship size	Y	X	Y	Y	Y	Y	Y	X	X	Y	X	X	X	Y	Y	X	Y	X	Y	X	11
Sib rank	Y	X	X	Y		Y	Y	X	X	X	X	X	X	X	X	X	X	X			4
Water source (tap water)	X	X	Y	Y	X	Y	Y	X	X	X	Y	Y	Y	Y	X	X	Y	X	X	Y	10
Bathroom sharing	X	X	X	X	X	X	X	X	X	X	X	Y	Y	Y	X	X	X	X	X	X	3
Toilet facility	Y	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	X	X	X	Y	3
Heated water	Y	X	X	Y	X	X	Y	X	X	X	X	Y	Y	Y	X	X	X	X	X	Y	7
Cigarette smoke exposure	Y	Y	X	Y	Y	Y	X	Y	X	X	X	Y	X	X	X	Y	X	X	X	X	8
Preschool/daycare attendance	Y	X	X	X	Y	X	X	X	X	X	X	Y	Y	X	X	Y	X	X	X	X	5
Physical activity	X	X	X	X	X	X	X	Y	X	Y	X	X	X	X	X	X	X	X	Y	X	4
Use of swimming pool	Y	X	X	Y	Y	X	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	4
Home refrigerator	X	X	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	X	X	X	1
Sand pit	X	X	X	Y	Y	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	2
Person to room ration in home	Y	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1
Number of house moves	Y	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	X	X	X	X	2
Number of family cars/Parents owned a car	Y	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	Y	X	X	X	3
Month and season of birth	X	X	Y	X	X	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	2
Feeding a pet	X	X	X	X	Y	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1
Having vegetable garden	X	X	X	Y	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1
Ethnicity	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Drinking unpasteurised milk	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	X	X	X	X	X	1
Eating chicken	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Type of toilet facility	X	X	X	X	X	X	X	X	X	X	X	Y	Y	X	X	X	X	X	X	X	2
Raw beef consumption	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	X	X	X	X	X	1
Home shared with other family	X	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	X	X	X	X	1
Central heating installed	X	X	X	Y	X	X	X	X	X	X	X	X	Y	Y	X	X	X	X	X	X	3
Delivery via caesarean section	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	X	1
Family income	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	1
Travelling abroad	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	1
Exposure to Allergens	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	1
Dental care history	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	Y	X	1
Proportion of carpeting in the home	X	X	X	X	Y	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	1
Parental occupation	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	Y	X	X	X	1

Table 10: Critical Appraisal Tool; Cohort Studies

SIGN	**Methodology Checklist 3: Cohort studies**

Study identification (*Include author, title, year of publication, journal title, pages*)

Guideline topic:	Key Question No:	Reviewer:

Before completing this checklist, consider:

1. Is the paper really a cohort study? If in doubt, check the study design algorithm available from SIGN and make sure you have the correct checklist.
2. Is the paper relevant to key question? Analyse using PICO (Patient or Population Intervention Comparison Outcome). IF NO REJECT (give reason below). IF YES complete the checklist.

Reason for rejection: 1. Paper not relevant to key question ☐ 2. Other reason ☐ (please specify):

Please note that a retrospective study (i.e. a database or chart study) cannot be rated higher than +.

Section 1: Internal validity

In a well conducted cohort study:		*Does this study do it?*
1.1	The study addresses an appropriate and clearly focused question.[i]	Yes ☐ No ☐ Can't say ☐
SELECTION OF SUBJECTS		
1.2	The two groups being studied are selected from source populations that are comparable in all respects other than the factor under investigation.[ii]	Yes ☐ No ☐ Can't say ☐ Does not apply ☐

1.3	The study indicates how many of the people asked to take part did so, in each of the groups being studied.[iii]	Yes □ No □ Does not apply □
1.4	The likelihood that some eligible subjects might have the outcome at the time of enrolment is assessed and taken into account in the analysis.[iv]	Yes □ No □ Can't say □ Does not apply □
1.5	What percentage of individuals or clusters recruited into each arm of the study dropped out before the study was completed.[v]	
1.6	Comparison is made between full participants and those lost to follow up, by exposure status.[vi]	Yes □ No □ Can't say □ Does not apply □
ASSESSMENT		
1.7	The outcomes are clearly defined.[vii]	Yes □ No □ Can't say □
1.8	The assessment of outcome is made blind to exposure status. If the study is retrospective this may not be applicable.[viii]	Yes □ No □ Can't say □ Does not apply □
1.9	Where blinding was not possible, there is some recognition that knowledge of exposure status could have influenced the assessment of outcome.[ix]	Yes □ No □ Can't say □ □
1.10	The method of assessment of exposure is reliable.[x]	Yes □ No □ Can't say □

1.11	Evidence from other sources is used to demonstrate that the method of outcome assessment is valid and reliable.[xi]	Yes □ No □ Can't say □ Does not apply□
1.12	Exposure level or prognostic factor is assessed more than once.[xii]	Yes □ No □ Can't say □ Does not apply □
CONFOUNDING		
1.13	The main potential confounders are identified and taken into account in the design and analysis.[xiii]	Yes □ No □ Can't say □
STATISTICAL ANALYSIS		
1.14	Have confidence intervals been provided?[xiv]	Yes □ No □
SECTION 2: OVERALL ASSESSMENT OF THE STUDY		
2.1	How well was the study done to minimise the risk of bias or confounding?[xv]	High quality (++) □ Acceptable (+) □ Unacceptable – reject 0
2.2	Taking into account clinical considerations, your evaluation of the methodology used, and the statistical power of the study, do you think there is clear evidence of an association between exposure and outcome?	Yes ☐ No ☐ Can't say ☐
2.3	Are the results of this study directly applicable to the patient group targeted in this guideline?	Yes □ No □
2.4	**Notes.** Summarise the author's conclusions. Add any comments on your own assessment of the study, and the extent to which it answers your question and mention any areas of uncertainty raised above.	

Unless a clear and well defined question is specified in the report of the review, it will be difficult to assess how well it has met its objectives or how relevant it is to the question you are trying to answer on the basis

of the conclusions.

This relates to selection bias.* It is important that the two groups selected for comparison are as similar as possible in all characteristics except for their exposure status, or the presence of specific prognostic factors or prognostic markers relevant to the study in question.

This relates to selection bias.* The participation rate is defined as the number of study participants divided by the number of eligible subjects, and should be calculated separately for each branch of the study. A large difference in participation rate between the two arms of the study indicates that a significant degree of selection bias* may be present, and the study results should be treated with considerable caution.

If some of the eligible subjects, particularly those in the unexposed group, already have the outcome at the start of the trial the final result will be subject to performance bias.* A well conducted study will attempt to estimate the likelihood of this occurring, and take it into account in the analysis through the use of sensitivity studies or other methods.

This question relates to the risk of attrition bias.*The number of patients that drop out of a study should give concern if the number is very high. Conventionally, a 20% drop out rate is regarded as acceptable, but in observational studies conducted over a lengthy period of time a higher dropout rate is to be expected. A decision on whether to downgrade or reject a study because of a high dropout rate is a matter of judgement based on the reasons why people dropped out, and whether dropout rates were comparable in the exposed and unexposed groups. Reporting of efforts to follow up participants that dropped out may be regarded as an indicator of a well conducted study.

For valid study results, it is essential that the study participants are truly representative of the source population. It is always possible that participants who dropped out of the study will differ in some significant way from those who remained part of the study throughout. A well conducted study will attempt to identify any such differences between full and partial participants in both the exposed and unexposed groups. This relates to the risk of attrition bias.* Any unexplained differences should lead to the study results being treated with caution.

This relates to the risk of detection bias.* Once enrolled in the study, participants should be followed until specified end points or outcomes are reached. In a study of the effect of exercise on the death rates from heart disease in middle aged men, for example, participants might be followed up until death, or until reaching a predefined age. If outcomes and the criteria used for measuring them are not clearly defined, the study should be rejected.

This relates to the risk of detection bias.* If the assessor is blinded to which participants received the exposure, and which did not, the prospects of unbiased results are significantly increased. Studies in which this is done should be rated more highly than those where it is not done, or not done adequately.

This relates to the risk of detection bias.* Blinding is not possible in many cohort studies. In order to assess the extent of any bias that may be present, it may be helpful to compare process measures used on the participant groups - e.g. frequency of observations, who carried out the observations, the degree of detail and completeness of observations. If these process measures are comparable between the groups, the results may be regarded with more confidence.

This relates to the risk of detection bias.* A well conducted study should indicate how the degree of exposure or presence of prognostic factors or markers was assessed. Whatever measures are used must be sufficient to establish clearly that participants have or have not received the exposure under investigation and the extent of such exposure, or that they do or do not possess a particular prognostic marker or factor. Clearly described, reliable measures should increase the confidence in the quality of the study

This relates to the risk of detection bias.* The primary outcome measures used should be clearly stated in the study. If the outcome measures are not stated, or the study bases its main conclusions on secondary

outcomes, the study should be rejected. Where outcome measures require any degree of subjectivity, some evidence should be provided that the measures used are reliable and have been validated prior to their use in the study.

This relates to the risk of detection bias.* Confidence in data quality should be increased if exposure level is measured more than once in the course of the study. Independent assessment by more than one investigator is preferable.

Confounding is the distortion of a link between exposure and outcome by another factor that is associated with both exposure and outcome. The possible presence of confounding factors is one of the principal reasons why observational studies are not more highly rated as a source of evidence. The report of the study should indicate which potential confounders have been considered, and how they have been assessed or allowed for in the analysis. Clinical judgement should be applied to consider whether all likely confounders have been considered. If the measures used to address confounding are considered inadequate, the study should be downgraded or rejected, depending on how serious the risk of confounding is considered to be. A study that does not address the possibility of confounding should be rejected.

Confidence limits are the preferred method for indicating the precision of statistical results, and can be used to differentiate between an inconclusive study and a study that shows no effect. Studies that report a single value with no assessment of precision should be treated with extreme caution.

Rate the overall methodological quality of the study, using the following as a guide: High quality (++): Majority of criteria met. Little or no risk of bias. Results unlikely to be changed by further research. Acceptable (+): Most criteria met. Some flaws in the study with an associated risk of bias, Conclusions may change in the light of further studies. Low quality (0): Either most criteria not met, or significant flaws relating to key aspects of study design. Conclusions likely to change in the light of further studies.

Table 11: Critical Appraisal Tool; Case-control Studies

SIGN	**Methodology Checklist 4: Case-control studies**		
Study identification (*Include author, title, year of publication, journal title, pages*)			
Guideline topic:		Key Question No:	Reviewer:
Before completing this checklist, consider: 1. Is the paper really a case-control study? If in doubt, check the study design algorithm available from SIGN and make sure you have the correct checklist. 2. Is the paper relevant to key question? Analyse using PICO (Patient or Population Intervention Comparison Outcome). IF NO REJECT (give reason below). IF YES complete the checklist.			
Reason for rejection: Reason for rejection: 1. Paper not relevant to key question ☐ 2. Other reason ☐ (please specify):			
Section 1: Internal validity			
In an well conducted case control study:			*Does this study do it?*
1.1	The study addresses an appropriate and clearly focused question.[xvi]		Yes ☐ No ☐ Can't say ☐
SELECTION OF SUBJECTS			
1.2	The cases and controls are taken from comparable populations.[xvii]		Yes ☐ No ☐ Can't say ☐
1.3	The same exclusion criteria are used for both cases and controls.[xviii]		Yes ☐ No ☐ Can't say ☐
1.4	What percentage of each group (cases and controls) participated in the study?[xix]		Cases: Controls:

1.5	Comparison is made between participants and non-participants to establish their similarities or differences.[xx]	Yes ☐ No ☐ Can't say ☐
1.6	Cases are clearly defined and differentiated from controls.[xxi]	Yes ☐ No ☐ Can't say ☐
1.7	It is clearly established that controls are non-cases.[xxii]	Yes ☐ No ☐ Can't say ☐
ASSESSMENT		
1.8	Measures will have been taken to prevent knowledge of primary exposure influencing case ascertainment.[xxiii]	Yes ☐ No ☐ Can't say ☐ Does not apply ☐
1.9	Exposure status is measured in a standard, valid and reliable way.[xxiv]	Yes ☐ No ☐ Can't say ☐
CONFOUNDING		
1.10	The main potential confounders are identified and taken into account in the design and analysis.[xxv]	Yes ☐ No ☐ Can't say ☐
STATISTICAL ANALYSIS		
1.11	Confidence intervals are provided.[xxvi]	Yes ☐ No ☐
SECTION 2: OVERALL ASSESSMENT OF THE STUDY		
2.1	How well was the study done to minimise the risk of bias or confounding? [xxvii]	High quality (++) ☐

		Acceptable (+) □ Unacceptable – reject 0 □	
2.2	Taking into account clinical considerations, your evaluation of the methodology used, and the statistical power of the study, do you think there is clear evidence of an association between exposure and outcome?	Yes ☐ Can't say ☐	No ☐
2.3	Are the results of this study directly applicable to the patient group targeted by this guideline?	Yes ☐	No ☐
2.4	**Notes.** Summarise the authors' conclusions. Add any comments on your own assessment of the study, and the extent to which it answers your question and mention any areas of uncertainty raised above.		

Unless a clear and well defined question is specified in the report of the review, it will be difficult to assess how well it has met its objectives or how relevant it is to the question you are trying to answer on the basis of the conclusions.

Study participants may be selected from the target population (all individuals to which the results of the study could be applied), the source population (a defined subset of the target population from which participants are selected), or from a pool of eligible subjects (a clearly defined and counted group selected from the source population. If the study does not include clear definitions of the source population it should be rejected.

All selection and exclusion criteria should be applied equally to cases and controls. Failure to do so may introduce a significant degree of bias into the results of the study.

Differences between the eligible population and the participants are important, as they may influence the validity of the study. A participation rate can be calculated by dividing the number of study participants by the number of eligible subjects. It is more useful if calculated separately for cases and controls. If the participation rate is low, or there is a large difference between the two groups, the study results may well be invalid due to differences between participants and non-participants. In these circumstances, the study should be downgraded, and rejected if the differences are very large.

Even if participation rates are comparable and acceptable, it is still possible that the participants selected to act as cases or controls may differ from other members of the source population in some significant way. A well conducted case-control study will look at samples of the non-participants among the source population to ensure that the participants are a truly representative sample.

The method of selection of cases is of critical importance to the validity of the study. Investigators have to be certain that cases are truly cases, but must balance this with the need to ensure that the cases admitted into the study are representative of the eligible population. The issues involved in case selection are complex, and should ideally be evaluated by someone with a good understanding of the design of case-control studies. If the study does not comment on how cases were selected, it is probably safest to

reject it as a source of evidence.

Just as it is important to be sure that cases are true cases, it is important to be sure that controls do not have the outcome under investigation. Control subjects should be chosen so that information on exposure status can be obtained or assessed in a similar way to that used for the selection of cases. If the methods of control selection are not described, the study should be rejected. If different methods of selection are used for cases and controls the study should be evaluated by someone with a good understanding of the design of case-control studies.

If there is a possibility that case ascertainment can be influenced by knowledge of exposure status, assessment of any association is likely to be biased. A well conducted study should take this into account in the design of the study.

The primary outcome measures used should be clearly stated in the study. If the outcome measures are not stated, or the study bases its main conclusions on secondary outcomes, the study should be rejected. Where outcome measures require any degree of subjectivity, some evidence should be provided that the measures used are reliable and have been validated prior to their use in the study.

Confounding is the distortion of a link between exposure and outcome by another factor that is associated with both exposure and outcome. The possible presence of confounding factors is one of the principal reasons why observational studies are not more highly rated as a source of evidence. The study should indicate which potential confounders have been considered, and how they have been allowed for in the analysis. Clinical judgement should be applied to consider whether all likely confounders have been considered. If the measures used to address confounding are considered inadequate, the study should be downgraded or rejected. A study that does not address the possibility of confounding should be rejected.

Confidence limits are the preferred method for indicating the precision of statistical results, and can be used to differentiate between an inconclusive study and a study that shows no effect. Studies that report a single value with no assessment of precision should be treated with extreme caution.

Rate the overall methodological quality of the study, using the following as a guide: High quality (++): Majority of criteria met. Little or no risk of bias. Results unlikely to be changed by further research. Acceptable (+): Most criteria met. Some flaws in the study with an associated risk of bias, Conclusions may change in the light of further studies. Low quality (0): Either most criteria not met, or significant flaws relating to key aspects of study design. Conclusions likely to change in the light of further studies.

CHAPTER 5: ENVIRONMENTAL EXPOSURES DURING CHILDHOOD AND THE SUBSEQUENT DEVELOPMENT OF CROHN'S DISEASE IN THE WESTERN CAPE, SOUTH AFRICA

5.1 Background

While the aetiology of CD remains unclear, several theories have emanated in a bid to understand the underlying pathogenesis of the disease. The most widely accepted is the 'hygiene hypothesis' and the theory has received much academic speculation and attention. Numerous studies have been conducted on the basis of the hygiene hypothesis, including the recent case control investigation conducted in the 2 largest tertiary referral IBD centres in Cape Town, South Africa by Basson A. (Basson A. 2014). The methodological approaches of the study were previously described in Chapter 2.

5.2 Aim and Objectives

Aim

The aim of the present study was to investigate the association between childhood environmental exposures and the risk of CD development in the Western Cape, South Africa based on a score analysis of a subset of data collected from the aforementioned completed study performed by Basson A. 2014.

Objectives

The objectives of the study were:

1. To perform a score analysis for the environmental exposures during the three age intervals; 0-5, 6-10 and 11-18 years
2. To investigate a number of childhood infections between the ages 0-20 years based on original data from a case control study design
3. To perform a score analysis on data pertaining to childhood infections between the ages 0-20 years
4. To compare the difference in score analysis between case and control subjects for objectives 1 and 2 above

5.3 Materials and Methods

5.3.1 Study Data

The results from Basson A of the multiple logistic regression analysis evaluating environmental risk factor exposure in 3 age groups (0-5 years, 6-10 years and 11-18 years) are shown in Table 12. Fourteen environmental exposures were investigated, these include: Primary source of drinking water, Hot piped tap water, Community type, Total number of people in household, Number of people sharing a bathroom, Number of bedrooms in home, Type of toilet facility, Household pets, Donkey/horse/cow/sheep on property, Cigarette smoke exposure, Unpasteurized milk consumption, Raw beef consumption, Helminth infection, Treatment for helminths.

5.3.1.1 Methodology

Environmental Exposures for the age intervals (0-5 years, 6-10 years and 11-18 years)

To evaluate the environmental exposures during the 3 age intervals based on a score analysis, the fourteen environmental exposure variables (presented in Table 12) three different approaches were undertaken. In the first analysis approach, all variables were assigned a weighting score value equal to one. In the second and third analytical approach, two Groups were formed: Group A and Group B. While both Group A and Group B consisted of all fourteen exposure variables, the subgrouping of exposure variables differed between Group A and Group B. Methodology for subgrouping: environmental exposure variables of similar nature were combined in 'subgroups' within Group A and B. For instance, within Group A, the environmental exposure variables 'water source' and 'hot water availability' were grouped together, to form one subgroup within Group A, while the exposure variables 'number of people in the household', 'number of bedrooms in the household' and 'pets in the home' were combined to form another subgroup within Group A. The weighting for each individual subgroup was equal to the value of one. Thus, the weighting for each individual exposure variable with the subgroups was dependent upon the number of variables within the subgroup. For instance, the weighting of each variable in the latter subgroup ('number of people in the household', 'number of bedrooms in the household' and 'pets in the home') was 1/3 + 1/3 + 1/3, whereas the weighting for the former subgroup ('water source' and 'hot water') was 1/2 + 1/2. The grouping of exposure variables has been explained under Point 5.3.2, and the weighting of subgroups with Group A and Group B are shown in Table 13 and Table 14, respectively.

The two different Groups (A and B) were created in an attempt to compare the difference in the

effect between the variations in weighting for the exposure variables within subgroups. It must be noted that for both group A and group B, the exposure variables 'never having consumed unpasteurized milk' and 'passive cigarette smoke exposure' were not combined with other exposures and their individual score remained equal to the value of one. As this component of the study was a post-hoc analysis of published data, this weighting was determined based on several factors, namely; 1) a significant association was identified for unpasteurized milk during all 3 age intervals, including the identification of an independent risk association for 6-10 and 11-18 years, and 2) a significant association was identified for passive cigarette smoke exposure during 2 age intervals, including the identification of an independent risk association for 11-18 years, as well as that smoking is a well-established risk factor in CD.

Group A and B consisted of individual subgroups and the lowest possible weighting within any subgroup was 1/5th, thus a subgroup consisted of no more than 5 exposure variables. Since the 'score value' for every subgroup was equal to the value of one, the total possible score for Group A and B was equivalent to the total number of subgroups contained within that group. For Group A, the total possible score was 8, and for Group B, the total possible score was five. A score analysis was performed based on these premises. The subgroups within Group A and Group B, respectively, were statistically analysed based on their single (i.e. unpasteurized milk) or pooled weighted score.

Comparisons were performed between the cases and the controls using these predefined Groups (A and B), and the score analysis for environmental risk factors was performed over the 3 childhood age intervals (0-5, 6-10 and 11-18 years). For each analysis (0-5, 6-10 and 11-18 years), score results have been represented as minimum, maximum, mean and median values for the case and control groups. The Odds Ratios and 95%CIs represents the significance of the difference

between the two groups, as well as the difference in proportion with regard to number of exposures based on the score.

Frequency of Childhood Infections between ages 0-20 years

While a similar methodological approach was used for the frequency of childhood infections, no grouping of exposure variables was performed (refer to Point 5.3.2.2; Objective 2).

5.3.2 Score Analysis: Grouping of Variables

5.3.2.1 Objective 1

Group A: Environmental Exposure Subgroups

Below represents the subgroups comprised within Group A (Table 13):

- Source of drinking water (Not piped) + Hot water source (No access)
- Number of people in home (Less than 3) + Number of bedrooms in home (3 or more) + pets in home
- Number of people sharing a bathroom (Less than 3) + Type of toilet facility (Bucket, pit latrine or no facility)
- Raw beef consumption (once per year or more)
- Unpasteurized milk consumption (once per year or more)
- Helminth infection (not exposed) + Treatment for helminths
- Community type (Suburban or Urban) + Donkey/horse/sheep/cow living permanently on property (not exposed)

- Passive Cigarette smoke exposure (exposure)

Group B: Environmental Exposure Subgroups

Below represents the subgroups comprised within Group B (Table 14):

- Source of drinking water (Not piped) + Hot water (No access) + Raw beef consumption (once per year or more)
- Number of people in a home (Less than 3) + Number of bedrooms in a home (3 or more) + Number of people sharing a bathroom (Less than 3) + Type of toilet facility (Bucket, pit latrine or no facility) + pets in home
- Community type (Suburban or Urban) + Donkey/horse/sheep/cow living permanently on property (no exposure) + Helminth infection (no exposure) + treatment for helminths
- Unpasteurized milk consumption (once per year or more)
- Passive Cigarette smoke exposure (exposure)

5.3.2.2 Objective 2

Frequency of Childhood Infections between Ages of 0-20 years

A subset of unused, unpublished data from the original research study focusing on childhood infections during the age intervals 0-12 and 12-20 years was used. Regarding the frequency of childhood infections during the age intervals 0-12 years and 13-20 years, the data was combined, and evaluated as 0-20 years, because of the small cell size of some variables. The standard for significance for all analysis was $P < 0.05$.

The frequency of 12 variables during the defined age intervals were evaluated on the basis of either ‘never’ or ‘once or more per year’, these included; tooth decay/cavity; periodontitis; sore

throat; respiratory infection; diarrhoea; gastritis; mouth ulcers; perianal disease; arthritis/arthralgia; eye disease (not myopia, hypermetropia, astigmatism or presbyopia); use of antibiotics; and the use of probiotics with or after antibiotic pills. For this score analysis, no grouping was performed and all variables were equal to the value of one. The reason for this is because the data collected was not supported by medical records and it was felt that recall bias was a strong limitation to the data.

5.3.2.3 Score Analysis; Statistical Approach

Demographic data for the cases and controls is presented as frequencies (percentages) for the childhood infection and as medians, and as medians (interquartile range (IQR)) and mean (standard deviation (SD)) for the environmental exposure variables (environmental risk factors). The score analysis was conducted for the environmental exposures over the three age intervals using logistics regression. The three age intervals were between 0 to 18 years (0-5 years, 6-10 years and 11-18 years) for environmental exposures. The logistics regression was also used to conduct the score analysis for the frequency of childhood infections between ages 0 to 20 years old.

5.4 Results

5.4.1 Score analysis for environmental risk factors during the 3 age intervals

The results of the score analysis evaluating environmental risk factor exposure in 3 age groups (0-5 years, 6-10 years and 11-18 years) are shown in Table 15. In this model, all exposures were equal to the value of one, thus the total possible score was 14, given that there were 14 variables (Table 12).

0-5 years

During the age interval 0-5 years, the mean and median scores for the case and control group were [4.39 (SD ± 1.93) vs 4.71 (SD ± 1.98); and 4.0 (IQR 3.0-5.0) vs 4.0 (IQR 3.0-6.0)], respectively. Both groups had a minimum score of zero, whereas cases had a maximum score of 10 and controls had a maximum score of 12. There was no significant difference in the exposure scores between the case and control groups (OR = 0.92; 95% CI, 0.83-1.02).

6-10 years

During the age interval 6-10 years, the mean and median scores for the case and control group were [4.39 (SD ± 1.80) vs 4.60 (SD ± 1.92); and 4.0 (IQR 3.0-6.0) vs 4.0 (IQR 3.0-6.0)], respectively. Both groups had a minimum score of zero, whereas cases had a maximum score of 9 and controls had a maximum score of 11. There was no significant difference in the exposure scores between the case and control groups (OR = 0.94; 95% CI, 0.80-1.02).

11-18 years

During the age interval 11-18 years, the mean and median scores for the case and control group were [3.77 (SD ± 1.54) vs 4.02 (SD ± 1.65); and 4.0 (IQR 3.0-5.0) vs 4.0 (IQR 3.0-5.0)], respectively. The minimum and maximum scores for cases were 0 and 9, respectively, whereas the minimum and maximum scores for controls were 1 and 9, respectively. There was no significant difference in the exposure scores between the case and control groups (OR = 0.90; 95% CI, 0.80-1.02).

5.4.2 Score Analysis for Environmental Risk Factors during the three Age Intervals: Group A and B

5.4.2.1 Group A

The results of the score analysis evaluating environmental risk factor exposure in 3 age groups (0-5 years, 6-10 years and 11-18 years) for Group A are shown in Table 16.

0-5 years

During the age interval 0-5 years, the mean and median scores for the case and control group were [2.08 (SD ± 0.98) vs 3.58 (SD ± 1.12); and 1.99 (IQR 1.33-2.66) vs 2.16 (IQR 1.66-3.16)], respectively. Both groups had a minimum score of zero, whereas cases had a maximum score of 5.16 and controls had a maximum score of 6.46. The maximum possible score for Group A was 8. There was a significant difference in exposure scores between the case and control groups (OR = 0.74; 95% CI, 0.62-0.92), thus indicating that cases had 26% less exposure during this age interval when compared to the controls.

6-10 years

During the age interval 6-10 years, the mean and median scores for the case and control group were [1.30 (SD ± 0.79) vs 1.64 (SD ± 1.03); and 1.50 (IQR 0.5-2.0) vs 1.50 (IQR 1.0-2.5)], respectively. Both groups had a minimum score of zero, whereas cases had a maximum score of 3.5 and controls had a maximum score of 6. There was a significant difference in exposure scores between the case and control groups (OR = 0.67; 95% CI, 0.53-0.83), thus indicating that cases

had 33% less exposure during this age interval when compared to the controls.

11-18 years

During the age interval 11-18 years, the mean and median scores for the case and control group were [1.81 (SD ± 0.85) vs 2.08 (SD ± 0.98); and 1.66 (IQR 1.16-2.33) vs 1.83(IQR 1.33-2.08)], respectively. The minimum and maximum scores for cases were 0 and 4.66, respectively, whereas the minimum and maximum scores for controls were 0.33 and 5.16, respectively. There was a significant difference in exposure scores between the case and control groups (OR = 0.72; 95% CI, 0.58-0.90), thus indicating that cases had 28% less exposure during this age interval when compared to the controls.

5.4.2.2 Group B

The results of the score analysis evaluating environmental risk factor exposure in 3 age groups (0-5 years, 6-10 years and 11-18 years) for Group B are shown in Table 17.

0-5 years

During the age interval 0-5 years, the mean and median scores for the case and control group were [1.20 (SD ± 0.63) vs 1.47 (SD ± 0.77); and 1.10 (IQR 0.73-1.60) vs 1.38 (IQR 0.85-1.98)], respectively. Both groups had a minimum score of zero, whereas cases had a maximum score of 3.13 and controls had a maximum score of 3.93. The maximum possible score for Group B was 5. There was a significant difference in exposure scores between the case and control groups (OR = 0.72; 95% CI, 0.58-0.90), thus indicating that cases had 42% less exposure during this age interval when compared to the controls.

6-10 years

During the age interval 6-10 years, the mean and median scores for the case and control group were [1.22 (SD ± 0.60) vs 1.33 (SD ± 0.78); and 1.19 (IQR 0.73-1.60) vs 1.33 (IQR 0.81-1.98)], respectively. Both groups had a minimum score of zero, whereas cases had a maximum score of 3.06 and controls had a maximum score of 4.26. There was a significant difference in exposure scores between the case and control groups (OR = 0.58; 95% CI, 0.43-0.78), thus indicating that cases had 42% less exposure during this age interval when compared to the controls.

11-18 years

During the age interval 11-18 years, the mean and median scores for the case and control group were [1.08 (SD ± 0.60) vs 1.31 (SD ± 0.71); and 0.93 (IQR 0.64-1.41) vs 1.19(IQR 0.73-1.80)], respectively. The minimum and maximum scores for cases were 0 and 3.13, respectively, whereas the minimum and maximum scores for controls were 0.2 and 3.63, respectively. There was a significant difference in exposure scores between the case and control groups (OR = 0.59; 95% CI, 0.43-0.79), thus indicating that cases had 41% less exposure during this age interval when compared to the controls.

5.4.3 Frequency of childhood infections between ages of 0-20 years

The results of the multiple logistic regression analysis evaluating frequency of infections before the age of 20 years are shown in Table 18. On multiple logistic regression analysis, subjects who never had tooth decay/cavity (OR = 1.78; 95% CI, 1.05-3.04), periodontitis (OR = 1.95; 95% CI, 1.10, 3.48), diarrhoea (OR = 2.71; 95% CI, 1.62-4.62), gastritis (OR = 2.13; 95% CI, 1.30-3.35),

or mouth ulcers (OR = 2.02; 95% CI, 1.12-3.70), at least once per year or more, were at an increased risk for later development of CD, when compared to those who were exposed to these infections at least once per year or more.

5.4.4 Score Analysis for the Frequency of Childhood Infections; 0-20 years

The results of the score analysis evaluating childhood infections during the age interval 0-20 years, is shown in Table 19. The mean and median scores for the case and control group were [6.43(SD ± 1.54) vs 7.54 (SD ± 3.26); and 7.0 (IQR 4.8-8.3) vs 8.0 (IQR 6.0-10.0)], respectively. Both groups had a minimum score of zero, as well as a maximum score of 12. The total possible score for this analysis was 12. There was a significant difference in exposure scores between the case and control groups (OR = 0.88; 95% CI, 0.82-0.94), thus indicating that cases had 12% less exposure to childhood infections from birth until the age of 20 years, when compared to the controls.

5.5 Discussion

The enteric flora is comprised of numerous microorganisms and the history of CD is “paved with” publications hypothesizing specific infectious agents behind the aetiology of CD. However the majority of studies have only focused on select environmental exposures during childhood, without accounting for the numerous potential confounding interactions which may compromise the true validity of what is being measured. Using a subset of previously collected data, the present study conducted a both a post-hoc score analysis, as well as a primary score analysis in an attempt to investigate the latter hypothesis, together with the paradigm of the hygiene hypothesis.

Results of the score analysis for the environmental exposures during 3 age intervals (0-5, 6-10 and 11-18 years) revealed no significant difference between the case and control groups (Table 15). Notably, all exposure variables were equal to a value of one, thus frequency of exposure was based on the individual effect of each variable. By contrast, a significant difference was observed during all 3 age intervals between the case and control groups, within both Group A and Group B, with cases having significantly lower exposure scores (approximately 30% and 40% lower, respectively), when compared with that of controls. These findings are in support of the hygiene hypothesis, and implicate the complex role in which multiple microbial-based environmental exposures function in the development of the intestinal immune system. The latter is further supported by the change in both the mean values and CIs observed between Group A and Group B, in that, during all 3 age intervals, the difference in mean values between the cases and controls in Group A, is consistently higher compared to the difference in mean values for cases and controls in Group B, as well as that smaller CIs are consistently observed for Group A, when compared with Group B, during all 3 age intervals.

In the present study, subjects who did not experience tooth decay, periodontitis, diarrhoea, gastritis, or mouth ulcers, at least once per year or more prior to the age of 20, were significantly more likely to develop CD. However, these findings do not necessarily indicate that the latter factors trigger CD, but rather, that the dysbacteriosis which these conditions are likely to induce, plays a role in the pathogenesis of CD. It is possible that these results indicate specific microorganisms, but this could not be investigated. In the present study, antibiotic pill use was not associated with risk for future CD development. This is at odds with previous studies which have shown early antibiotic use in children to confer an increased risk for CD development (Kronman MP *et al.* 2012; Shaw SY *et al.* 2010), as well as that the strongest risk may be conferred by

several antibiotic courses, suggesting a dose-dependent effect (Virta L *et al*. 2012; Hviid A *et al*. 2011). Furthermore, antibiotics used within the two to five years prior to CD diagnosis were associated with increasing risk of disease by 1.3-fold (Shaw SY *et al.* 2010; Card T *et al*. 2004).

One possible explanation for the present findings is the broad age interval (0-20 years) in which antibiotic use was evaluated; in addition, antibiotic use was self-reported and not confirmed by medical records: it is thus subject to recall bias. Nevertheless, results of the score analysis suggest that cases experienced significantly fewer childhood infections (12%) when compared to controls. In the latter score analysis, all exposure variables were assigned a weighting value equal to one; it is possible that these positive results reflect the broader age range evaluated as a result of having combining the original data sets (0-12 years and 12-20 years).

Findings from the score analysis performed in the present study provide insight into the 'compound' effects from environmental risk factors in the pathogenesis of CD. This has important implications for future IBD-related studies as it demonstrates the importance of accounting for environment as a 'whole' during epidemiological research, as opposed to the impact of individual factors. Indeed, it is likely that certain environmental risk factors may hold a greater 'weight' with regard to their effect on the gut microbiota in disease pathogenesis, together with timing of exposure.

Another important caveat underlying the development of the gut microbiome is CD susceptibility mutations. While it is recognized that the gut microbiome is profoundly shaped by the microbial environment, there is now evidence that the converse is also true. For instance, it has been shown that the intricate array of PPRs, including the VDR, which equip the immune system to recognize microbial molecular patterns, have an impressive effect on both the diversity and functionality of

the gut microbiota (Cantorna MG *et al* 2014; Ooi JH *et al* 2013) Therefore, genetic alterations in these PPRs may influence this bidirectional interaction, in which immune activity can either suppress or promote pathogenic microbial blooms, in turn, affecting homeostasis of host intestinal immunity and the convergence of disease susceptibility. The NOD-like or nucleotide oligomerization domain receptors (encoded by NOD2 gene) is a pattern recognition receptor (PRR) that recognizes intracellular bacterial products and variants in the CARD15/NOD2/IBD1 locus are associated with the development and phenotypic patterns of CD. The likelihood of a NOD2-dysregulating microbial community in immune-mediated disorders, such as CD is strengthened by data from recent NOD2-/- mice, in which unregulated inflammation of the gut results taxonomic shifts in bacterial phyla characteristic of the change in the microbiome during inflammation, and similar to those described in IBD patients compared with healthy controls.

Tables Referred to in Chapter 5:

The following tables from this chapter are represented:

Table 12: Environmental risk factors over three age intervals; 0-5 years, 6-10 years and 11-18 years (Basson A. 2014)

Table 13: Score analysis for group A

Table 14: Score analysis for group B

Table 15: Score analysis for environmental risk factors over three age intervals

Table 16: Score analysis for environmental risk factors over three age intervals for group A

Table 17: Score analysis for environmental risk factors over three age intervals for group B

Table 18: Frequency of childhood infections between ages of 0-20 years

Table 19: Score analysis for the frequency of childhood infection between ages 0-20 years

Table 12: Environmental risk factors over three age intervals; 0-5 years, 6-10 years and 11-18 years (Basson A, 2014)

	0-5 years			6-			11-18 years		
	Cases n (%)	Control n (%)	Adjusted OR (95% CI)*	Cases n (%)	Control n (%)	Adjusted OR (95% CI)*	Cases n (%)	Control n (%)	Adjusted OR (95% CI)*
Primary source of drinking water									
Piped or bottled water	162 (84)	172 (80)	**2.10 (1.20, 4.00)**	171 (88)	183 (86)	**2.05 (1.10, 4.10)**	94 (194)	194 (91)	1.92 (0.80, 4.60)
Outside tap/borehole/well/river/dam	31 (16)	42 (20)		23 (12)	31 (14)		6 (19)	19 (9)	
Hot piped tap water									
Access	59 (32)	85 (40)	1.18 (0.71,	77 (40)	99 (46)	1.52 (0.92,	112 (58)	129 (60)	1.52 (0.93, 2.51)
No access	128 (68)	127 (60)		116 (60)	114 (54)		82 (42)	85 (40)	
Community type									
Suburban or Urban	136 (76)	146 (72)	1.21 (0.71,	147 (80)	156 (74)	1.58 (0.91,	152 (80)	168 (80)	0.97 (0.55, 1.70)
Rural or farm or informal settlement	43 (24)	58 (28)		36 (20)	54 (26)		38 (20)	42 (20)	
Total number of people in household									
5 or less	77 (41)	123 (59)	0.68 (0.43,	79 (41)	130 (62)	0.67 (0.42,	100 (52)	129 (61)	1.05 (0.66, 1.69)
6 or more	111 (59)	86 (41)		113 (59)	81 (38)		94 (48)	82 (39)	
Number of people sharing a									
3 or less	31 (17)	59 (29)	**0.55 (0.31, 0.97)**	27 (15)	62 (30)	**0.51 (0.28, 0.90)**	36 (19)	65 (31)	0.65 (0.38, 1.11)
4 or more	151 (83)	147 (71)		159 (85)	146 (70)		157 (81)	147 (69)	
Number of bedrooms in home									
3 or more	82 (44)	110 (52)	0.87 (0.69,	87 (46)	116 (55)	0.87 (0.69,	110 (57)	131 (61)	0.91 (0.72, 1.14)
2 or less	104 (56)	101 (48)		102 (54)	94 (45)		83 (43)	83 (39)	
Type of toilet facility									
Flush (own family or shared)	154 (81)	187 (89)	1.35 (0.69,	166 (86)	190 (90)	1.62 (0.78,	180 (94)	203 (95)	1.76 (0.60, 5.08)
Bucket, pit latrine, no facility	36 (19)	24 (11)		26 (14)	21 (10)		11 (6)	10 (5)	
Household pets									
No	95 (51)	81 (39)	1.47 (0.92,	91 (48)	96 (45)	1.13 (0.71,	88 (46)	102 (48)	1.01 (0.64, 1.59)
Yes	93 (49)	126 (61)		100 (52)	116 (55)		103 (54)	112 (52)	
Donkey/horse/cow/sheep on property									
No	172 (90)	179 (85)	1.67 (0.83,	178 (94)	179 (84)	**3.10 (1.42,**	187 (97)	199 (93)	**4.31 (1.36, 16.14)**
Yes	19 (10)	32 (15)		12 (6)	34 (16)		5 (3)	15 (7)	

Table 12 (*continued*) Environmental risk factors over three age intervals; 0-5 years, 6-10 years and 11-18 years

	0-5 years			6-10			11-18 years		
	Cases n (%)	Control n (%)	Adjusted OR (95% CI)*	Cases n (%)	Control n (%)	Adjusted OR (95% CI)*	Cases n (%)	Control n (%)	Adjusted OR (95% CI)*
Cigarette smoke exposure									
Yes	145 (78)	150 (71)	**1.71 (1.01, 2.94)**	149 (78)	158 (74)	1.63 (0.95, 2.83)	155 (80)	149 (70)	**2.03 (1.20, 3.48)**
No	42 (22)	60 (29)		42 (22)	55 (26)		39 (20)	65 (30)	
Unpasteurized milk consumption									
Never	169 (96)	139 (78)	**8.02 (3.19, 23.28)**	169 (93)	149 (73)	**6.43 (3.02, 14.81)**	178 (93)	179 (85)	**2.69 (1.23, 6.17)**
Once per year or more	7 (4)	39 (22)		12 (7)	54 (27)		13 (7)	32 (15)	
Raw beef consumption									
Never	164 (95)	163 (86)	**2.84 (1.17, 7.56)**	171 (92)	173 (85)	**2.31 (1.00, 5.80)**	174 (92)	184 (86)	1.48 (0.69,3.29)
Once per year or more	9 (5)	27 (14)		11 (96)	29 (15)		15 (8)	29 (14)	
Helminth infection									
No	82 (52)	107 (56)	0.87 (0.53, 1.42)	104 (58)	125 (63)	0.85 (0.53, 1.37)	166 (88)	171 (81)	**1.90 (1.00, 3.71)**
Yes	77 (48)	84 (44)		74 (42)	75 (38)		23 (12)	40 (19)	
Treatment for helminths									
No	72 (48)	88 (47)	0.99 (0.60, 1.63)	98 (57)	105 (53)	1.17 (0.73, 1.89)	152 (82)	155 (74)	1.70 (0.95, 2.97)
Yes	79 (52)	98 (53)		74 (43)	95 (48)		33 (18)	55 (26)	

*OR odds ratio adjusted for age at study enrolment, ethnicity and gender, and 95% confidence interval.

Subjects who responded ‘do not know’ were excluded from analysis

Table 13: Score analysis for group A

Environmental Risk Factor Combination	**Score for each factor**	**Total score for category**
Source of drinking water (Not piped) + Hot water source (No access)	½+½	1
Number of people in home (Less than 3) + Number of bedrooms in home (3 or more) + pets in home	1/3+1/3+1/3	1
Number of people sharing a bathroom (Less than 3) + Type of toilet facility (Bucket, pit latrine or no facility)	½+½	1
Raw beef consumption (once per year or more)	1	1
Unpasteurized milk consumption (once per year or more)	1	1
Helminth infection (not exposed) + Treatment for helminths	½+½	1
Community type + Donkey/horse/sheep/cow on property	½+½	1
Passive Cigarette smoke exposure (exposure)	1	1
Total Possible Score	**8**	**8**

Table 14: Score analysis for group B

Environmental Risk Factor Combination	Score for each factor	Total score for category
Source of drinking water (Not piped) + Hot water (No access) + Raw beef consumption (once per year or more)	1/3+1/3+1/3	1
Number of people in a home (Less than 3) + Number of bedrooms in a home (3 or more) + Number of people sharing a bathroom (Less than 3) + Type of toilet facility (Bucket, pit latrine or no facility) + pets in home	1/5+1/5+1/5+1/5+1/5	1
Community type (Suburban or Urban) + Donkey/horse/sheep/cow living permanently on property (no exposure) + Helminth infection (no exposure) + treatment for helminths	¼+¼+¼+¼	1
Unpasteurized milk consumption (once per year or more)	1	1
Passive Cigarette smoke exposure (exposure)	1	1
Total Possible Score	**5**	**5**

Table 15: Score analysis for environmental risk factors during the 3 age intervals

0-5 Age group

	Cases	Control	Overall	OR (95% CI)
Mean	4.39	4.71	4.56	
Median, IQR	4 (3.0,5.0)	4 (3.0,6.0)	4 (3.0, 6.0)	
SD	1.93	1.98	1.96	0.92 (0.83, 1.02)
Min	0	0	0	
Max	10	12	12	

6-10 Age group

	Cases	Control	Overall	OR (95% CI)
Mean	4.39	4.6	4.5	
Median, IQR	4 (3.0, 6.0)	4 (3.0, 6.0)	4 (3.0, 6.0)	
SD	1.80	1.92	1.86	0.94 (0.80, 1.02)
Min	0	0	0	
Max	9	11	11	

11-18 Age group

	Cases	Control	Overall	OR (95% CI)
Mean	3.77	4.02	3.90	
Median, IQR	4 (3.0, 5.0)	4 (3.0, 5.0)	4 (3.0, 5.0)	
SD	1.54	1.65	1.60	0.90 (0.80, 1.02)
Min	0	1	0	
Max	9	9	9	

Table 16: Score analysis for environmental exposures during 3 age intervals; Group A

0-5 years

	Cases	Control	Overall	OR (95% CI)
Mean	2.08	3.58	3.40	
Median, IQR	1.99 (1.33, 2.66)	2.16 (1.66, 3.16)	2.16 (1.49, 2.83)	
SD	0.98	1.12	1.07	**0.74 (0.62, 0.90)**
Min	0	0	0	
Max	5.16	6.49	6.49	

6-10 years

	Cases	Control	Overall	OR (95% CI)
Mean	1.30	1.64	1.48	
Median, IQR	1.5 (0.5, 2.0)	1.5 (1.0, 2.5)	1.5 (0.5, 2.0)	
SD	0.79	1.03	0.94	**0.67 (0.53, 0.83)**
Min	0	0	0	
Max	3.5	6	6	

11-18 years

	Cases	Control	Overall	OR (95% CI)
Mean	1.81	2.08	1.95	
Median, IQR	1.66 (1.16, 2.33)	1.83 (1.33, 2.08)	1.83 (1.33, 2.49)	
SD	0.85	0.98	0.93	**0.72 (0.58, 0.90)**
Min	0	0.33	0	
Max	4.66	5.16	5.16	

Table 17: Score analysis for environmental exposures during 3 age intervals; Group B

0-5 years

	Cases	Control	Overall	OR (95% CI)
Mean	1.20	1.47	1.34	
Median, IQR	1.10 (0.73, 1.6)	1.38 (0.85, 1.98)	1.22 (0.8, 1.78)	
SD	0.63	0.77	0.72	**0.58 (0.43, 0.77)**
Min	0	0	0	
Max	3.13	3.93	3.93	

6-10 years

	Cases	Control	Overall	OR (95% CI)
Mean	1.22	1.48	1.36	
Median, IQR	1.19 (0.73, 1.60)	1.33 (0.81, 1.98)	1.23 (0.76, 1.8)	
SD	0.6	0.78	0.71	**0.58 (0.43, 0.78)**
Min	0	0	0	
Max	3.06	4.26	4.26	

11-18 years

	Cases	Control	Overall	OR (95% CI)
Mean	1.08	1.31	1.21	
Median, IQR	0.93 (0.64, 1.41)	1.19 (0.73, 1.8)	0.98 (0.73, 1.65)	
SD	0.60	0.71	0.67	**0.59 (0.43, 0.79)**
Min	0	0.2	0	
Max	3.13	3.63	3.68	

Table 18: Frequency of childhood infections before the age of 20 years

	Cases n (%)	Controls n (%)	OR (95% CI)*
Tooth decay/cavity			
Never	51 (29)	73 (37)	**1.78 (1.05, 3.04)**
Once per year or more	127 (71)	126 (63)	
Periodontitis			
Never	114 (68)	157 (81)	**1.95 (1.10, 3.48)**
Once per year or more	54 (32)	38 (19)	
Sore Throat			
Never	45 (25)	43 (20)	1.26 (0.70, 2.27)
Once per year or more	138 (75)	167 (80)	
Respiratory infection			
Never	88 (51)	123 (62)	1.20 (0.74, 1.94)
Once per year or more	84 (49)	75 (38)	
Diarrhoea			
Never	56 (31)	91 (44)	**2.71 (1.62, 4.62)**
Once per year or more	123 (69)	115 (56)	
Gastritis			
Never	72 (40)	103 (51)	**2.13 (1.30, 3.35)**
Once per year or more	106 (60)	98 (49)	
Mouth ulcers			
Never	126 (81)	173 (79)	**2.02 (1.12, 3.70)**
Once per year or more	29 (19)	46 (21)	
Perianal Disease			
Never	163 (92)	200 (98.5)	**9.08 (2.66, 42.31)**
Once per year or more	14 (8)	3 (1.5)	
Arthritis/Arthralgia			
Never	158 (88)	201 (98)	**6.89 (2.32, 25.64)**
Once per year or more	21 (12)	4 (2)	
Eye Disease			
Never	163 (93)	187 (94)	1.22 (0.47, 3.15)
Once per year or more	12 (7)	12 (6)	
Antibiotic pills			
Never	93 (55)	108 (54)	1.45 (0.88, 2.41)
Once per year or more	76 (45)	93 (46)	
Probiotics use after antibiotic pills			
Never	132 (89)	154 (90)	1.28 (0.56, 2.91)
Once per year or more	16 (11)	17 (10)	

*Adjusted for gender and ethnicity.

Table 19: Score analysis for the frequency of childhood infection between ages 0-20 years

	Cases	Control	Overall	OR (95% CI)
Mean	6.43	7.54	7.01	
Median, IQR	7 (4.8, 8.3)	8 (6.0, 10,0)	7 (5.0, 9.0)	
SD	1.54	3.26	2.67	**0.88 (0.82, 0.94)**
Min	0	0	0	
Max	12	12	12	

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APPENDIX I

Title of Project

The association between serum pro-inflammatory cytokines with 25(OH)-Vitamin D concentrations in Crohn's disease patients in the Western Cape, South Africa

THE PROTOCOL: Clinical

Background and Literature

Crohn's disease (CD) is a subtype of inflammatory bowel disease (IBD) characterized by a pronounced inflammation of the intestinal lining due to a deregulated immune response against antigenic stimulation that occurs in genetically predisposed individuals. The abnormal immune profile is characterized by an up-regulation of the type 1 T helper (Th1) cell, down-regulation of the type 2 T helper (Th2) cell and a deregulated type 17 T helper (Th17) cell immune-response (Basson A. 2014). This deregulation leads to an increased production of pro-inflammatory cytokines, tumour necrosis factor alpha (TNFα) and interferon gamma (INF-γ), which directly contribute to damage to the intestinal mucosa (Cantorna et al. 2004). Vitamin D is a steroid-like hormone that is metabolized in the body from 25-hydroxy vitamin D (25[OH]D), the major circulating form, to '1,25-hydroxy vitamin D' [$1,25(OH)_2D_3$ or $1,25(OH)_2D$], the bioactive forms, both of which perform a number of biological functions in the body via their genomic actions on the vitamin D receptor (VDR) (Cantorna et al. 2010). It is well known that vitamin D plays a significant role in bone health and up to 70% CD patient will experience a vitamin D deficiency at some point during the disease course (Basson et al. 2014). Recently however accumulating evidence suggests that the vitamin also plays a crucial role in the regulation of the general immune

system (Basson et al. 2014). The bioactive form of vitamin D regulates the functional differentiation of CD4+ T cells, as well as the differentiation of monocytes into macrophages. In vitro $1,25(OH)_2D$ has been shown to down-regulate the production of Th-1 associated pro-inflammatory cytokines such as INF-γ and interleukin-2 (IL-2 produced by CD 4+ cells, (Cantorna and Mahon. 2004). In experimental IBD VDR knock out (KO) mice, meaning that vitamin D signalling was inhibited developed an accelerated form of IBD. An increase in the production of pro-inflammatory Th-1 response associated cytokine INF- γ and a subsequent decrease in the production of the anti-inflammatory Th-2 response cytokines interleukin-4 (IL-4) and interleukin-5 (IL-5) was observed in the KO mice, when compared to the wild type mice (Cantorna et al. 2004). Similar findings were observed in interleukin-10 (IL-10) KO mice. IL-10 is an anti-inflammatory cytokine produced by the Th-2 cells which has an inhibitory effect on Th-1 cell mechanism. IL-10 KO mice develop IBD within 5-6 weeks exhibiting severe symptoms of enterocolitis and a 30% mortality rate. However, upon administration of $1,25(OH)_2D_3$, the mice exhibited a drastic reduction of the inflammation, an improvement on the IBD symptoms and a decreased mortality rate (Garg et al. 2012). In humans a number of studies have found a significant association between low serum 25(OH)-vitamin D with increased disease activity (Joseph et al. 2009; Grzanka et al. 2014; Javorsky et al. 2006). However there is limited data evaluating the association between pro-inflammatory cytokine levels and serum vitamin D in the CD patient.

Aims and Objectives

Aim

The aim of this study is thus to evaluate the association between serum 25(OH)D concentration with 3 pro-inflammatory cytokines TNFα, IFNγ and IL-2 and their association with 25(OH)D concentrations in CD patients in the Western Cape, South Africa.

APPENDIX I

Primary Objectives

To evaluate the association between serum 25(OH)-vitamin D concentrations with serum TNF-α concentrations

To evaluate the association between serum 25(OH)-vitamin D concentrations with serum IL-2 concentrations

To evaluate the association between serum 25(OH)-vitamin D concentrations with serum IFN-γ concentrations

Secondary Objectives

To evaluate the association between serum 25(OH)-vitamin D concentrations with serum CRP concentrations

To compare serum 25(OH)-vitamin D and cytokine concentrations in CD patients with healthy controls

To evaluate if vitamin D deficiency is independently associated with; disease duration, body mass index (BMI), oral contraceptive use, smoking, medication use (immunosuppressants, biologics, corticosteroids), surgical resection, season of study enrolment and ethnicity

Hypothesis to be tested

Hypothesis 1: Lower serum 25(OH)-vitamin D concentration is associated with higher serum cytokine levels.

Hypothesis 2: The former hypothesis will remain significant after adjusting for any significant independent predictors of vitamin D deficiency.

APPENDIX I

Summary of the Proposed Project

The proposed research will use a subset of data collected from a larger case control study supervised by Dr. Gillian Watermeyer. Briefly, 186 CD patients and 199 healthy controls between the ages of 18-67 currently residing in the Western Cape were recruited. At time of study enrolment, demographic and clinical variables were extracted using an investigator administered questionnaire, a review of medical and pharmacy records and clinical examination by a consulting gastroenterologist. In addition, blood samples were collected. The investigators of the original project have granted permission for the current investigator to use a subset of the data for the proposed project. Analysis of 25(OH)-vitamin concentrations has been performed. The investigator will further analyse the serum samples for the proposed inflammatory markers, as well as investigate independent factors related to low serum 25(OH)D.

Benefits of the study

This research is not designed to explore the aetiology of CD and to help the principal researcher and gastrointestinal academic community understand more about the pathogenesis of CD in relation to vitamin D within the defined population. Outcomes from this study will add insight into the understanding of inflammatory bowel disease worldwide. In addition, the relapse-remitting nature of the disease is such that it demands expensive medications which often have many undesirable side effects and negatively impact the patients' quality of life. Therefore exploring the role of vitamin D in the pathogenesis of CD is of paramount importance.

Institutions where project would occur

Groote Schuur Hospital (GSH) / Tygerberg Hospital (TBH) / University of Western

Cape (UWC).

Definition of existing resources

As per the original research, consecutive CD patients seen at GSH and TBH gastrointestinal clinics during normally scheduled appointments between September 2011 and January 2013 were recruited. Subjects with active disease, or inactive CD (i.e., 'remission') were included. The diagnosis of CD was defined according to the European consensus guidelines set out by the European Crohn's and Colitis Organization (ECCO) (Stange, Travis et al. 2006).

All participants provided written informed consent. Permission from the principal investigator of the original research project has been given allowing the use of a subset of the original data. The objectives of the current proposal are separate from those of the original research; however do fall under proposed outcomes of the original study. The outcome of the present proposal was included in the participant consent form and does not require further retrospective ethical approval. The researcher has been granted access to UWC laboratory within the Medical Biosciences department for blood sample analysis. In addition, the Medical Biosciences department makes provision for desk space and computer use for all postgraduate students. Due to funding limitations, for the proposed research 160 patients and 120 controls will be included.

Overview of available data from original research

Complete data relating to disease characteristics, vitamin D supplementation and medication use for the subjects is available. This data has been cleaned and coded. Supplemental vitamin D is prescribed by GSH and TBH gastrointestinal clinics as weekly dose units of 50,000 IU calciferol for 8 weeks. Information on medical management included the recent or current use of; corticosteroids, immunomodulators (azathioprine, 6-mercaptopurine, methotrexate), anti-tumour

necrosis factor inhibitors (infliximab, adalimuamab), or 5-aminosalicylates. Seasonal variation at study enrolment was categorized as either "summer" or "winter", and based on the average Ultraviolet Index (UVI) for Cape Town, South Africa (latitude 34ºS) (World Health Organization). The 'summer months' included September through March (UVI; 6-10) and 'winter months' included April through August (UVI; 2-5). Body mass index (BMI) was calculated as weight/height in meters squared; values >25 kg/m² were classified as overweight.

Patients with a prior diagnosis of tuberculosis, kidney disease and liver disease were excluded. Only patients with complete data at diagnosis were included. Patients were excluded if disease duration was less than 5 years, or had a prior diagnosis of intestinal tuberculosis. In accordance with the paper published by Epstein et al. 2007 there is no gold standard in the differential diagnosis between CD and intestinal tuberculosis however as per the algorithm suggested by these authors every attempt was made to exclude a diagnosis of tuberculosis.

Controls

One hundred and ninety nine, healthy controls unrelated to the CD cases were included and identified in 3 ways; namely, 1) visiting family and friends of patients admitted to the referral based spinal injury ward of GSH and TBH, 2) outpatients from GSH and TBH orthopaedic wards, and 3) porter and security personnel of GSH. Two members of the same family were not included. Controls were excluded if any of the following were present; prior or current diagnosis of tuberculosis, IBD or any other gastrointestinal illness, any immune-mediated disease, or a family history of IBD.

Blood Samples

For the proposed research one 2ml aliquot Micro Tube currently being stored at minus 80°Celcius

is available to the researcher. The samples to be used in the proposed research have not been previously thawed for analysis.

Statistical Analysis

The statistical analysis will be performed under the guidance of a professional statistician from the Medical Research Council of South Africa.

Ethical clearance

Ethical clearance was accorded by the Ethics Research committee of the University of the Western Cape (UWC) (Reg No.11/3/16), the Human Research Ethics Committee of the University of Cape Town (UCT) (HREC REF: 122/2011), the Provincial Ethics Committee of the Department of Health as well as both the ethics committees of GSH and TGH. The ethical clearance permissions were accorded annually when required. The research was conducted according to the guidelines for ethics on medical research as published by the Medical Research Council of South Africa.

Informed Consent

After full disclosure of study details, written informed consent was obtained from participants prior to data collection. Only participants who fully understood the consent form and the conditions of the study were enrolled. Participation was voluntary and participants were informed of their right to withdraw from the study at any time, without any consequences. In the event that the participant decided to withdraw and questionnaires and samples had already been collected, questionnaires and blood samples would be appropriately discarded. Two different versions of the consent forms were available, one for the cases and one for the controls. Informed consent was obtained from all participants. If a signed consent was not provided, no data would be collected and the subject would be excluded from the study.

Participant Anonymity

All materials were obtained for research purposes only, and all clinical and personal data was kept in strict confidence and remained anonymous. Removing identifiers from questionnaires was done to maintain anonymity and confidentiality of the participant information. Information sharing was strictly limited to co-investigators only on a need-to-know basis and no sharing of was acceptable for medical information given by a participant or acquired from medical records with other participants, with relatives or any other persons. Instead of participant identifiable information, questionnaires were coded using a unique number serving as an identifier. Identifiable information of participant was only found on the front page of the questionnaire and the rest of the questionnaire pages only contained the participant study code. The front page of the questionnaire was detached from the questionnaire and kept under lock and key to maintain the strictest patient confidentiality. Only the principle researcher had access to patient names and identifier code key which was at all times kept separately from the questionnaires in a locked file cabinet located in a locked office and maintained with the strictest confidentiality precautions of the principal researcher. Coded data was entered into a password protected computer.

Methodology

From the original research: Serum 25(OH)D concentrations have been measured using the SIEMENS CENTAUR XP immunoassay. The method was standardized to LC/MS/MS and performance was monitored according to the DEQAS external quality control.

ADVIA Centaur® XP Vitamin D Assay System

Validation of an assay is a process that determines whether the assay measures what it is intended to measure and includes estimates of the analytical and diagnostic performance characteristics of

a test (ed. Wild 2005). The analytical specificity, analytical sensitivity, linearity, dilution recovery and interferences of an assay are the primary parameters which determine the validity of an assay. These parameters have been described below as per the manufacturer specifications (Basson A. 2014).

Analytical Specificity

The analytical specificity is the ability of the assay to distinguish the target analyte from other closely related substances (including matrix components), and represents the degree to which the assay does not cross-react with other analytes (ed. Wild 2005).

The analytical specificity for the ADVIA Centaur® Vitamin D Assay System was tested with total 25(OH)-Vitamin D concentrations of 20 and 50 ng/mL, for the following structurally similar compounds: 1,25$(OH)_2$Vitamin D_2, 1,25$(OH)_2$ Vitamin D_3, Paricalcitol, 3-epi-25-OH- Vitamin D_3, Vitamin D_2, Vitamin D_3, 25(OH)-Vitamin D_2 and 25(OH)-Vitamin D_3.

The percent change was calculated as:

Percent cross-reactivity = (corrected assay value / amount of compound spiked) x 100

The results are depicted in FIGURE 2.3 (located at the end of this chapter), which indicates that the assay has a high specificity for 25(OH)-Vitamin D_2 (106.2%) and 25(OH)-Vitamin D_3, with minimal interference from structurally related substances at equimolar amounts (97.4%) [Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA].

Analytical Sensitivity

APPENDIX I

The analytical sensitivity of an assay is the smallest amount of the analyte that the assay is able to detect. The detection limit is assessed in three ways: limit of blank (LoB); limit of detection (LoD); and limit of quantification (LoQ) (Westgard 1999). The LoB is the highest apparent measurement of an analyte that is likely to be observed for a blank sample, and is typically estimated as a 95% one-sided confidence limit by the mean blank value, plus 1.65 times the standard deviation of the blank (Armbruster and Pry 2008; Westgard 1999). The LoD is the lowest concentration of analyte in a sample that can be reliably distinguishable from the LoB, and at which detection is possible, and is estimated as a 95% one-sided confidence limit by the mean of the blank, plus 1.65 times the standard deviation of the blank, plus 1.65 times the standard deviation of a low concentration sample (Armbruster and Pry 2008; Westgard 1999). The LoQ is the lowest concentration at which the analyte can be reliably detected but at which some predefined goals for bias and imprecision are met, under stated experimental conditions; the analyte concentration at which the 95% limit of the total error (bias plus 2 times the standard deviation) meets the required or stated goal for allowable error (Armbruster and Pry 2008; Westgard 1999). The 'functional sensitivity' of an assay is defined as the analyte concentration at which results in a CV are equal to 20 percent (Westgard 1999). While the LoQ is somewhat similar to the functional sensitivity, estimating the LoQ is more complicated because it is more difficult to estimate method bias.

The LoB, LoD, and LoQ for the ADVIA Centaur® Vitamin D Assay System were determined as described in the CLSI document EP17-A (Clinical and Laboratory Standards Institute [formerly NCCLS] 2004c). The ADVIA Centaur® Vitamin D Total Assay has an LoB of 1.6 ng/mL, an LoD of 3.20 ng/mL and a LoQ of 3.52 ng/mL. The LoD is defined as the lowest concentration of 25(OH)-Vitamin D that can be detected with 95% probability [Siemens Healthcare Diagnostics

Inc., Tarrytown, NY, USA].

ADVIA Centaur® XP Vitamin D assay has a functional sensitivity of 3.33 ng/mL. The functional sensitivity was established using multiple samples in the range of 2 to 10 ng/mL. All samples were assayed twice a day, in replicates of 4 over 10 days, using two lots (n = 320 for each sample) of ADVIA Centaur® Vitamin D Total reagents [Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA].

Linearity

The linearity of an analytical method is the ability to obtain test results which are directly proportional to the concentration of analyte in the sample (ed. Wild 2005). For the ADVIA Centaur® Vitamin D Assay, linearity was evaluated according to the CLSI protocol EP6-A (Clinical and Laboratory Standards Institute [formerly NCCLS] 2003). A sample containing high concentrations of total 25(OH)-Vitamin D was mixed in various proportions with a sample containing low concentrations of total 25(OH)-Vitamin D. The resulting sample mixtures were assayed for total vitamin D. On the ADVIA Centaur® System, the vitamin D assay is linear from 3.7 ng/mL to 150 ng/mL, with an R^2 value of 0.9945 [Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA].

Dilution Recovery

Five serum samples in the range of 118 ng/mL to 154 ng/mL of total 25(OH)-Vitamin D were diluted 1: 2, with the ADVIA Centaur® Vitamin D diluent and assayed for recovery and parallelism. The recoveries ranged from 83% to 100% with a mean of 93.5% [Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA].

Interference

Using the ADVIA Centaur® Vitamin D Assay, interfering substances were tested as described in the CLSI Document EP7-A2 (Clinical and Laboratory Standards Institute [formerly NCCLS] 2005). Interference was evaluated for specimens that are haemolysed, lipemic or icteric, as well as for specimens containing cholesterol, uric acid or human immunoglobulin. For haemolysed, lipemic and icteric (conjugated and unconjugated bilirubin) specimens, a ≤10% change in results was demonstrated for concentrations up to; 155 mg/dL of haemoglobin; 360 mg/dL of triglycerides; 40 mg/dL of conjugated bilirubin; and 40 mg/dL of unconjugated bilirubin, respectively. For samples containing cholesterol, uric acid and human immunoglobulin, a ≤10% change in results was demonstrated for concentrations up to: 236 mg/dL; 20 mg/dL; and 6 mg/dL, respectively[Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA].

Key References:

1) Basson. A. Vitamin D and Crohn's Disease in the Adult Patient: A Review: JPEN 2013; 1-21.

2) Loftus EV. Clinical Epidemiology of Inflammatory Bowel Disease: Incidence, Prevalence and Environmental Influences. Gastroenterology 2004; 1504-1517.

3) Cantorna MT, Zhu Y, Froicu M and Wittke A. Vitamin D Status, 1,25-Dihydroxyvitamin D_3 and the Immune system. Journal of Clin Nut 2004; 1717-20.

4) Kriegel MA, Manson JE, Constenbader KH. Does Vitamin D affect risk of Developing Autoimmune Disease? A Systematic review. Semin Athritis Rheum 2011; 512-531.

5) Jorgensen SP, Hvas CL, Agnholt J, Christensen LA, Heickendorff L, Dahlerup JF. Active Crohn's diseases is associated with low vitamin D levels. J Crohn's Colitis 2013; viewed on 21 May 2014, from http://dx.org/10.1016/j.crohn's.2013.01.12.

APPENDIX II

FACULTY OF LIFE SCIENCES

DEPARTMENT OF MEDICAL BIOSCIENCES

DATE

My name is Dr. Abigail Raffner Basson and I am a lecturer at the University of the Western Cape. I am writing this letter on behalf of my Masters student Mr. Victor Sabe who is conducting a systematic review titled 'The Association between Childhood Environmental Exposures and the Subsequent Development of Crohn's Disease: A Systematic Review'. The student intends to submit the completed manuscript for peer-review process to the Journal of PLOS ONE.

The primary aim of the paper is to focus on a number of exposures during various age intervals during 'childhood'. We would like to include your paper titled "……" in our review and respectfully request for you to send us further information pertaining to the details of your study methodology, as well as all statistical results which may not have been included in your final publication. We would also greatly appreciate it if you could inform us about any additional variables, relative to our systematic review topic, which may have been considered during your study. .

We thank you very much for your help, and look forward to your response.

Kindest Regards,

Dr. Abigail Raffner Basson and Mr. Victor
Sabe University of the Western Cape
Medical Biosciences Department

Dr Abigail R. Basson, R.D. (RSA/USA)
Lecturer and Hospital Internship
Supervisor Office Tel: +27 21 959 2159
Email: 311599@myuwc.ac.za
ORCID ID: orcid.org/0000-0002-8369-919X

Mr Victor T. Sabe
Postgraduate Medical Biosciences
Student Number 3410048

APPENDIX III

DEPARTMENT of HEALTH

GROOTE SCHUUR HOSPITAL

REFERENCE: Abigail Basson/GIT
ENQUIRIES: Dr Bhavna Patel

Mrs A. Basson
School of Public Health
Dietetics Department
University of the Western Cape
Private Bag X17
BELLVILLE
7535

E-mail: abbasson@uwc.ac.za

Dear Mrs Basson

PROJECT TITLE: Environmental Risk Factors & the Serological Immune Marker Prevalence Associated with Crohn's Disease Development & Phenotype in the Western Cape Populations, South Africa

Your recent letter to the Hospital refers.

You are hereby granted permission to proceed with your research.

Please note the following:

a) Your research may not interfere with normal patient care
b) Hospital staff may not be asked to assist with the research.
c) No hospital consumables and stationary may be used.
d) No patient folders may be removed from the premises or be inaccessible.
e) Please introduce yourself to the person in charge of an area before commencing.

I would like to wish you every success with the project.

Yours sincerely

Patel

DR BHAVNA PATEL
SENIOR MANAGER: MEDICAL SERVICES

APPENDIX IV

UNIVERSITY OF THE WESTERN CAPE

Private Bag X 17, Bellville 7535, South Africa

Tel: +27 21-959 2458, Fax: 27 21-959 2679, Cel: 079 135 4543

E-mail: abbasson@uwc.ac.za

PARTICIPANT INFORMATION DOCUMENT (Cases)

Project Title:

Environmental risk factors and the serological immune marker prevalence associated with Crohn's disease development and phenotype in the Western Cape population, South Africa.

What is the study about?

This is a research project being conducted by Mrs. Abigail Basson, a lecturer from the University of the Western Cape. We are inviting you to participate in this research project because you are currently residing in the Western Cape, between the ages of 18-70 and have been diagnosed with

Crohn's Disease between the years 1994 and 2005 inclusive, and have recently been seen by your gastrointestinal doctor at either Groote Schuur Hospital or Tygerberg Hospital Gastrointestinal clinic.

Crohn's disease causes inflammation that can affect many areas of the gut, in particular the small and large intestine. This is a poorly understood illness and it is unclear why some people are affected but not others. Because of this it is important to perform testing on individual population groups in defined geographical areas.

What will I be asked to do if I agree to participate?

If you agree to take part in this study you will be asked to:

1. Answer a detailed questionnaire with help of a trained study interviewer

2. Allow us to draw samples of your blood: This will involve having 1-2 tubes of blood drawn from your arm (up to 10mls, or approximately two teaspoons worth).

APPENDIX V

WE ARE RECRUITING STUDY PARTICIPANTS

We are recruiting individuals for a research study looking at Inflammatory Bowel Disease in the Western Cape, South Africa

YOU CAN PARTICIPATE IF YOU ARE:

Between the ages of 18-70, with NO previous medical history of: Tuberculosis, Crohn's disease, Ulcerative Colitis, Irritable bowel Syndrome, Rheumatoid arthritis, Psoriasis, Lupus, and NO family history of Inflammatory Bowel Disease.

IF YOU PARTICIPATE YOU WILL BE REQUIRED TO:

1. Complete a questionnaire.
2. Allow us to take a blood sample.

Participation will take approximately 45 minutes

APPENDIX VI

UNIVERSITY OF THE WESTERN CAPE

Private Bag X 17, Bellville 7535, South Africa

Tel: +27 21-959 2458, Fax: 27 21-959 2679,Cel: 079 135 4543

E-mail: abbasson@uwc.ac.za

CONSENT FORM: ADULTS OVER 18 YEARS (Cases)

Title of Research Project: Environmental risk factors and the serological immune marker prevalence associated with Crohn's disease development and phenotype in the Western Cape population, South Africa.

What does your signature on this consent form mean?

- You give us permission to enrol you into this study
- You give us permission to administer the study questionnaire:

The questionnaire will include questions on: General Information, Family History, Medical and Crohn's disease History, Environmental Questions, and Smoking History.

- Permission for the researcher to access your patient records for additional medical information.

- You give us permission to draw samples of your blood. This will involve having 1-2 tubes of blood drawn from your arm (10 mls in total). This will be performed using sterile techniques by a trained nurse. The risks of drawing blood from a vein (the same procedure used in all laboratories) may be slight pain from the needle, and possibly some mild bruising. In rare instances redness of the skin may occur at the site. Some individuals may faint during blood taking.

Firmly pressing down on the site where the needle entered the skin using the index and middle fingers over top of a clean gauze can reduce chance of bruising. If the sight of blood makes you want to faint, make sure you are sitting down during the blood draw procedure and turn your face away while they draw the blood. Also, make sure you are

Participant Code:

APPENDIX VII

CONFIDENTIAL STUDY QUESTIONNAIRE

ALL INFORMATION WILL BE KEPT STRICTLY CONFIDENTIAL, AND WILL IN NO WAY AFFECT HOSPITAL TREATMENTS OR PAYMENTS	
Name of Study Interviewer:	
Name of Study Translator (If	
Applicable): Date of Interview:	
MEDICAL CHART NUMBER:	

PARTICIPANT DETAILS

Title:	
Initials:	
First	
Name:	
Surname:	

Postal Address:		
		Postal Code:

Physical Address:		
		Postal

Daytime Telephone Number:	( )
Work Telephone Number:	( )
Cell Phone Number:	( )

Code:

PARTICIPANT EMERGENCY CONTACT DETAILS

Primary Emergency Contact Person Name:	
Relation to Participant:	
Emergency Contact Person CELL Phone:	( )
Emergency Contact Person HOME Phone:	( )

APPENDIX VIII

MEDICAL CHART REVIEW FORM: SECTION 1

PARTICIPANT CODE

NAME OF CHART REVIEWER:	
TODAYS DATE:	
Participant appointment date:	

Height (cm):

Weight (kg):

PARTICIPANT DETAILS		
CHART NUMBER		
Surname		**Initials:**
First Name		
Date of Birth	**Day:** **Month:** **Year:**	
Gender		

CROHN'S DISEASE MEDICAL HISTORY: AT DIAGNOSIS			
Date of Diagnosis:	**Day:**	**Month:**	**Year:**
Diagnosis of TB or amoebiasis (circle one):	**Yes**	**No**	

METHOD OF DIAGNOSIS (circle all that apply):				
Gastroscopy	Colonoscopy	CT scan	MRI	Ultrasound
Endoscopy with biopsy	Endoscopy without biopsy	Double Balloon Enteroscopy (DBE)	Small bowel enema (SBE) with biopsy	Small bowel enema (SBE) without biopsy
Other:				

DISEASE LOCATION :_____(write name) (circle all that apply)				
DISEASE BEHAVIOR: Ileal (Distal Small Intestine)	Colonic:	Ileocolonic	Isolated Upper Disease (write name) (excludes disease distal to jejunum):	Perianal
Comments:				

APPENDIX IX

Western Cape Government
Health

TygerbergHospital and
MitchellsPlain&Tygerberg Oral Health Centres
REFERENCE: Researches
ENQUIRIES:Dr M A Mukosi
TELEPHONE: 021 938-5966

ETHICS NO: 122/2011 (University of Cape Town)

Environmental risk factors and the Serological Immune marker prevalence associated with Crohn's Disease development and Phenotype in the Western Cape Population, South Africa.

Dear Ms A Basson

PERMISSION TO CONDUCT YOUR RESEARCH AT TYGERBERG HOSPITAL

In accordance with the Provincial Research Policy and Tygerberg Hospital Notice No 40/2009, permission is hereby granted for you to conduct the above-mentioned research here at Tygerberg Hospital.

APPENDIX IX

OFFICE OF THE DEAN
DEPARTMENT OF RESEARCH DEVELOPMENT

Private Bag X17, Bellville 7535
South Africa
Telegraph: UNIBELL
Telephone: +27 21 959-2948/2949
Fax: +27 21 959-3170
Website: www.uwc.ac.za

21 April 2011

To Whom It May Concern

I hereby certify that the Senate Research Committee of the University of the Western Cape has approved the methodology and ethics of the following research project by: Mrs. A Basson (School of Public Health)

Research Project: Environmental risk factors and serological immune marker prevalence associated with Crohn's disease development and phenotype in the Western Cape population, South Africa

Registration no: 11/3/16

Mr. Peter Syster
Research Development: Manager
University of the Western Cape

APPENDIX IX

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

Annual Progress Report

Date	28/02/2012
HREC REF Number	122/2011
Protocol number (if applicable) & Protocol title	Environmental risk factors and the serological immune marker prevalence associated with Crohn's disease development and phenotype in the western cape population, South Africa
Principal Investigator	Mrs. Abigail Basson
Department / Office Internal Mail Address	UWC, Dietetics Department, Private Bag X17, Bellville 7535

List of documentation

See Amendment form FHS006 Ethical clearance regarding the proposal amendment to included 25(OH)-vitamin D in the blood serum analysis has been granted by the Research Ethics Committee of the University of the Western Cape. The proposal and title amendment has been approved by the Higher degrees of the University.

The protocol amendment was approved by the Human Research Ethics Committee of the University of Cape town in 2013.

HUMAN RESEARCH ETHICS COMMITTEE
05 MAY 2014

All approval documentation has been provided.

The current amendment is only a title change, in order to appropriately reflect the proposal amendment (to included 25(OH)-vitamin D) which was approved in 2013 by the above committees.

HREC office use only (FWA00001637; IRB00001938)

☑ Approved	This serves as notification of annual approval, including all documentation described above
☐ Not approved	See attached comments.

Type of review	☑ Expedited	☐ Full committee	
Expiry date	30 MAY 2015		
Signature Chairperson of the HREC		Date	5/5/14

APPENDIX IX

UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

Amendment Form

Date	03/04/2014
HREC REF Number	122/2011
Protocol number (if applicable) & Protocol title	Environmental risk factors and the serological immune marker prevalence associated with Crohn's disease development and phenotype in the western cape population, South Africa
Principal Investigator	Mrs. Abigail Basson
Department / Office Internal Mail Address	UWC, Dietetics Department, Private Bag X17, Bellville 7535

List of Proposed Amendments with Revised Version Numbers and Dates

Amended title: The association between Crohn's disease activity, serum 25(OH)-vitamin D status, the disease-associated environmental risk factors and the variability of Crohn's disease phenotype in the Western Cape population, South Africa.

I have not attached a revised protocol as it is only a title change, however the 2013 approval forms have been attached.

HUMAN RESEARCH ETHICS COMMITTEE
05 MAY 2014
HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN

HREC office use only (FWA00001637; IRB00001938)

☑ Approved ☑ Type of review: Expedited ☐ Full committee

This serves as notification that all changes and documentation described above are approved.

Signature Chairperson of the HREC		Date	5/5/2014

APPENDIX IX

DEPARTMENT of HEALTH
Provincial Government of the Western Cape

GROOTE SCHUUR HOSPITAL

REFERENCE: Abigail Basson/GIT
ENQUIRIES: **Dr Bhavna Patel**

Mrs A. Basson
School of Public Health
Dietetics Department
University of the Western Cape
Private Bag X17
BELLVILLE
7535

E-mail: abbasson@uwc.ac.za

Dear Mrs Basson

PROJECT TITLE: **Environmental Risk Factors & the Serological Immune Marker Prevalence Associated with Crohn's Disease Development & Phenotype in the Western Cape Populations, South Africa**

Your recent letter to the hospital refers.

You are hereby granted permission to proceed with your research.

Please note the following:

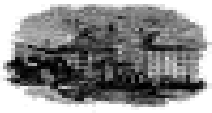
a) Your research may not interfere with normal patient care
b) Hospital staff may not be asked to assist with the research.
c) No hospital consumables and stationary may be used.
d) No patient folders may be removed from the premises or be inaccessible.
e) Please introduce yourself to the person in charge of an area before commencing.

I would like to wish you every success with the project.

Yours sincerely

Patel

DR BHAVNA PATEL
SENIOR MANAGER: MEDICAL SERVICES
Date: 28th September 2011



Groote Schuur Hospital
Private Bag,
Observatory, 7935
Telephone : 021 404-9111

APPENDIX X

CROHN'S DISEASE PARTICIPANT CONTACT TELEPHONE SCRIPT

Hello, may I please speak to Mr/Mrs _____

Hello my name is _____, and I am calling you from the gastrointestinal
at Groote Schuur Hospital/Tygerberg Hospital. I am calling to confirm you appointment
the clinic tomorrow at _____, is that correct?

I would also like to inform you that we are currently conducting a study regarding Crohn
disease and the environmental risk factors and blood markers that may be linked to the
disease development.

I would like to also tell you that your participation is voluntary, will in no way affect you
treatment at the clinic. You are eligible to participate in the study, and we would like to
know if you are interested?

(If No):

Thank you for your time, we see you tomorrow for your regularly scheduled appointmen
Feel free to let us know if you change your mind!

(If yes)

That's great!
If you participate you will be asked complete a questionnaire and allow us to take some
blood... I would like to also tell you that your participation is voluntary, will in no way a
your treatment at the clinic, and all information will be kept strictly confidential.
Are you willing to participate?

(If yes)

Since you have a _____ (am/pm) appointment tomorrow at the clinic, would
like to schedule something either before or after that time? The appointment will take
approximately 40-45 minutes.

(Schedule appointment time)

Your appointment will be with Mrs/Mr _____ tomorrow. Please le
receptionist know of your appointment when you arrive and they will direct you where t

Thank you for agreeing to participate! We will see you tomorrow!

APPENDIX XI: Studies investigating childhood environmental risk factors using defined age intervals

Author	Study Centre	Study Design	Data Collection	Age Range	Categories (Age)	Subjects	Control selection	Variables
Basson A 2014	Major Tertiary health referral centres GSH and TBH South Africa	Population based Case-control	Retrospective Questionnaire Interviewer administered and medical records	18-64	0-5 6-10 11-18	N=194 CD 213 controls	Controls: orthopaedic outpatients, hospital porters/security and family friends visiting spinal injury patients	**Source of drinking water** **Hot piped drinking water** **Suburban /urban/Rural /farm/ informal settlement** **Number of people in household** **Number of people sharing a bathroom** **Number of bedrooms in a home** **Type of toilet facility** **Household pets** **Farm animals** **Cigarette smoke exposure** **Unpasteurized milk consumption** **Raw beef consumption** **Helminth infection** **Treatment for helminths** **Breastfeeding** **Day care attendance**
Gearry RB *et al* 2009	Canterbury New Zealand	Population based Case-control	Retrospective Questionnaire Self – administered	20-59	0-5 6-11 12-16	N=638 CD N=653 UC 600 controls	Randomly selected from the Electoral Roll for Electorates corresponding with the Canterbury district health Board Age and sex matched controls.	**Birth place/ geography – local/ overseas** **Upbringing**- city/town/country **Immunizations/medications-** measles/mumps/TB **Antibiotics >4x/year** **Operations/ medical conditions** **Household characteristics: bedroom sharing bedroom number, sibling number, older sibling number, number of home inhabitants , farm animal contact, pets in home, sandpit, swimming pool** Vegetable garden **Home-heating (presence of each type of heating compared with absence)** Wood/Coal/electricity **Immunizations/medications** **Breastfeeding** **Exposure to smoke**
Han DY *et al* 2009	North Island New Zealand	Case-control cohort	Questionnaire Self-reported And medical records	5-86	0-5 6-11 12-15	N=315 CD 536controls	Volunteers who responded to media publicity	**Antibiotic Use: Taking 4< antibiotics in a year** **Taking regular medications** **Having pet at home** **Regularly feed pets** **Proportion of carpeting in the home** **Swam in public swimming pool as a child:** **Lived in a house with a public swimming pool for greater than a year** **Immunization in infancy:** measles/mumps/TB **Had a sandpit in the garden** **Breastfeeding in infancy:** yes/no **Medication (regular) use** **Birth order** **Number of siblings** **Early exposure to allergens and microbes** **Exposure to smoke** **Attending preschool**
Wurzelmann JI *et al* 1994	Membership roll North Carolina USA	Case-control	Questionnaire Self-administered	N.S	0-5 6-11 12-15	N=322 CD N= 262 controls	Controls were the patient's closest neighbour	Place of upbringing: Rural/Small town/Suburb/City Childhood infections: Bacterial infections/Viral infections Antibiotic treatment by illness: Otitis/Pharyngitis/Tonsillitis/Colds Urban living relative to farm living: Rural/Small town/Suburb/City Breastfeeding/bottle-feeding Surgical history (tonsillectomy/Appendectomy) Childhood Hospitalization Bedroom sharing **Sibship Month and season of birth**

APPENDIX XII: Studies broadly investigating childhood environmental exposures during 'childhood' (0-18 years)

Author	Study Centre	Study Design	Data Collection	Age Range	Categories (Age)	Subjects	Control selection	Variables
Bernstein CN *et al* 2006	Manitoba Health Registry Canada	Population based Case-control	Questionnaire (multi-item) Self-administered	18-50	0-12 0-5 for cat as a pet	N=364 CD N=217 UC	Controls selected from the Manitoba health population registry	**Taking regular medications** **Urban/rural** **Family size** **Birth order** **Primary water source** **Appendectomy** **Breast feeding** **Vaccination** **Exposure to smoke** **Ethnicity** **Household pet** (Cat) **Lived on a farm (Y/N) and main farm type :** Cattle/Pig/Poultry **Primary source of water being a well/ lake/other non-tap source as a child** **Frequency of drinking unpasteurized milk as a child:** Low/Medium/High **Frequency of eating chicken as a child:** Low/Medium/High
Castiglione F *et al* 2011	Multi-Centre Southern Italy	Case-control Prospective	Questionnaire	18-61 CD 16-63 UC 18-66 Controls	N.S	N=468 CD N=527 UC 562 controls	Controls were physicians, nurses and support service professionals Age/sex matched	**Helminth infection** **Pet exposure in childhood** **Antibiotic use in childhood** **Breastfeeding** **Family size** **Sibship** **Vaccinations** **Allergens** **Urban/rural dwelling** **Dental care history**
Duggan AE *et al* 1998	Nottingham University hospital United Kingdom	Case-control	Questionnaire Self-administered	15-65	0-11	N=110 CD N=213 UC 337 controls	Controls were surgical inpatients with no history of IBD, having elective surgery Sex matched controls	**Childhood hygiene** **H pylori status** **Surgical history** **Antibiotic use** **Bed shared with other child:** Always or sometimes/Never **Bedroom shared with other child:** Always or sometimes/Never **Running hot water:** Never or sometimes/Always **Fixed bath or shower:** Sometimes or never/Always **Inside Toilet:** Sometimes or never/Always **Home shared with other family:** Always or sometimes/Never **Central heating installed:** Sometimes or never/Always **Parents owned a car:** Sometimes or never/Always **Nursery school attendance**
Feeney MA *et al* 2002	District general hospital United Kingdom	Case-control	Questionnaire Self-administered	16-45	0-5	N=139 CD N=137 UC	Outpatient controls Sex/Age matched	**Medical History:** Helicobacter pylori/ Hepatitis A Ig G status Appendectomy/ Tonsillectomy <17 years Eczema/ Hay fever <10 years **Family characteristics:** Number of siblings Number of older siblings Parental smoking Person to room ratio in home Social class **Childhood circumstances:** Hot water tap Number of indoor toilets Nursery attendance Using swimming pool Number of family cars Number of house moves Environment (urban/ rural) Pets in home(cat, dog, bird, rodent)

APPENDIX XII: Studies broadly investigating childhood environmental exposures during 'childhood' (0-18 years)

Author	Study Centre	Study Design	Data Collection	Age Range	Categories (Age)	Subjects	Control selection	Variables
Hampe J *et al* 2003	German Crohn's and Colitis foundation Germany	Population based Case-control Double blinded	Questionnaire Interviewer administered	N.S	0-3	N=1468 CD N=651 UC N=232 IC 3364 controls	Controls were unaffected family members	**Childhood hygiene:** tap water/warm tap water/water toilet/ central heating **Sibship size:** 2/>2 siblings **Birth rank:** 1/2/3/4/>4 **Community size for childhood and current place:** Village <3000/town 3000-100000/city>100000
Hansen TS *et al* 2011	Herlev University hospital Denmark	Case-control	Questionnaire Self-administered	N.S	<20	N=123 CD N=144 UC 267 controls	Orthopaedic controls Age/sex matched	**Breastfeeding:** ever/>6months **Operation:** tonsillectomy/appendectomy <20yrs **Vaccination:** diphtheria/pertussis/measles/polio/rubella/tetanus/TB **Infections:** mumps/pertussis/rubella/scarlatina/chicken pox <20 **Running water**
Hlavaty T *et al* 2013	Bratislava University Hospital Slovakia	Case-control	Questionnaire Self-administered	0-41+	N.S	N=190 CD N=148 UC 355 controls	Controls were healthy volunteers Age/sex matched	**Breastfeeding duration (months)** 0-5 months/6-12 months/12+ months **Size Family in childhood** People in family/Children in family/Older siblings **Infections in childhood (yes/no)** Frequent respiratory/Ascaris infections/Any parasitic infection **Settlement in childhood** Village/Town <20, 000/City >20, 000 **Contact with animals in childhood** Dogs/Cats/Poultry/Cattle/Horses/Pigs/Small rodents **Sporting activity in childhood** Never/Once per week or less/Twice and more per week **Allergic disease/ Concomitant medication** **Surgical history (Appendectomy/tonsillectomy)** **Housing type**
Lopez-Serrano P *et al* 2010	Spanish University hospital Spain	Hospital-based Case-control	Questionnaire and medical records Self-administered	18-80	N.S	N=124 CD and 235 controls N=146 UC and 238 controls	Controls chosen from GI clinic Age/sex matched	**Season of birth** **Viral infections** **Hospitalization during childhood** **Breastfeeding** **Age of gluten introduction to diet** **Number of siblings** **Shared bedroom** **Household pet** **Owned cars** **Urban/Rural residence** **Appendectomy** **Parental occupation** **Family income**
Malekzadeh F *et al* 2009	Tertiary referral clinics in Tehran Iran	Case-control	Questionnaire Interviewer administered	N.S	N.S	N=199 CD 207 controls	Controls were irritable bowel syndrome (IBS) patients Age matched	Domestic hygiene/social status: Refrigerator/Freezer/Runningwater/WC?/Hottapwater/Separatebathroom/Television/Washingmachine/Car/Centralheating/Computer/Micro-wave oven Urban/rural Pets No of siblings No of sibs in the bedroom Comfort at home

APPENDIX XII: Studies broadly investigating childhood environmental exposures during 'childhood' (0-18 years)

Author	Study Centre	Study Design	Data Collection	Age Range	Categories (Age)	Subjects	Control selection	Variables
Ng SC *et al* 2014	Multi-Centre Asia-Pacific Across 9 countries	Population based Case-control	Questionnaire Interviewer administered	25-50 cases 26-53 controls	N.S	N=186 CD N=256 UC 940 controls	Asymptomatic subjects randomly selected and invited from streets or stores within the same residential areas as cases Age/ sex/ ethnicity and geographic location matched	**Breastfeeding** **Pet ownership:** **Dog/cat/rodents/birds/aquarium fish** **Antibiotic use <15yrs** **Surgical History:** **Tonsillectomy/Appendectomy/Eczema** **Vaccination:** **diphtheria/pertussis/measles/polio/rubella/tetanus/TB/polio/diphtheria** **Childhood Infections:** **mumps/pertussis/rubella/scarlatina/chicken pox/ mumps/ scarlet fever/ measles** **Sanitary conditions:** **In-house water tap/hot water tap/flush toilet** **Physical activity**
Persson PG *et al* 1993	Stockholm County population Sweden	Case-control	Questionnaire and medical records Self-administered	15-79	0-15	N=152 CD N=145 UC 305 controls	Controls randomly selected via population register Age/sex matched	**Infections in childhood (GI infection 0-10)** **Sibship (rank and size)** **Breastfeeding** **Physical activity** **Household pets** **Preschool attendance**
Timm S *et al* 2014	Multi-centre Northern Europe	Cohort	Questionnaire Self-administered	20-44	0-5	Pop N=10864 N=49 CD N=140 UC N=10 IC	N.S	Place of upbringing (0-5years) Farm with livestock Farm without livestock Village in rural area Small town Suburb of City Inner City Smoking status

*N.S denotes Not Specified

APPENDIX XIII: Studies that were eliminated from the selection

Author	Study Centre	Study Design	Data Collection	Age Range	Categories (Age)	Subjects	Control selection	Variables
Corrao G *et al* 1998	Multiple centre study Italy	Population based Case-control	Questionnaire	18-65	N.S	N=225 CD N=594 UC	Controls had acute disease not related to smoking, OC use or immunological disorder Age/sex matched	**Breastfeeding** **Physical activity** **Dietary factors** **Illness history (such as psoriasis)** **Early infections** **Absence of appendectomy** **Contact with animals** **Urban/rural** **Exposure to smoke**
Guo AY *et al* 2014	Massachusets general hospital USA		Questionnaire and medical records	N.S	N.S	N=333 CD N=270 UC	N.S	**Breastfeeding** **Antibiotic use** **Delivery via caesarean section** **Hospitalization before the age of 5** **Childhood pets** **Exposure to farm animals** **Exposure to day-care during childhood** **Exposure to cigarette smoke**
Halfvarson J *et al* 2006	Orebro University hospital Sweden	Population based cohort Case-control	Questionnaire	N.S	<20	N=317 twin pairs	Controls were the co-twin Age/sex matched	**Infections (GI & RT)** **Surgical History:** Tonsillectomy/Cholecystectomy/Appendectomy Oral Contraceptive use **Travelling abroad** **Swimming habits** **Childhood infections** **Exposure to pets** **Antibiotic therapy** **Vaccinations** **Physical activity**
Hildebrand H *et al* 2008	Multicentre Sweden	Case-control	Medical birth register records (Swedish population registers)	0-15 16-24 0-5 (antibiotic use)	N.S	N=1098 CD 6550 controls 590ped onset 508adlt onset	Cases were matched with controls at random among those without a diagnosis of IBD Age/sex matched	Antibiotic therapy Childhood infections: (pneumonia/otitis/appendicitis/IRDS/sepsis/cystitis/tonsillitis)

*N.S denotes Not Specified

APPENDIX XIV: Availability of Tap Water and Heated Tap Water

	0-5	6-10	11-18	'childhood' (0-18)
Basson	**Having piped tap or bottled water was associated with CD development** OR=2.10 95% CI (1.20-4.00), (K=0.63; 95% CI, 0.37-0.89)	**Having piped tap or bottled water was protective of CD development** OR=2.05 95% CI (1.10-4.10), (K=0.69; 95% CI, 0.69-1.00)	**No significant association** OR=1.92 95% CI (0.80-4.60)	**N/A**
	No significant association OR=1.18 95% CI (0.71-2.00)	**No significant association** OR=1.52 95% CI (0.92-2.54)	**No significant association** OR=1.52 95% CI (0.93-2.51)	**N/A**
Bernstein *et al*	**N/A**	**N/A**	**N/A**	**0-12 years: Primary source of water being well, lake or other non-tap source is not associated with CD development** OR=0.77 95% CI (0.56-1.06) p=0.11
Duggan *et al*	**N/A**	**N/A**	**No significant association** OR=0.84 95% CI (0.3-1.4) Unadjusted OR=1.74 95% CI (0.8-3.8) Adjusted	**0-11 years and 11-16 years: Availability hot tap water associated with CD** After adjustment for age and sex only it was a significant independent association at 5% level Unadjusted OR=0.55 95% CI (0.3-0.9) Adjusted OR=0.56 95% CI (0.3-0.9) p=0.02
Feeney *et al*	**Significantly associated with developing CD** Was reported by over 95% of all subjects (data not shown)	**N/A**	**N/A**	**N/A**
Hampe *et al*	**N/A**	**N/A**	**N/A**	**No significant association** OR=0.94 95% CI (0.53-1.66)
	No significant association OR=1.25 95% CI (0.78-2.00) However a trend towards a high risk was associated	**N/A**	**N/A**	**N/A**
Hansen *et al*	**N/A**	**N/A**	**N/A**	**No significant association** OR=0.50 95% CI (0.05-5.51)
Malekzadeh *et al*	**N/A**	**N/A**	**N/A**	**No significant association** (data not shown)
Ng *et al*	**N/A**	**N/A**	**N/A**	**No significant association** OR=1.208 95% CI (0.803-1.819) p=1.208 Asia only unadjusted OR=0.763 95% CI (0.477-1.222) p=0.364 Asia only adjusted OR=0.847 95% CI (0.533-1.344) p=0.48 Asia and Australia
	N/A	**N/A**	**N/A**	**Significantly associated with CD** OR=1.478 95% CI (1.028-2.125) p=0.035

***** Non-shaded areas denote farm animal contact; shaded areas denote place of upbringing.

APPENDIX XV: Household Pets

	0-5 years	6-10 years	11-18 years	'childhood' (0-18)
Basson	**No significant association** OR=1.47 95% CI (0.92-2.34)	**No significant association** OR=1.13 95% CI (0.71-1.79)	**No significant association** OR=1.01 95% CI (0.64-1.59)	**N/A**
Bernstein *et al*	**Having pet cat protective against CD** OR=0.66 95% CI (0.46-0.96) p=0.03 CD patients less likely to have had a household pet than controls (71.9%) OR =0.73, 95% CI (0.53-1.0) p=0.05 Fewer CD patients had pet cats (33.8%) than controls (44.1%) OR = 0.68 95% CI (0.50-0.92, p=0.012 Among those with pets, CD patients (50.8%) were less likely to have cats than controls (61.3%) but the result was not significant OR = 0.72, 95% CI (0.51-1.03) p=0.07	**N/A**	**N/A**	**N/A**
Castiglione *et al*	**N/A**	**N/A**	**N/A**	**No significant association** OR=0.96 95% CI (0.75-1.24)
Feeney *et al*	**No significant association with having a pet in the home** **Cat** OR=0.81 95% CI (0.41-1.63) p=0.561 **Dog** OR=0.65 95% CI (0.34-1.24) p=0.190 **Bird** OR=1.76 95% CI (0.75-4.14) p=0.194 **Rodent** OR=0.89 95% CI (0.43-1.81) p=0.743	**N/A**	**N/A**	**N/A**
Gearry *et al* **CD=638**	**No significant association** (data not shown)	**No significant association** (data not shown)	**No significant association** (data not shown)	**N/A**
Han *et al*	**No significant association** OR=1.26 95% CI (0.92-1.73)	**Having a pet showed significant increased risk of developing CD** OR=1.98 95% CI (1.28-3.06) p=0.002 **Regularly feeding pets was not sufficient to offer protection but somehow showed some decrease in risk**	**Having a pet showed significant increased risk of developing CD** OR=1.61 95% CI (1.07-2.42) p=0.02 **Regularly feeding pets was not sufficient to offer protection but somehow showed some decrease in risk**	**N/A**
Hlavaty *et al*	**N/A**	**N/A**	**N/A**	**Not having a pet cat showed significant increased risk of developing CD** OR=0.6 95% CI (0.4-0.9) p<0.03 CD patients had less frequent contact with dogs p=0.02, cats p=0.049 and cattle p=0.049
Lopez *et al*	**N/A**	**N/A**	**N/A**	**Having a pet showed significant increased risk of developing CD** OR=0.5 95% CI (0.3-0.9) p=0.001
Malekzadeh *et al*	**N/A**	**N/A**	**N/A**	**No significant association** (data not shown)
Ng *et al*	**N/A**	**N/A**	**N/A**	**Having dogs showed significant decreased risk of developing CD before 15 among Asians** OR=0.54 95% CI (0.43-0.91) Cats, rodents, birds and aquarium fish had no significant association

APPENDIX XVI: Farm Animal Contact and Place of upbringing*

	0-5 years	6-10 years	11-18 years	'childhood' (0-18)
Basson	**No significant association** OR=1.67 95% CI (0.83-3.45)	**Never having had farm animals increased the risk of CD** OR=3.10 95% CI (1.42-7.21) K=0.84; 95% CI (0.12-1.00)	**Never having had farm animals increased the risk of CD** OR=4.31 95% CI (1.36-16.14) K=1.00; 95% CI (1.00-1.00)	**N/A**
	No significant association OR=1.21 95% CI (0.71-2.10)	**No significant association** OR=1.58 95% CI (0.91-2.76)	**No significant association** OR=0.97 95% CI (0.55-1.70)	**N/A**
Bernstein *et al*	**N/A**	**N/A**	**N/A**	**Not having farm animal contact increased the risk of CD** OR=0.62 95% CI (0.46-0.85)
	N/A	**N/A**	**N/A**	**Lower likelihood of farm living associated with CD** OR=0.62 95% CI (0.46-0.85)
Castiglione *et al*	**N/A**	**N/A**	**N/A**	**Insignificant association** OR=0.73 95% CI (0.52-1.02) [urban upbringing]
Feeney *et al*	**No significant association** OR=0.59 95% CI (0.28-1.24) [urban environment]	**N/A**	**N/A**	**N/A**
Gearry *et al*	**No significant association** (data not shown) (farm animal contact >4x/week)	**No significant association** (data not shown) (farm animal contact >4x/week)	**No significant association** (data not shown) (farm animal contact >4x/week)	**N/A**
	No significant association Town OR=0.88 95% CI (0.67-1.16) Country OR=0.86 95% CI (0.65-1.14)	**Town and country dwellers were at a significantly decreased rate of developing CD than city dwellers** Town OR=0.69 95% CI (0.51-0.94) adjusted Country OR=0.67 95% CI(0.50-0.90)unadjusted Country OR=0.64 95% CI (0.46-0.89) adjusted	**Town and country dwellers were at a significantly decreased rate of developing CD than city dwellers** Town OR=0.69 95% CI (0.51-0.94) adjusted Country OR=0.67 95% CI(0.50-0.90)unadjusted Country OR=0.64 95% CI (0.46-0.89) adjusted	**N/A**
Hlavaty *et al*	**N/A**	**N/A**	**N/A**	**Having no farm animals increased the risk of CD** CD patients had less frequent contact with dogs p=0.02, cats p=0.049 and cattle p=0.049
Lopez *et al*	**N/A**	**N/A**	**N/A**	**Urban dwelling associated with risk of CD** OR=4.58 95% CI (2.17-10.0)
Malekzadeh *et al*	**N/A**	**N/A**	**N/A**	**No significant association** Urban/rural Cases 89%/11% Controls 85%/15%
Timm *et al*	**Living on a livestock farm was protective compared to city and village life** OR=0.54 95% CI (0.31-0.94)	**N/A**	**N/A**	**N/A**
Wulzermann *et al*	**City dwellers significantly associated with CD development** OR=1.80 95% CI (1.1-3.0) p=0.031	**Suburbanites most significantly associated with CD development** OR=1.70 95% CI (1.0.-3.0) p=0.048	**City dwellers significantly associated with CD development** OR=2.0 95% CI (1.1-3.8) p=0.047	**N/A**

* Non-shaded areas denote farm animal contact; shaded areas denote place of upbringing.

APPENDIX XVII: Sibship Size

	0-5 years	6-10 years	11-18 years	'childhood' (0-18)
Bernstein *et al*	**N/A**	**N/A**	**N/A**	CD patients were less likely to have **brothers** (mean=1.67) than controls (mean=2.12) OR=0.78, 95% CI (0.67-0.9) p<0.0001 or **sisters** (1.77) than controls (mean=2.11) OR=0.82 95% CI (0.7-0.96) p=0.0126
Castiglione *et al*	**N/A**	**N/A**	**N/A**	**No significant association** Number of brothers /sisters (≤1vs≥2) OR=1.02 (0.76-1.37)
Feeney *et al*	**No significant association** OR=0.59, 95% CI (0.28-1.24) p=0.164	**N/A**	**N/A**	**N/A**
Gearry *et al*	**No significant association** (data not shown)	**No significant association** (data not shown)	**No significant association** (data not shown)	**N/A**
Hampe *et al*				**No significant association** X^2 =0.48, 1df, *P*>0.20
Han *et al*	**No significant association** (data not shown)	**No significant association** (data not shown)	**No significant association** (data not shown)	**N/A**
Hlavaty *et al*	**N/A**	**N/A**	**N/A**	**CD patients had significantly less number of siblings compared to controls** 1.6+/-1.3 vs 1.9+/-1.3 respectively at p<0.05
Lopez *et al*	**N/A**	**N/A**	**N/A**	**No significant association** OR 0.6, 95% CI (0.2-1.7) p=0.31
Malekzadeh *et al*	**N/A**	**N/A**	**N/A**	**No significant association** Median of CD patients vs controls 5(4-7) and 6(5-7) respectively
Persson *et al*	**N/A**	**N/A**	**N/A**	**No significant association** OR 1.8, 95% CI (1.0-3.4)

APPENDIX XVIII: PRISMA-E 2012 Checklist

Checklist of Items for Reporting Equity-Focused Systematic Reviews			
Section	**Item**	**Standard PRISMA Item**	**Extension for Equity-Focused Reviews**
Title			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	Identify equity as a focus of the review, if relevant, using the term equity
Abstract			
Structured summary	2	2. Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	State research question(s) related to health equity.
	2A		Present results of health equity analyses (e.g. subgroup analyses or meta-regression).
	2B		Describe extent and limits of applicability to disadvantaged populations of interest.
Introduction			
Rationale	3	Describe the rationale for the review in the context of what is already known.	Describe assumptions about mechanism(s) by which the intervention is assumed to have an impact on health equity.
	3A		Provide the logic model/analytical framework, if done, to show the pathways through which the intervention is assumed to affect health equity and how it was developed.
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	Describe how disadvantage was defined if used as criterion in the review (e.g. for selecting studies, conducting analyses or judging applicability).
	4A		State the research questions being addressed with reference to health equity
Methods			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	
Eligibility criteria	6	6. Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	Describe the rationale for including particular study designs related to equity research questions.
	6A		Describe the rationale for including the outcomes - e.g. how these are relevant to reducing inequity.
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	Describe information sources (e.g. health, non-health, and grey literature sources) that were searched that are of specific relevance to address the equity questions of the review.
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Describe the broad search strategy and terms used to address equity questions of the review.
Study	9	State the process for selecting studies (i.e., screening, eligibility, included in	

selection		systematic review, and, if applicable, included in the meta-analysis).	
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	List and define data items related to equity, where such data were sought (e.g. using PROGRESS-Plus or other criteria, context).
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	Describe methods of synthesizing findings on health inequities (e.g. presenting both relative and absolute differences between groups).
Risk of bias across studies	15	15. Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	Describe methods of additional synthesis approaches related to equity questions, if done, indicating which were pre-specified
Results			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Present the population characteristics that relate to the equity questions across the relevant PROGRESS-Plus or other factors of interest.
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	Present the results of synthesizing findings on inequities (see 14).

Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	Give the results of additional synthesis approaches related to equity objectives, if done, (see 16).
Discussion			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	Present extent and limits of applicability to disadvantaged populations of interest and describe the evidence and logic underlying those judgments.
	26A		Provide implications for research, practice or policy related to equity where relevant (e.g. types of research needed to address unanswered questions).
Funding			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	

APPENDIX XIX: What question (PICO) did the systematic review address?	
What is best?	Where do I find the information?
The main question being addressed should be clearly stated. The exposure, such as a therapy or diagnostic test, and the outcome(s) of interest will often be expressed in terms of a simple relationship.	The ***Title, Abstract*** *or final paragraph of the* ***Introduction*** should clearly state the question. If you still cannot ascertain what the focused question is after reading these sections, search for another paper!
This paper: Yes ☐ No ☐ Unclear ☐ Comment:	
F - Is it unlikely that important, relevant studies were missed?	
What is best?	Where do I find the information?
The starting point for comprehensive search for all relevant studies is the major bibliographic databases (e.g., Medline, Cochrane, EMBASE, etc) but should also include a search of reference lists from relevant studies, and contact with experts, particularly to inquire about unpublished studies. The search should not be limited to English language only. The search strategy should include both MESH terms and text words.	The ***Methods*** section should describe the search strategy, including the terms used, in some detail. The ***Results*** section will outline the number of titles and abstracts reviewed, the number of full-text studies retrieved, and the number of studies excluded together with the reasons for exclusion. This information may be presented in a figure or flow chart.
This paper: Yes ☐ No ☐ Unclear ☐ Comment:	
A - Were the criteria used to select articles for inclusion appropriate?	
What is best?	Where do I find the information?
The inclusion or exclusion of studies in a systematic review should be clearly defined a priori. The eligibility criteria used should specify the patients, interventions or exposures and outcomes of interest. In many cases the type of study design will also be a key component of the eligibility criteria.	The ***Methods*** section should describe in detail the inclusion and exclusion criteria. Normally, this will include the study design.
This paper: Yes ☐ No ☐ Unclear ☐ Comment:	

APPENDIX XIX

A - Were the included studies sufficiently valid for the type of question asked?	
What is best?	Where do I find the information?
The article should describe how the quality of each study was assessed using predetermined quality criteria appropriate to the type of clinical question (e.g., randomization, blinding and completeness of follow-up)	The ***Methods*** section should describe the assessment of quality and the criteria used. The ***Results*** section should provide information on the quality of the individual studies.
This paper: Yes □ No □ Unclear □ Comment:	
T - Were the results similar from study to study?	
What is best?	Where do I find the information?
Ideally, the results of the different studies should be similar or homogeneous. If heterogeneity exists the authors may estimate whether the differences are significant (chi-square test). Possible reasons for the heterogeneity should be explored.	The ***Results*** section should state whether the results are heterogeneous and discuss possible reasons. The forest plot should show the results of the chi-square test for heterogeneity and if discuss reasons for heterogeneity, if present.
This paper: Yes □ No □ Unclear □ Comment:	

"The ultimate measure of a man is not where he stands in moments of comfort and convenience, but where he stands at times of challenge and controversy."

Dr Martin Luther King Jr