The Gastrointestinal Microbiota and Immunity with Treatment in Paediatric Inflammatory Bowel Disease

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Declaration

I, Intan Faizura Yeop, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed

Date

……July 2019
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Abstract

Intestinal microbiota is strongly implicated in IBD pathogenesis. These microbes exert their influence directly and indirectly through their metabolites which include short chain fatty acids (SCFA). These interactions can be mutually beneficial but can also be detrimental to the host. Combined with a dysregulated immune system, IBD manifests itself in genetically susceptible individuals.

These 3 interrelated factors, intestinal microbiota, SCFA and host immunity, were investigated to explore the extent of their associations. Intestinal luminal (stool and duodenal lavages) and mucosal biopsies were collected prospectively from treatment-naive IBD patients, some before diagnosis. Their clinical histories, results and dietetic assessment were also analysed. Dynamic changes in the intestinal microbial profiles with inflammation and treatment were compared to healthy and non-inflammatory control children. The corresponding alterations in stool SCFA, plasma cytokine and vascular profiles were identified.

Dysbiosis was characterised at phyla level, and disease-specific microbial profiles in Crohn’s Disease (CD) and Ulcerative colitis (UC) were recorded. The relative abundances of the 3 main phyla (Bacteroidetes, Firmicutes and Proteobacteria) varied with disease activity. Proteobacteria abundance increased with inflammation.

Distinct differences in SCFA profiles were observed between control groups and IBD, with distinguishing features evident between CD, UC and ileostomy samples. Inflammation appears to influence SCFA production, particularly butyrate. In addition, pro-inflammatory cytokines and vascular markers mirrored trends in clinical inflammatory markers, and some correlated with SCFA percentages.

In conclusion, alterations in intestinal microbial and SCFA profiles appear to influence the systemic cytokine milieu. Larger cohort studies are needed to confirm and further explore these findings if we are to utilise microbial and immune profiles in our quest for personalised treatment of children with IBD.
Impact Statement

Inflammatory Bowel Disease (IBD), encompassing Crohn’s Disease (CD) and Ulcerative Colitis (UC), is a chronic gastrointestinal inflammatory condition, affecting genetically susceptible individuals with varying severity. Approximately £470 million/year was spent treating the 620,000 IBD patients, of which half was spent on the small number of patients with severe disease. This underestimated cost has likely increased further with the rising incidence.

Paediatric IBD (PIBD), with an incidence of 10.5 per 100 000/year, accounts for 25% of all IBD patients. Disease extent and severity are generally greater in children, and affect their health, growth, development, education and wellbeing.

IBD occurs in individuals with various genetic mutations, different risk factors and manifests in varying ways, reflecting the diverse pathophysiology of IBD. Yet there are just two treatment guidelines for managing PIBD. Personalised medicine, tailoring treatment depending on risk stratification, is the obvious direction for future IBD treatment.

Intestinal microbiota is implicated in many aspects of IBD. Published data provides insight of events occurring at the mucosal level. However, patient numbers are small and the different methodologies used can influence results. In addition, the various centres report alterations in the lower taxa e.g. family, genera and species. These changes in abundances may be useful biomarkers, but occur as part of wider shifts in the microbial community, leaving the bigger picture of IBD pathogenesis incomplete.

This longitudinal study set out to understand these microbial shifts with disease activity and treatment in PIBD. Luminal and mucosal microbiota were analysed for potential associations with microbial metabolites short chain fatty acids (SCFA), inflammation and treatments. Clinical and dietetic factors were also investigated. Samples collected from control groups were analysed concurrently.

The main finding of the present study was the shifts in the three main bacterial phyla, which altered depending on the disease, disease severity and in response to treatment. Additional highlights included:

1. Improved characterisation of stool versus mucosal profiles in CD and UC. Confirmation in a bigger cohort would be needed for its potential use in clinical practice for predicting and monitoring disease course.
2. The significant impact of commonly-used antibiotics on butyrate-producing microbes, serving as a prompt for the medical community to consider how antibiotics can be better prescribed
3. The possible influence of food quality on IBD risk, thus suggesting potential benefits with more dietetic involvement in IBD management
4. The potential contribution of stress as a trigger for IBD

This study has contributed to our understanding of;
1. UC pathogenesis, highlighting potential benefits of butyrate in UC and the defunctioned colon post-surgery
2. Application of Exclusive Enteral Nutrition as a means to re-programme intestinal microbiota

This study highlighted factors worth considering in IBD research;
1. Non-IBD control patients e.g. with Irritable Bowel Syndrome, are also in an inflammatory state, with microbial dysbiosis and altered SCFA profile
2. Stool and mucosal microbial profiles differ in individual IBD patients

With limited NHS resources, individuals are more responsible for their health. It is thus imperative that the knowledge gained from this study is disseminated widely, targeting those most likely to benefit.
Acknowledgements

My life is blessed with some of the most amazing people.

Thank you for being part of the journey.

It has indeed been an incredible journey. I would like to thank my supervisors, Dr Mona Bajaj-Elliott and Prof Nigel Klein, for giving me this opportunity. I am grateful for their time, guidance and support throughout the years, and appreciate their willingness for my independence to develop this study.

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<td>Acetyl Coenzyme A</td>
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<tr>
<td>AHR</td>
<td>Aryl hydrocarbon ligands</td>
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<tr>
<td>A-IBS</td>
<td>Alternate IBS</td>
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<tr>
<td>AIEC</td>
<td>Adherent intestinal E. coli</td>
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<tr>
<td>AIEC</td>
<td>Adherent-invasive <em>Escheria coli</em></td>
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<td>AMP</td>
<td>Anti-microbial protein</td>
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<td>AMP</td>
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<td>Anti-Saccharomyces cerevisiae antibodies</td>
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<tr>
<td>ATG16L1</td>
<td>Autophagy-related 16-like 1</td>
</tr>
<tr>
<td>B.</td>
<td>Bacteroides</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>B:P</td>
<td>Bacteroidetes:Proteobacteria</td>
</tr>
<tr>
<td>BCFA</td>
<td>Branched chain fatty acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>Breg</td>
<td>Regulatory B cells</td>
</tr>
<tr>
<td>BSPGHAN</td>
<td>British Society of Paediatric Gastroenterology, Hepatology, and Nutrition</td>
</tr>
<tr>
<td>C.</td>
<td>Candida</td>
</tr>
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<td>C.</td>
<td>Clostridioides</td>
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<tr>
<td>CAPS</td>
<td>Cryopyrin-Associated Periodic Syndrome</td>
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<tr>
<td>CARD15</td>
<td>Caspase recruitment domain-containing protein 15</td>
</tr>
<tr>
<td>Cav-1</td>
<td>Caveolin-1</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's Disease</td>
</tr>
<tr>
<td>CDAI</td>
<td>Crohn's disease activity index</td>
</tr>
<tr>
<td>CDED</td>
<td>Crohn's Disease exclusion diet</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming Unit</td>
</tr>
<tr>
<td>CH₄</td>
<td>Methane</td>
</tr>
<tr>
<td>C-IBS</td>
<td>IBS with constipation</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxymethyl cellulose</td>
</tr>
<tr>
<td>CMPA</td>
<td>Cow’s milk protein allergy</td>
</tr>
<tr>
<td>CMPA</td>
<td>Cow’s milk protein allergy</td>
</tr>
<tr>
<td>CoeD</td>
<td>Coeliac disease</td>
</tr>
<tr>
<td>COSHH</td>
<td>Control of Substances Hazardous to Health</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>COSHH</td>
<td>Control of Substances Hazardous to Health</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>C-RPMI</td>
<td>Complete RPMI</td>
</tr>
<tr>
<td>CT</td>
<td>Cycling Threshold</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>Cholesterol 7 α-hydroxylase</td>
</tr>
<tr>
<td>D</td>
<td>Duodenum</td>
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<tr>
<td>DC</td>
<td>Dendritic cells</td>
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<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>D-IBS</td>
<td>IBS with diarrhoea</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DP-IBS</td>
<td>Diarrhoea-predominant irritable bowel syndrome</td>
</tr>
<tr>
<td>DR3</td>
<td>Death domain receptor 3</td>
</tr>
<tr>
<td>EAR</td>
<td>Estimated Average Requirement</td>
</tr>
<tr>
<td>EC</td>
<td>Extraction Control</td>
</tr>
<tr>
<td>EEN</td>
<td>Exclusive Enteral nutrition</td>
</tr>
<tr>
<td>EHF</td>
<td>Extensively hydrolysed formula</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EN</td>
<td>Enteral nutrition</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EOIBD</td>
<td>Early onset IBD</td>
</tr>
<tr>
<td>EPS</td>
<td>Exo-polysaccharide</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERA</td>
<td>Enthesitis-related arthritis</td>
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<tr>
<td>ESPGHAN</td>
<td>European Society of Paediatric Gastroenterology, Hepatology, and Nutrition</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
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<tr>
<td>F.</td>
<td>Faecalibacterium</td>
</tr>
<tr>
<td>F:P</td>
<td>Firmicutes:Proteobacteria</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>Ffar</td>
<td>Free fatty acid receptor</td>
</tr>
<tr>
<td>FMT</td>
<td>Faecal microbial transplantation</td>
</tr>
<tr>
<td>FODMAP</td>
<td>Fermentable Oligo-, Di-, Mono-saccharides And Polyols</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>FUT2</td>
<td>Fucosyltransferase 2</td>
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<tr>
<td>GALT</td>
<td>Gut associated lymphoid tissue</td>
</tr>
<tr>
<td>GBA</td>
<td>Gut-brain axis</td>
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<tr>
<td>GC</td>
<td>Glucocorticosteroids</td>
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<tr>
<td>GF</td>
<td>Germ-free</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GIt</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
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<td>GLP-2</td>
<td>Glucagon-like peptide 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>-------------</td>
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</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<td>GOSH</td>
<td>Great Ormond Street Hospital</td>
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<tr>
<td>GPRs</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptors</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>H+</td>
<td>Hydrogen ion</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferases</td>
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<td>HC</td>
<td>Healthy Control</td>
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<td>HDACs</td>
<td>Histone deacetylases</td>
</tr>
<tr>
<td>HI</td>
<td>Healthy Infant</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HIMEC</td>
<td>Human intestinal microvascular endothelial cells</td>
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<tr>
<td>HMGCR</td>
<td>3-hydroxy-3-methylglutaryl-CoA reductase</td>
</tr>
<tr>
<td>HMGCS</td>
<td>Hydroxymethylglutaryl-CoA synthase</td>
</tr>
<tr>
<td>HPA axis</td>
<td>Hypothalamus-pituitary-adrenal axis</td>
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<td>HSCT</td>
<td>Haematopoietic stem cell transplantation</td>
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<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
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<tr>
<td>IBD-AID</td>
<td>IBD-anti-inflammatory diet</td>
</tr>
<tr>
<td>IBDU</td>
<td>Inflammatory Bowel Disease Undetermined</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable Bowel Syndrome</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICH</td>
<td>Institute of Child Health</td>
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<tr>
<td>IEC</td>
<td>Intestinal epithelial cell</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IgG1</td>
<td>Immunoglobulin G1</td>
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<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IRGM</td>
<td>Immunity-related GRP as family M</td>
</tr>
<tr>
<td>JIA</td>
<td>Juvenile Idiopathic Arthritis</td>
</tr>
<tr>
<td>JIA</td>
<td>Juvenile idiopathic arthritis</td>
</tr>
<tr>
<td>L.</td>
<td>Lactobacillus</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic-acid-producing bacteria</td>
</tr>
<tr>
<td>LC</td>
<td>Left colon</td>
</tr>
<tr>
<td>LLOD</td>
<td>Lower limit of detection</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lower limit of quantification</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propia</td>
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<tr>
<td>LPS</td>
<td>Liposaccharide</td>
</tr>
<tr>
<td>M cell</td>
<td>Microfold cell</td>
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<tr>
<td>MAdCAM-1</td>
<td>Mucosal addressin cell adhesion molecule-1</td>
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<tr>
<td>MAMP</td>
<td>Microbial-associated molecular patterns</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MCFA</td>
<td>Medium chain fatty acid</td>
</tr>
<tr>
<td>MD-index</td>
<td>Microbial Dysbiosis Index</td>
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<td>MDX</td>
<td>Maltodextrin</td>
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<td>MHC</td>
<td>Major histocompatibility</td>
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MLN  Mesenteric lymph node
MSD  MesoScale Discovery
mTOR  Mammalian target of rapamycin
MUC-2  Mucin-2
Na  Sodium
nf-H₂O  Nuclease-free water
NHE3  Na hydrogen exchanger-3
NHS  National Health Service
NIC  Non-inflammatory control
NK cell  Natural Killer cell
NKG2D–MICA  Natural killer-activating receptor-major histocompatibility-class I chain-related gene A
NLR  Nucleotide binding domain and leucine-rich repeat-containing receptors
NMDS  Non-metric multidimensional scaling
NOD2  Nucleotide-binding oligomerization domain-containing protein 2
NRES  National Research Ethics Service
OD  Optical Density
OD  Optical density
OPA  Orthophosphoric acid
osc/min  Oscillations per minute
OUT  Operational Taxonomic Unit
P.  Pseudomonas
P.  Pneumocystis
pAMPK  Phosphorylated adenosine monophosphate-activated protein kinase
PBMC  Peripheral blood mononuclear cells
PBS  Phosphate buffer solution
PCDAI  Paediatric Crohn's Disease Activity Index
PCR  Polymerase Chain Reaction
PEN  Partial Enteral Nutrition
PGC  Proliferator-activated receptor gamma coactivator
PGE1  Prostaglandin E1
PIBD  Paediatric Inflammatory Bowel Disease
PMA  PMAPhorbol 12-myristate 13-acetate
PPI  Proton-pump inhibitor
PPI  Proton-pump inhibitor
PRR  Pattern recognition receptor
PSA  Polysaccharide A
PUCAI  Paediatric Ulcerative Colitis Activity Index
PUFA  Polyunsaturated fatty acids
PYY  Peptide-YY
R.  Ruminicoccus
R.  Rumunicoccus
R²  Coefficient of Determination
RA  Rheumatoid Arthritis
RC  Right colon
rDNA  Ribosomal DNA
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Real-time PCR</td>
<td>Real-time polymerase chain reaction</td>
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<td>REC</td>
<td>Research Ethics Committee</td>
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<tr>
<td>Reglllc</td>
<td>Regenerating protein-IIIc</td>
</tr>
<tr>
<td>RELMβ</td>
<td>Resistin-like molecule β</td>
</tr>
<tr>
<td>RNI</td>
<td>Reference Nutrient Intake</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute (Culture medium)</td>
</tr>
<tr>
<td>S.</td>
<td>Streptococcus</td>
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<tr>
<td>SCBU</td>
<td>Special Care Baby Unit</td>
</tr>
<tr>
<td>SCD</td>
<td>Specific carbohydrate diet</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short chain fatty acids</td>
</tr>
<tr>
<td>SFB</td>
<td>Segmented filamentous bacteria</td>
</tr>
<tr>
<td>slgA</td>
<td>Secretory IgA</td>
</tr>
<tr>
<td>SMCT1</td>
<td>Sodium-dependent monocarboxylate transporter 1</td>
</tr>
<tr>
<td>SMD</td>
<td>Small molecule drug</td>
</tr>
<tr>
<td>sp.</td>
<td>species (singular)</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
<tr>
<td>spp.</td>
<td>species (plural)</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>tDC</td>
<td>Tolerogenic dendritic cells</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TI</td>
<td>Terminal ileal</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>TL1A</td>
<td>TNF-like ligand 1A</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
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<td>TMAO</td>
<td>Trimethylamin oxide</td>
</tr>
<tr>
<td>TMP</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>tTG</td>
<td>Tissue transglutaminase</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>UCH</td>
<td>University College Hospital</td>
</tr>
<tr>
<td>ULOD</td>
<td>Upper limit of detection</td>
</tr>
<tr>
<td>ULOQ</td>
<td>Upper limit of quantification</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEOIBD</td>
<td>Very Early Onset IBD</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous thromboembolism</td>
</tr>
<tr>
<td>wPCDAI</td>
<td>Weighted Paediatric Crohn's Disease Activity Index</td>
</tr>
</tbody>
</table>
1. Chapter 1

Introduction

1.1 Introduction

We coexist with a complex community of commensal, symbiotic and pathogenic microorganisms that reside on and within us. These bacteria, archaea, protists, fungi and viruses make up our unique microbiome. Their number exceeds our human cells by a factor of 10 (1), contributing to several immunological, hormonal and metabolic functions in beneficial and sometimes in harmful ways. It is therefore unsurprising that the human microbiome has been referred to as an organ in itself.

1.1.1 The History of Gastrointestinal Microbiota

Long before Antoni van Leeuwenhoek discovered microbes, “God’s smallest creatures”, in his stool, stool has been used for medicinal purposes. The earliest documented treatment utilising stool was Yellow Soup administered to patients with severe food poisoning and diarrhoea in the 4th century in China (2). In the 16th century, Li Shizhen, a Chinese doctor, included Yellow Soup in his compilation of remedies used for gastrointestinal (GI) conditions such as diarrhoea, vomiting and constipation. These conditions were reported to be effectively treated with suspensions of fresh, fermented and dry stool from adults and infants (3)(Ben Cao Gang Mu, Compendium of Materia Medica). Similarly, Bedouins used fresh, warm camel stool for gastroenteritis. Egyptian
doctors have also used animal stool to ward off evil spirits. Such treatments occasionally resulted in tetanus but were largely considered beneficial.

Further west, in the 17th century, an Italian surgeon introduced the concept of transferring GI contents from a healthy animal to a sick one, a procedure referred to as transfusionation. Rumen transfusionation was used to treat cows with mastitis, and caecal transfusionation was used to treat horses with chronic diarrhoea. A German physician, Christian Franz Paullini (Heilsame Dreck-Apotheke or Salutary Filth-Pharmacy), reported on medicinal properties of bodily secretions including stool for treating dysentery. Russian zoologist, Metchnikoff, introduced fermented products into this diet after hearing of longevity in Bulgarian farmers. The subsequent improved general health was hypothesised to be due to increased lactic acid bacteria, *Lactobacillus bulgaricus*, which was successfully marketed as the first documented probiotics.

In the 1940s, when German soldiers of the Afrikakorps were dying of dysentery, German scientists observed the locals improving after consuming fresh camel stools. Their analysis led to the isolation of *Bacillus subtilis*, which was cultured and administered to good effect. The practice of faecal transplantation was still unacceptable in the west. In the early 1950s, recognising the side effects of antibiotics, bacteriologist Stanley Falkow used pre-procedure stool to prepare pills that were subsequently prescribed to half the patients post-operatively, with the aim of restoring pre-procedural microbial population. The results were never published but anecdotal evidence indicated better outcomes in those treated, however the discovery of this trial by the administrative authorities led to his dismissal.

The tide started to change following this. Stool transfer evolved to faecal microbiota transplantation (FMT). In the 1950s, Dr Eiseman, a Colorado surgeon, resorted to stool of healthy donors delivered as an enema for 4 patients who were critically ill with refractory pseudomembranous colitis, now known to be caused by *Clostridium difficile*. The immediate and complete recovery of these patients were later confirmed with 16 further patients (4). Several subsequent studies on FMT have reported similarly remarkable outcomes (5-7), with the most notable being the Dutch study, which was stopped earlier than planned in 2013 due to reports of 81% resolution after 1 FMT and over 90% resolution after a second (8).

Following this success, FMT has been trialled in other conditions (9, 10) including IBD. Thomas Barody started treating IBD patients with FMT in 1988, starting with a UC patient who then achieved long-term remission (11). The numbers extended to 55 patients with
UC and CD as well as constipation, diarrhoea and abdominal pain (12). Following FMT, 20 patients of undefined groups were reportedly “cured” in addition to 9 who had improved symptoms (13).

Centuries after Antoni van Leeuwenhoek discovered stool microbes, there was significant investments to characterise the human microbiota including the microbiota within the GI tract. The Human Microbiome Project boosted our awareness of these microbes within us, spearheading thousands of subsequent studies in the quest of understanding the effects of the commensal microbiota on the human body and functions.

1.1.2 The Evolution of the Hygiene Hypothesis

The Hygiene Hypothesis was first described in 1989 by Strachan (14). Children with more siblings were less likely to develop hayfever or eczema, suggesting that infections in early childhood contracted from siblings may protect against these conditions. Opportunities for infections have reduced with smaller family sizes, improved household amenities and personal hygiene.

The Hygiene Hypothesis was further strengthened by the concept of postnatal immune deviation. The neonatal immune system is dominated by Th2 (allergy-promoting) than Th1 (allergy-inhibiting) responses but this can be shifted to more prominent Th1 responses with infections in early life (15). Subsequently, allergic diseases have been linked to mode of delivery, infant feed, exposure to pets and antibiotic use; all are factors known to modulate the gut microbiota. The Hygiene Hypothesis thus evolved into the Microbiota Hypothesis (16) and has been applied to the risk of developing other immune diseases such as IBD.

1.2 Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is a spectrum of heterogeneous, chronic GI inflammatory conditions predominantly consisting of Crohn’s Disease (CD), Ulcerative Colitis (UC) and Inflammatory Bowel Disease Undetermined (IBDU). CD can occur anywhere in the GI tract whereas UC predominantly affects the colon. When colitis is atypical of CD or UC, IBDU is diagnosed.
Paediatric IBD (PIBD) accounts for 25% of all IBD patients (17). The incidence of PIBD continues to increase, currently reported as 10.5 per 100 000 per year (18). Worldwide, paediatric CD incidence was estimated as 2.5 – 11.4 per 100 100/year, with a prevalence of 58 per 100 000/year (19, 20). The incidence of paediatric UC is less, estimated at 1-4 per 100 000/year, accounting for 15-20% of all UC patients in North America and European regions (20).

With an estimated 620,000 people affected in the UK (21), the NHS spends £470 million annually (22), half of which is spent on inpatient management of a minority of patients (23). Given that disease extent and severity are greater in children than adults, children with IBD are more likely to be hospitalised and require treatment escalation (24-28).

1.2.1 Presentation of Inflammatory Bowel Disease

Children with IBD are diagnosed at a median age of 13.5 years (IQR 11.08-15.14 years) (18), with a greater proportion of Asian children having UC than other children (29). PIBD can be more aggressive and extensive, and can be associated with significant morbidity (24).

In UC, continuous mucosal inflammation extends proximally from the distal colon. In contrast, the transmural inflammation in CD is associated with strictures, ulcers, fistulae and granulomas, and can occur anywhere in the GI tract. Extra-intestinal manifestations such as arthritis, uveitis and skin changes can coexist with IBD (Figure 1.1). Patients can present with generalised symptoms such as anorexia, lethargy, pyrexia and unintentional weight loss in addition to GI symptoms of abdominal pain, cramping, diarrhoea, blood per rectum and stooling urgency. In CD, patients may also present with mouth ulcers, constipation, fistula and anaemia. UC patients also report tenesmus and may have associated sclerosing cholangitis.
1.2.2. Pathogenesis of Inflammatory Bowel Disease

Despite extensive research in the field, IBD pathogenesis remains elusive. What is known, however, is that IBD occurs in genetically susceptible individuals with compromised mucosal barrier function, impaired bacterial handling and inappropriate immune responses to pathogenic bacteria. In addition, with greater understanding of the gut microbiota, it is now well-accepted that gut dysbiosis is a major factor in IBD pathogenesis (30, 31).

1.2.2.1 Genetic Susceptibility

Population-based genome-wide association studies (GWAS) have shed light on more than 160 IBD-associated loci (71 in CD and 47 in UC) (32), accounting for 23% and 16% of disease heritability respectively (33, 34). These discoveries have reiterated the important roles of barrier function, bacterial handling and immune cell signalling (35, 36). These studies have also led to new genes and pathways in IBD pathogenesis such as autophagy and the IL-23 signalling pathway.

In addition, IBD GWAS have revealed significant overlap between loci for CD and UC as well as IBD and other immune-mediated diseases (Figures 1.2.,1.3)(37). There have
also been studies investigating monogenic IBD presenting in early childhood such as mutations in the IL-10 pathway. Gut inflammation is one of the manifestations of these diseases of immune dysregulation.

The relatively low percentage of patients with affected relatives (6-33%) and low concordance between monozygotic twins in UC (16%) and CD (35%)(38), suggests other factors are at play.

1.2.2.2 Barrier function

There is a single layer of epithelial cells separating the host from the intestinal intraluminal contents. The mucus layer has several roles in maintaining barrier integrity but is known to be altered in IBD patients (39). This may be associated with MUC19 gene and certain bacterial species such as Akkermansia muciniphila and Enterorhabdus mucosicola, which degrades the mucus layer (40, 41). With compromised barrier function and altered luminal bacterial composition, IBD patients are susceptible to invasion by pathogenic bacteria.

Figure 1. 2: Genetic Mutations in IBD with Overlapping Loci in CD and UC, as adapted from Kautnitz et al. (42)
1.2.2.3 Bacterial handling

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) gene (or CARD15), was the first gene to be associated with increased susceptibility to CD (44). It is involved in bacterial recognition and stimulating the immune system in the presence of bacterial-derived peptidoglycans as well as autophagy. Since then, several NOD2 mutations have been recognised as being pathogenic in CD (40, 45).

IL-23 has more recently been recognised to play a major role in coordinating a range of innate and adaptive immune responses at the GI mucosal surfaces, especially against bacterial infections (46). It has also been found to regulate repair of the intestinal epithelium but when sustained at high levels, it can lead to chronic inflammatory responses seen in IBD (47), highlighting the importance of immune regulation and homeostasis.
1.2.2.4 Microbial Dysbiosis

The gut microbiome includes bacteria, viruses, archaea and eukaryotic cells. Our current understanding of the bacterial population is considerably more than that of other organisms. This thesis focuses on the microbial dysbiosis in IBD, which is discussed in further detail in Chapter 3, Section 3.1.

1.2.2.5 Immune Dysregulation

The immune dysregulation seen in IBD includes an exaggerated response to pathogens leading to epithelial damage. Impaired epithelial barrier can lead to further invasion of pathogenic and opportunistic pathogens present in the gut lumen. The composition of luminal microbiota is thus an important factor, with dysbiosis being a major contributing factor to IBD. It is now well established that on-going exposure to the bacteria, the resulting inflammation and chronic state of immune dysregulation is a central feature central to IBD pathogenesis.

The chronic inflammatory state can also be caused by impaired host sensing of bacterial products and metabolites via defects in SLC22A5A, G protein-coupled receptor 35 (GPR35) or GPR65 (48). In addition, impaired autophagy may result in commensal organisms being seen as pathogenic by the host. Autophagy is a regulated process of removing and degrading unnecessary or dysfunctional cellular components in the body. Mutations in loci containing immunity-related GRP as family M (IRGM), autophagy-related 16-like 1 (ATG16L1) and NOD2 are associated with increased CD risk (40, 49). The role of immune dysregulation in IBD is discussed further in Chapter 5.

1.2.3 Risk factors for Developing Inflammatory Bowel Disease

Although the cause of IBD remains unknown, several risk factors have been identified. As mentioned above, family history of IBD i.e. genetic mutations increase the risk of developing IBD. In addition, environmental factors play a major part in contributing to disease development, trigger and progression (Table 1.1).

These factors are at play from birth. Perinatal infections are positively associated with IBD development. Vaginal mode of delivery and breastfeeding have been reported as protective of IBD (50). Fruit, vegetables and fibre are beneficial in CD, whilst sugars and
fats increase the risk of IBD. Interestingly, smoking increases the risk of CD but protects against UC (51). These factors are known to modulate the gut microbiota.

Commonly-used medications can also contribute to the development of IBD. Antibiotics, such as Metronidazole, Sulphonamides, Trimethoprim, Macrolides, Tetracyclines and Cephalosporin, have been reported to increase the risk of IBD, especially in early childhood (52, 53). More recent evidence suggests that antibiotics per se does not increase the risk of CD or UC but its use under 5 years of age may increase the risk of CD (54). Antibiotics have been demonstrated to lead to dysbiosis lasting for weeks/months (55, 56), thus repeated courses in early childhood may well affect the intestinal microbiota and immune development. Non-steroidal anti-inflammatory agents and oral contraceptive pills also increase the risk.

Interestingly, appendectomy is protective of UC but increases the risk of CD (51, 57, 58). Although the aetiology of this is unknown, there are a few theories that may explain this. The appendix, being an important part of the gut-associated lymphoid system and morphologically similar to Peyer’s patches, is a reservoir of enteric bacteria and involved in the recognition of luminal antigens and development of mesenteric lymph nodes (59). Without an appendix, experimental rabbits were found to have less IgA-producing plasma cells and IgA secretion (60) thus subsequently affecting bacterial handling. Appendicitis, possibly a Th1-mediated immune response, may contribute to a shift towards CD, a predominantly Th1-mediated disease, rather than UC, which is predominantly Th2-mediated (61, 62). Potentially, suppressor cells generated during appendicitis may later suppress mucosal inflammation in those susceptible to UC (63). It may also be possible that subclinical CD was not recognised and misdiagnosed as appendicitis (62).

Specific infective organisms have been associated with CD but in both, CD and UC, preceding gastroenteritis is a known factor for developing and triggering disease. These are likely to disrupt the gut microbiome thus potentially leading to the dysbiosis recognised to predispose susceptible individuals to IBD (51).
Table 1: Environmental Factors in Inflammatory Bowel Disease, as adapted from Molodecky et al. (51, 64)

<table>
<thead>
<tr>
<th>Environmental Factors</th>
<th>Crohn’s Disease</th>
<th>Ulcerative Colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breastfeeding</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sugars</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fats</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fruit and vegetables</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fibre</td>
<td>-</td>
<td>Null</td>
</tr>
<tr>
<td>Smoking</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Medications/Treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsteroidal anti-inflammatory drugs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oral contraceptive pill</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Appendectomy</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Infections</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior gastroenteritis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mycobacterium avium paratuberculosis</td>
<td>+</td>
<td>Null</td>
</tr>
<tr>
<td>Adherent invasive Escherichia coli</td>
<td>+</td>
<td>Null</td>
</tr>
<tr>
<td>Psychrotrophic bacteria</td>
<td>+</td>
<td>Null</td>
</tr>
<tr>
<td>Perinatal infections</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Hygiene Hypothesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family size</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number of siblings</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Birth order</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pets</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urban environment</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Helicobacter pylori</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Helminths</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Stress</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depression</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anxiety</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

With better understanding of the gut microbiome, the Hygiene Hypothesis has resurfaced. Bigger families, more siblings, higher birth order and pets are home environmental factors that increase exposure to dirt and organisms in early childhood thus influencing immune development during this critical period. Colonisation with *Helicobacter pylori* and helminths are also protective against IBD (51).

Psychological stress is increasingly recognised as an exacerbating factor and a potential trigger for IBD and disease relapse, perhaps through altering the immune function.
Studying this effect is a challenge and observational studies have been inconsistent thus most reported studies are on mice models. Evidence thus far suggests inflammation, the hypothalamus-pituitary axis and microbiota-gut-brain axis playing central roles in disease activation and progression (64).

1.2.4 Diagnosis of Inflammatory Bowel Disease

The diagnosis of IBD is confirmed based on histology of mucosal biopsies obtained during endoscopic assessment (Table 1.2). Endoscopic findings in CD include transmural skip lesions and fissure ulceration causing the typical cobblestone appearance, while in UC there is continuous colitis often worse distally.

Histologically, there are cryptitis, crypt abscesses, goblet cell depletion and distorted crypt architecture in UC. There is uniformly increased lymphocytic infiltrate in the mucosal layer and little submucosal inflammation. On the other hand, characteristic inflammatory changes in CD include patchy, heavy lymphocytic infiltrate, presence of granulomas, preserved crypt architecture and disproportionate submucosal inflammation.

<table>
<thead>
<tr>
<th>Histological Features</th>
<th>Crohn’s Disease</th>
<th>Ulcerative Colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytic infiltration</td>
<td>Patchy, increased</td>
<td>Uniformly increased</td>
</tr>
<tr>
<td>Crypt abscess</td>
<td>Scanty</td>
<td>Diffusely seen</td>
</tr>
<tr>
<td>Crypt architecture</td>
<td>Preserved</td>
<td>Distorted</td>
</tr>
<tr>
<td>Goblet cells</td>
<td>Normal</td>
<td>Depleted</td>
</tr>
<tr>
<td>Submucosal inflammation</td>
<td>Disproportionately increased</td>
<td>Little</td>
</tr>
<tr>
<td>Granulomas</td>
<td>Present</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Table 1.2: Diagnostic Histological Findings in Crohn’s Disease and Ulcerative Colitis, adapted from Magro et al. (65)

1.2.5 Treatment and Management of Inflammatory Bowel Disease

The aim of treatment in IBD is to induce and maintain remission. Escalation of treatment occurs, as per guidelines, to achieve deep mucosal healing (66-70).
Exclusive enteral nutrition (EEN) is the first-line therapy for CD patients but if EEN is unsuitable, corticosteroids may be used in moderate to severe disease and Budesonide may be used for mild to moderate ileocecal CD (70). For induction of remission in UC and IBDU, 5-aminosalicylic acid (5-ASA) can be used for mild to moderate disease and corticosteroids for moderate to severe disease (69). Patients often require an immunomodulator, such as Azathioprine or 6-Mercaptopurine, to maintain remission. For patients unresponsive to these or continue to relapse despite these, treatment is escalated to additional biologic agents, commonly anti-TNF agent Infliximab, Adalimumab or their biosimilars. Patients with fistulating CD also proceed to biologic agents. Newer biologic agents are increasingly being trialled in patients with IBD. This is further discussed in Chapter 5.

In severe disease, there may be further escalation to Sirolimus, Tacrolimus and Thalidomide in CD, and Cyclosporin or Tacrolimus, both calcineurin inhibitors, in UC. Rarely, patients refractory to medical therapies proceed to surgical resection of the inflamed bowel.

1.2.6 Monitoring Inflammatory Bowel Disease

Patients with IBD are monitored regularly to ensure continuing growth and development, treatment compliance and prompt treatment of relapses. Disease activity scores, weighted Paediatric Crohn’s Disease Activity Index (wPCDAI) and Paediatric Ulcerative Colitis Activity Index (PUCAI), are used to objectively assess these patients (Tables 1.3, 1.4).

Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are two commonly used clinical markers of systemic inflammation. ESR is increased in inflammation due to red cells sticking to the increased fibrinogen in blood. The levels can remain elevated for days until the excess fibrinogen is removed. CRP binds to phosphocholine expressed on dead or dying cells leading to activation of the complement pathway/ immune system and phagocytosis. CRP rises when there is increased rate of production, a reflection of the severity of the cause. Thus, CRP is a more sensitive marker of acute phase inflammation while ESR is more useful in chronic inflammation. Although these are commonly used for disease monitoring, it is not a sensitive marker of disease activity.
### Table 1.3: Weighted Paediatric Crohn’s Disease Activity Index (wPCDAI)

Remission - < 12.5, Mild - 12.5-40, Moderate – 41-57.5, Severe - >57.5

<table>
<thead>
<tr>
<th>Item Points/Score</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abdominal pain:</strong></td>
<td></td>
</tr>
<tr>
<td>No pain</td>
<td>0</td>
</tr>
<tr>
<td>Mild: Brief, does not interfere with activities</td>
<td>10</td>
</tr>
<tr>
<td>Moderate/Severe: Daily, longer lasting, affects activities, nocturnal</td>
<td>20</td>
</tr>
<tr>
<td><strong>Patient Functioning, General Well-Being</strong></td>
<td></td>
</tr>
<tr>
<td>No limitation of activities, well</td>
<td>0</td>
</tr>
<tr>
<td>Occasional difficulty in maintaining age appropriate activities, below par</td>
<td>10</td>
</tr>
<tr>
<td>Frequent limitation of activity, very poor</td>
<td>20</td>
</tr>
<tr>
<td><strong>Stools (per day)</strong></td>
<td></td>
</tr>
<tr>
<td>0–1 liquid stools, no blood</td>
<td>0</td>
</tr>
<tr>
<td>Up to 2 semi-formed with small blood, or 2-5 liquid</td>
<td>7.5</td>
</tr>
<tr>
<td>Gross bleeding, or &gt; 6 liquid, or nocturnal diarrhoea</td>
<td>15</td>
</tr>
<tr>
<td><strong>Erythrocyte Sedimentation Rate</strong></td>
<td></td>
</tr>
<tr>
<td>&lt; 20 mm/hr</td>
<td>0</td>
</tr>
<tr>
<td>20-50 mm/hr</td>
<td>7.5</td>
</tr>
<tr>
<td>&gt; 50 mm/hr</td>
<td>15</td>
</tr>
<tr>
<td><strong>Albumin</strong></td>
<td></td>
</tr>
<tr>
<td>&gt; 3.5 g/dL</td>
<td>0</td>
</tr>
<tr>
<td>3.1-3.4 g/dL</td>
<td>10</td>
</tr>
<tr>
<td>&lt; 3.0 g/dL</td>
<td>20</td>
</tr>
<tr>
<td><strong>Weight</strong></td>
<td></td>
</tr>
<tr>
<td>Weight gain or voluntary weight stable/loss</td>
<td></td>
</tr>
<tr>
<td>Involuntary weight stable, weight loss 1-9%</td>
<td>5</td>
</tr>
<tr>
<td>Weight loss &gt; 10%</td>
<td>10</td>
</tr>
<tr>
<td><strong>Perirectal Disease</strong></td>
<td></td>
</tr>
<tr>
<td>None, asymptomatic tags</td>
<td>0</td>
</tr>
<tr>
<td>1-2 indolent fistula, scant drainage, no tenderness</td>
<td>7.5</td>
</tr>
<tr>
<td>Active fistula, drainage, tenderness, or abscess</td>
<td>15</td>
</tr>
<tr>
<td><strong>Extra-intestinal Manifestations</strong></td>
<td></td>
</tr>
<tr>
<td>(fever &gt; 38.5 °C for 3 days over past week, definite arthritis, uveitis, E. nodosum, P. gangren)</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>One or more</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 1.4: Paediatric Ulcerative Colitis Activity Index (PUCAI)

<table>
<thead>
<tr>
<th>Item Points/ Score</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Abdominal pain:</td>
<td></td>
</tr>
<tr>
<td>No pain</td>
<td>= 0</td>
</tr>
<tr>
<td>Pain can be ignored</td>
<td>= 5</td>
</tr>
<tr>
<td>Pain cannot be ignored</td>
<td>= 10</td>
</tr>
<tr>
<td>2. Rectal bleeding</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>= 0</td>
</tr>
<tr>
<td>Small amount only, in less than 50% of stools</td>
<td>= 10</td>
</tr>
<tr>
<td>Small amount with most stools</td>
<td>= 20</td>
</tr>
<tr>
<td>Large amount (&gt;50% of the stool content)</td>
<td>= 30</td>
</tr>
<tr>
<td>3. Consistency of most stools</td>
<td></td>
</tr>
<tr>
<td>Formed</td>
<td>= 0</td>
</tr>
<tr>
<td>Partially formed</td>
<td>= 5</td>
</tr>
<tr>
<td>Completely unformed</td>
<td>= 10</td>
</tr>
<tr>
<td>4. Number of stools per 24 hours</td>
<td></td>
</tr>
<tr>
<td>0–2</td>
<td>= 0</td>
</tr>
<tr>
<td>3–5</td>
<td>= 5</td>
</tr>
<tr>
<td>6–8</td>
<td>= 10</td>
</tr>
<tr>
<td>&gt;8</td>
<td>= 15</td>
</tr>
<tr>
<td>5. Nocturnal stools (any episode causing wakening)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>= 0</td>
</tr>
<tr>
<td>Yes</td>
<td>= 10</td>
</tr>
<tr>
<td>6. Activity level</td>
<td></td>
</tr>
<tr>
<td>No limitation of activity</td>
<td>= 0</td>
</tr>
<tr>
<td>Occasional limitation of activity</td>
<td>= 5</td>
</tr>
<tr>
<td>Severe restricted activity</td>
<td>= 10</td>
</tr>
<tr>
<td>Total Score</td>
<td></td>
</tr>
</tbody>
</table>

Calprotectin has been increasingly used as a biomarker of localised gut inflammation. Calprotectin is a zinc- and calcium-binding heterodimer that is released by polymorphic nucleated cells and monocytes following activation at sites of active inflammation. The release of calprotectin in plasma and stool correlates with the number of polymorphonuclear leukocytes migrating into the gut lumen and is proportionate to the degree of inflammation (71). It has been found to correlate with disease activity, endoscopic and histological evidence of mucosal inflammation in children and adults with IBD therefore can be helpful as a marker of disease severity and for monitoring disease activity (72-75). In addition, it has been found to precede disease relapse in CD and UC thus predicting a relapse before clinical symptoms develop (76-80).
Calprotectin is resistant to bacterial degradation and is evenly distributed in the stool. It is stable at room temperature for up to a week thus is user-friendly in the clinical setting. In normal individuals, calprotectin concentrations are generally less than 50 µg/g. Concentrations less than 100 µg/g are indicative of remission and values greater than 250 µg/g are suggestive of mucosal inflammation (69). For patients being monitored with stool calprotectin, the degree of increase in concentration is usually more useful than absolute values (76).

1.2.7 Complications of Inflammatory Bowel Disease

This life-long disease can be more aggressive and extensive in the paediatric population (24). It can be complicated by ulcers, abscesses, fissures, fistulae and strictures in CD, and toxic megacolon and bowel perforation in UC. The chronic inflammation can impair growth and development in CD (81). Thrombosis and anaemia are possible complications of CD and UC.

In addition, general ill health and episodes of relapses impair their ability to fully engage with education. These can also significantly affect psychological well-being and the quality of life of these children and their families.

1.3 Other Gastrointestinal Inflammatory Conditions

This study also investigated patients with other GI inflammatory conditions discussed below. In addition, there was a patient with CD and Coeliac Disease, and another patient with IBD and Granulomatosis Polyangiitis.

1.3.1 IL-10 Pathway Mutations

IL-10 is a major anti-inflammatory, regulatory cytokine with multi-systemic pleotropic effects, thus it is unsurprising that mutations in the IL-10 pathway result in multi-systemic unregulated, inflammation. IL-10 is also known as human cytokine synthesis inhibitory factor (CSIF). It is produced mostly by monocytes but also lymphocytes in response to bacterial invasion. IL-10 binds to the IL-10 receptor, which consists of 2 ligand-binding alpha subunits (IL-10RA) and 2 beta (IL-10RB) subunits. This leads to the
phosphorylation of JAK1 and TyK2, then subsequently the phosphorylation of cytoplasmic tails of IL-10R, triggering recruitment and phosphorylation of STAT3. Homodimer formation and nuclear translocation takes place resulting in transcription of IL-10 responsive genes.

Genetic mutations in humans causing IL-10 deficiency and IL-10 (alpha and beta) receptor mutations were first reported in 2009 and 2010 (82, 83). It provided a definitive diagnosis for a very small group of patients presenting with severe intractable ulcerating, fistulating panenteric inflammation in infancy. With its pleotropic effects, these patients suffered with GI as well as extra-intestinal manifestations (Figure 1.4). Some patients underwent subtotal colectomy in an attempt to minimise the inflammation which was refractory to medical treatments. The identification of these genetic mutations has resulted in quicker diagnosis and treatment with haematopoietic stem cell transplantation (HSCT) for these patients. Despite the risks, HSCT is potentially curative (84).

Patients with severe IBD develop complications seen in IL-10-deficient patients, thus these unique patients offer insight into potential sequelae of severe, refractory IBD. These patients are, however, rare and have had several treatments and surgeries. Separating the effects of these from the underlying disease can be challenging. These patients are referred to Great Ormond Street Hospital (GOSH) for HSCT.

IL-10 knock-out (KO) mice first described by Kuhn et al. in 1993 were reported to develop chronic enterocolitis (85). Since then, this model has been widely-used to study the various factors influencing IBD. IBD occurs in genetically susceptible individuals with intestinal dysbiosis, impaired mucosal barrier and immune dysregulation, all replicable in the IL-10 KO murine model.
1.3.2 Cow’s Milk Protein Allergy

Cow’s milk protein allergy (CMPA), is a common condition in UK children. In 2008, it was reported to affect 2.3% of 1-3 year old children (87). Infants can present with classical IgE-mediated hypersensitivity reactions (immediate-onset of urticaria, diarrhoea, vomiting, abdominal pain, anaphylaxis), or non-IgE-mediated or Type IV hypersensitivity reactions (delayed-onset of eczema, abdominal pain, loose frequent stools/constipation, blood/mucus in stools, pallor). For both groups, dietary elimination of dairy is the main treatment focus, followed by gradual reintroduction of dairy as tolerated. About 80-90% of children develop tolerance to dairy by 3 years of age (88).

IgE-mediated CMPA reactions have been better characterised than non-IgE-mediated CMPA, the latter being mediated by Th1 cells and interactions between T lymphocytes, mast cells and neurons which affect intestinal smooth muscles and motility (89). IgE-mediated CMPA consists of 2 stages, namely sensitization and activation. Infants are susceptible to CMPA as their digestive enzymatic activity and secretory IgA system are immature. In addition, there is increased mucosal permeability shortly after birth (90, 91).

In genetically susceptible infants, early exposure to CMP may lead to passage of undigested proteins, priming the immune system. Similarly, proton-pump inhibitors, used...
to reduce gastric acid secretion in children with gastroesophageal reflux symptoms, compromise gastric protein digestion thus enabling CMP to enter the duodenum partially digested or undigested (92, 93).

Approximately 50% of protein absorption occurs in the duodenum, with the proteins being taken up by transcytosis, via M-cells or by paracellular diffusion through the epithelial layer (94, 95). Antigens in the mucosal layer are taken up by antigen presenting cells (APC) which then present the antigenic fragments to T helper cells via MHC class II molecules. This activates an inflammatory response including cytokine secretion and antibody production. Inflammatory cytokines may exacerbate gut permeability (96), further facilitating the sensitisation stage.

Deficient regulation and polarisation of cow’s milk-specific T cells towards Th2 cells lead to B-cell signalling to produce CMP-specific IgE (97, 98). The secreted IgE antibodies against CMP bind to mast cells and basophils. On exposure to CMP, the activation stage is triggered, releasing histamine, platelet-activating factor (PAF) and inflammatory mediators of the allergic response (99).

Treg dysfunction plays a prominent role in CMPA, thus it is unsurprising that tolerance to CMPA in children coincides with development of Treg cells (100, 101). Treg cells are involved in clonal deletion (removal of B and T cells expressing receptors for self before developing into fully immunocompetent lymphocytes thus preventing recognition) and immune suppression (102, 103).

Commensal microbiota has been increasingly recognised to play a major role in allergic diseases through its influence on intestinal IgA production and immune regulation. Some Toll-like receptor (TLR) agonists activate Treg cells that ameliorate these diseases (104). Encouragingly, probiotic supplementation with *Lactobacillus Reuteri* and *L. Casei* have been shown to stimulate dendritic cells to increase Treg cell production. This in turn leads to increased IL-10 production (58, 59).

**1.3.3 Cryopyrin-Associated Periodic Syndrome (CAPS)**

Cryopyrin-associated periodic syndrome is a group of rare autoinflammatory diseases associated with a gain-of-function missense mutations in exon 3 of NLRP3, the gene encoding cryopyrin, a major component of the IL-1β inflammasome (105).
Cryopyrin is expressed in monocytes and neutrophils (106, 107). It is part of the inflammasome complex that facilitates activation of caspase-1 to cleave pro-IL-1β (108). In CAPS, the monocytes have impaired redox homeostasis and response to oxidative stress. This leads to increased IL-1β secretion and the inflammatory symptoms reported in these patients (109).

Prevalence is estimated at approximately 1 to 3 per 1 million (110). Patients usually present early in life with severe symptoms, commonly pyrexia, painful urticaria-like rash, conjunctivitis, headaches and limb pain and swelling from the IL-1β-mediated systemic inflammation (Levy, 2015 #468). Most patients report daily symptoms with episodes of flares. These patients also report GI symptoms, although this is less well-recognised. Upon diagnosis, these patients are treated with Canakinumab, a human monoclonal antibody targeting IL-1β.

### 1.3.4 Granulomatosis with Polyangiitis

Formerly known as Wegener's Granulomatosis, this rare multisystem autoimmune disease of unknown aetiology results in destructive inflammation of mainly small arteries. This Antineutrophil cytoplasmic antibodies (ANCA)-associated necrotising vasculitis can also affect the joints, lungs, kidneys, eyes, nose and ears.

Treatment is often with Cyclophosphamide and corticosteroids to control active disease, and long-term immunosuppression with Methotrexate, Azathioprine and calcineurin inhibitors to maintain remission (111). Presentation is usually with respiratory or renal symptoms but can present with GI symptoms.

### 1.3.5 Coeliac Disease

Coeliac disease (CoeD) is an autoimmune disease affecting an estimated 1 in 100 people in the UK (112, 113). These patients react to gliadin, a protein in gluten, which can be found in wheat, barley and rye. CoeD can present with abdominal pain, diarrhoea, flatulence, bloating, constipation, poor growth and delayed puberty in children. Treatment involves avoidance of gluten and ensuring good nutrition, growth and development of children.
Most patients with CoeD are positive for HLA-DQ2 and HLA-DQ8 heterodimers, which are expressed on antigen presenting cells (APC) and present gliadin to T cells (114). An estimated 30% of the population carry one of these susceptibility genes i.e. 20-30% of general population carry DQ2 and 10% carry DQ8 (115). A family history of biopsy-proven CoeD appears to be an important factor with approximately 20% of first-degree relatives developing the disease (113).

In addition, GWAS has identified 39 non-HLA risk loci in patients (116). Many of these genes are associated with immune regulation i.e. T cell activation and development via TLR7 and TLR 9. In addition, the fucosyltransferase 2 (FUT2) gene encodes enzymes that controls antigens in mucus and intestinal secretions. These antigens act as microbial anchors (117).

Host genetics appear to select early intestinal microbiota composition. Coupled with early life risk factors (Table 1.5), this combination promotes the characteristic dysbiosis seen in these children. Elective Caesarean section, antibiotics in childhood and higher socioeconomic status (hygiene hypothesis) have been reported to increase disease risk (118-121). The combination of early introduction of gluten and formula milk feeding are additional factors increasing the risk (122, 123). Medications such as proton-pump inhibitors and iron supplementation in pregnancy also increase risk (114, 124). In addition, infections such as Rotavirus and Campylobacter have been associated with increased risk of CoeD whilst colonisation with Helicobacter pylori reduces the risk (124-127).

Gliadin, zonulin and intestinal dysbiosis have been hypothesised to play major roles in the pathogenesis of CoeD (113, 128, 129). Gliadin passage across the mucosal barrier occurs via 3 known mechanisms:

1. Gliadin is a potent stimulant for the release of zonulin, an intracellular protein that regulates paracellular tight junctions i.e. gut permeability, in individuals with and without CoeD. Other triggers include dysbiosis such as small bowel bacterial overgrowth, candida and fungal overgrowth and parasitic infections. Zonulin release by epithelial cells and lamina propia (LP) macrophages leads to opening of the tight junctions, enabling passage of gliadin peptides across the tight junction barriers and into the LP.

2. Transcytosis, or

3. Retrotranscytosis of secretory IgA via apically-expressed CD71 receptor. The secretory IgA-gliadin complex binds to the transferrin receptor CD71. This
enables protected transport of gliadin peptides via a transcellular route into the LP (113).

In the lamina propria, tissue transglutaminase (tTG) deamidates or crosslinks gliadin which then binds to HLA-DQ2 and HLA-DQ8 on APC. Gliadin presentation to CD4+ T cells leads to the production of pro-inflammatory Th1 cytokines which stimulates CD8+ T cells and NK cells. Concurrent production of Th2 cytokines stimulates B cells, leading to their clonal expansion, differentiation into plasma cells and the production of anti-tTG and anti-gliadin antibodies measured in the clinical setting.

Subsequently, epithelial apoptosis occurs through these mechanisms:

1. CD8+ T cells activate the Fas/ Fas ligand system, which in turn initiate apoptosis via a caspase cascade.
2. Natural killer-activating receptor-major histocompatibility-class I chain-related gene A (NKG2D–MICA) signalling pathway, which targets intestinal epithelial cells leading to their destruction.
3. IL-15, secreted by mononuclear phagocytes following viral infections, induces the proliferation of NK cells and induces the expression of NKG2D. IL-15-NKG2D signalling pathway leads to epithelial cell death (130).
4. Perforin, a protein released by cytotoxic T lymphocytes and NK cells on degranulation, binds to epithelial cell membrane forming pores.
5. The interaction between the extracellular tTG and anti-tTG-autoantibody may induce epithelial damage.

Consequently, multiple cellular events contribute to increased intraepithelial lymphocytes, hyperplastic crypts and villous atrophy seen on histology of duodenal mucosal biopsies.

<table>
<thead>
<tr>
<th>Risk Factors</th>
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<td><em>Helicobacter pylori</em> colonization</td>
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<tr>
<td>Antibiotics in childhood</td>
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<td>Iron supplementation in pregnancy</td>
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<td>Use of proton-pump inhibitors</td>
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<td>Higher socioeconomic status – hygiene</td>
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<tr>
<td>Formula milk during infancy</td>
<td>(122, 123)</td>
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<tr>
<td>Early introduction of gluten</td>
<td>(132)</td>
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Table 1. 5: Risk Factors for Developing Coeliac Disease, adapted from Lebhowl et al. (124)
1.4 The Microbiome of the Gastrointestinal Tract

Whilst recognising that the microbiome includes bacteria, archaea, protists, fungi and viruses, we have a better understanding of the bacteria within us compared to other organisms. There are numerous factors that shape our unique microbiota throughout our lives. These microbes can coexist as commensals with symbiotic or pathogenic effects on our functioning.

1.4.1 Gastrointestinal Microbiota in Health

The GI bacterial population predominantly belong to 2 phyla, Bacteroidetes and Firmicutes, coexisting in a symbiotic relationship with its host in health. In return for residence and energy source, these microbes play a crucial role in modulating host immunity, barrier function and metabolism. Their composition varies depending on the luminal environmental conditions thus different microbes exist in different regions of the GI tract (Figure 1.5).

A critical window of opportunity exists in early childhood for the gut microbiota to influence the developing immune system. Therefore, factors influencing intestinal colonisation also affect immunity.
There is increasing evidence that colonisation of the GI tract begins in utero. Healthy placental microbiota varies with maternal gestational weight gain, and interestingly resembles the oral microbiota more than the vaginal or gut microbiota (134, 135). In the intrapartum period, prophylactic antibiotics for Group B *Streptococcus* are known to influence the infant microbiota composition for the first few weeks of life (136, 137). Interestingly, after adjusting for intrapartum antibiotics, Group B *Streptococcus* presence itself appears to affect infant intestinal microbiota (138).

The mode of delivery greatly influences the infant microbiota. Infants born vaginally are predominantly colonised with *Escherichia coli/Shigella, Bacteroides* and *Bifidobacterium*, and those born by caesarean section are colonised with skin flora, typically *Staphylococcus* and *Corynebacterium* (139). The risk of allergic diseases are higher in infants born by caesarean section, in whom there is delayed colonization with *Bacteroides* (139-142). Interestingly, the microbiota of caesarean-born infants can be altered to resemble the microbial profile of maternal vagina but whether this results in health benefits remains unproven (143). This is supported by murine studies. Using a mice model, Cahenzil *et al.* reported raised IgE levels in germ-free mice. The group went on to compare mice exposed to normal microbiota prior to 35 days of life with mice exposed to normal microbiota after this period and found that the later condition resulted in high IgE levels (144).

The effects of delivery mode appear to extend into early childhood. Bentley *et al.* studied various factors affecting hospitalisation for acute gastroenteritis in Australian children (145). They reported a 20% increased risk of hospitalisation for infants born by caesarean section, after controlling for other variables. Exposure to bacteria colonising the birth canal and perianal region during vaginal delivery appear to greatly influence subsequent colonisation on the infant gut, favouring the functionally beneficial *Bifidobacteria sp*. In contrast, infants born by caesarean section had lower numbers of *Bifidobacteria* and more often colonised by *C. difficile* (146). The group also reported further increased risk of hospitalisation to 62-78% for infants born prematurely by caesarean section and received formula milk. In a Scandinavian birth cohort study, elective, rather than emergency, caesarean section was associated with higher risk of lower respiratory tract infections, juvenile idiopathic arthritis (JIA) and asthma (140).

We continue to discover beneficial properties of the human breast milk (147). In addition to nutritional content, it is known to have probiotic and prebiotic properties that influence colonisation of the infant GI tract (148-151). Firmicutes and Proteobacteria, the
predominant bacteria in human breast milk, and prebiotics such as oligosaccharides assist in shaping the microbial community (152, 153). Breast-feeding is known to protect against childhood gastroenteritis, respiratory infections, allergies and autoimmune disease such as IBD and JIA (154-158).

The introduction of food into the infant diet generally occurs between 4-6 months of age. The timing and type of weaning food is thought to influence risk of diseases. An example of this is the Swedish Epidemic (discussed in Coeliac Disease Section 3.1.7.1). The earlier weaning age and increased gluten content in the infant diet resulted in an increase of children diagnosed with Coeliac Disease (159, 160). In addition, the risk of type 2 Diabetes Mellitus was reportedly increased in those weaned too early (before 4 months) or too late (after 6 months) (161).

As the infant grows into childhood, the diet continues to play a significant role in the development of the mature microbiota. With various influences, the microbial composition fluctuates in early childhood and stabilises at 1 year of age, when weaning completes and the diet is more like that of an adult (162-164). The microbiota continues to evolve until maturation into an adult profile at about 3 years of age (165) (166)(Figure 1.6). The microbiota was not concurrently analysed in these studies but solid food would inevitably alter the microbiota composition (167).
Relative abundances, as reported in the literature, are shown with individual abundances normalised to an abundance sum for all bacterial families at each month. The period of weaning is highlighted by the grey background at 4-6 months, and alpha diversity is shown as a greyscale gradient (white=low diversity, black=high diversity).

In a day, 6 to 12 month old infants are estimated to ingest 60 mg of dust and soil (168), with more hand-to-mouth activity in younger children and when indoors (169). It is thus unsurprising that the environment in which the child is raised also influences microbiota composition. Geographical differences exist (170) but there are also differences seen between children raised in rural and urban areas (171). Other factors are mentioned as part of the Hygiene/Microbiome Hypothesis above (Section 1.1.2).

It is inevitable that antibiotics would cause disruption of the microbiota but it is now recognised that this disruption can last weeks to months (172-174). Repeated antibiotic use in early childhood would thus impede the development of normal microbiota, disrupting the developing immune system. Several studies have demonstrated clear associations between antibiotics and immune-mediated diseases such as IBD and JIA (175, 176).

With age, the microbial diversity and commensal populations, namely Bacteroides, Bifidobacteria and Lactobacilli reduce whilst opportunistic microbes such as Enterobacteria, C. perfringens and C. difficile increase. However, these alterations vary depending on the many environmental, lifestyle and geographic factors. Nevertheless, these changes are associated with reduced microbe-related metabolic functions increasing the risk of many known age-related diseases (177-180).

1.4.2 Functions of the Intestinal Microbiota

In health, a collaborative relationship exists between intestinal microbiota and the human host. In exchange for substrate and residence, GI microbiota assists the human host in a list of functions that continue to expand.
Microbes play a significant role in the development, programming and function of the immune system through varied interactions with the human host. The host and its resident microbiota employ different strategies to shape the microbial community and accommodate the commensal microbiota in return for the microbial involvement in host metabolism and defence against pathogenic organisms.

1.4.2.1 Host Protection

Intestinal epithelial cells (IECs) and the mucus layer separate the majority of intraluminal contents from the host, balancing the need for absorption of nutrients and electrolytes with self-protection. Commensals modulate the immune system, triggering a self-limiting, non-pathogenic inflammation without tissue damage. This results in a state of immunological tolerance enabling its colonisation whilst creating a hostile environment for pathogenic organisms.

One such example is Regenerating protein-IIIc (RegIIIc). Its production by purine Paneth cells is stimulated by the intestinal microbiota. It is an antibacterial lectin that binds to pathogenic bacteria, limiting bacterial penetration and protecting the host from infection (181-184). This is particularly important during tissue injury, when RegIIIc prevents opportunistic penetration of commensal bacteria during wound healing (185).

1.4.2.1.1 Detection of Microbes by Pattern-recognition Receptor Systems

There are 2 major pattern-recognition receptor (PRR) systems, Toll-like receptors (TLR) and nucleotide-binding oligomerization domain/caspase recruitment domain isoforms (NOD/CARD). TLRs, which are expressed on and in IECs and immune cells, are stimulated by microbes, initiating a complex signalling cascade leading to the nuclear translocation of NF-kB thus promoting subsequent transcription of a wide variety of cytokines, chemokines, acute-phase proteins and cell adhesion molecules (186). In contrast to membrane-bound TLRs, NODs are cytoplasmic microbial detectors which when activated result in inflammation and antigen presentation (187). Activation of NF-kB requires NOD1 signalling in some Gram-negative bacterial infections that bypass TLR activation (188). NOD2 is expressed in monocytes/macrophages and DCs, detects muramyl dipeptide of Gram-positive and Gram-negative bacteria. NOD signalling results in innate immune activation.
1.4.2.1.2 Commensal Microbiota induces Immune Homeostasis

Microbial products of commensal bacteria, known as microbial-associated molecular patterns (MAMPs) stimulate IL-10 production to protect the host. Bacteroides fragilis produces Polysaccharide A (PSA), which triggers TLR2 signalling to induce IL-10 production, Treg polarisation and inhibit pathogenic Th17 responses. This induces mucosal tolerance enabling colonisation (189). Via this mechanism, PSA also protects the host against encephalomyelitis and colitis (190, 191). Another MAMP, flagellin, activates TLR5 signalling promoting Th1 and Th17 polarisation as well as B cell differentiation into IgA-producing plasma cells (192). In addition, pro-IL-1β and pro-IL-18 production, induced by intestinal microbiota, can lead to their conversion into mature IL-1β and IL-18 with infection to assist pathogen elimination (193, 194). Also, bacterial metabolites such as short chain fatty acids (SCFA), also modulate host immunity to induce Treg cell differentiation (195, 196).

Intestinal microbiota influences T helper cell development (197). Microbe-bearing DC induces IL-6 and TGF-β leading to Th17 production (198). Colonization with segmented filamentous bacteria (SFB) induces the differentiation of Treg cells into Th17 cells. Th17 cells produce IL-17 ad IL-22, which together increase anti-microbial protein (AMP) production and pathogen clearing (199), protecting against Citrobacter rodentium infection (200).

The intestinal microbiota is involved in the differentiation of T cells into IL-10 producing Treg cells (189, 197), which in turn plays a significant role in controlling the richness and balance of bacterial composition thus intestinal homeostasis. Follicular Treg stimulated by commensal bacteria such as Firmicutes determine the selection and diversification of IgA+ B cells thus influencing immune tolerance (201). A subset of Treg cells, called Tr1 subset, is known to produce high levels of IL-10, which is essential for self-tolerance. This subset is a target for microbial metabolites such as SCFA, which modulate Treg homeostasis (195).

IL-10 can be produced by T cells and B cells, but is mainly produced by macrophages in the colon in the presence of commensal bacteria (202-204). IL-10 inhibits maturation of dendritic cells (DC) and blocks mature DC function. DC, especially tolerogenic DC (tDC) can be induced to produce IL-10 by probiotics (205). IL-10 can, however, be manipulated by pathogens to hamper immune responses, enabling infection of the host (206-208).
1.4.2.1.3 Microbial Regulation of B cell Responses

Regulatory B cells (Breg) have recently been identified and found to express IL-10, thus also known as B10 cells. These Breg cells also produce IgM, CD1d, CD21, CD24 (209, 210) and CD5 in mice (211). Breg cells are thought to play a role in intestinal homeostasis, independent of Treg, and likely through down-regulation of Th1, Th17 activation and production of pro-inflammatory cytokines (212-214). Cytokines involved in Breg function include TGF-β (215), B cell activating factor (BAFF) (216), and IL-35 (217) as well as IL-10.

Intestinal microbiota is involved in regulating IgA responses (201). Interestingly, once released secretory IgA (sIgA) in the intestinal lumen plays an important role in modulating microbiota composition available for interaction with the host (214). Bacterial-induced IgA production also occurs in the small intestine (218, 219). It is a significant mucosal defence against pathogens as IgA entrap bacteria, thus blocking their pro-inflammatory epitopes, minimizing bacterial-epithelial contact and preventing microbial translocation across the epithelial layer (201, 218-222). The small proportion of bacteria that penetrates the IEC are retained by DCs, promoting B cell capture of antigens and further production of IgA by lamina propia B cells (218).

Commensal colonic bacteria negatively regulate intestinal macrophage inflammatory status (223). Commensal microbes such as indigenous Clostridia species have been reported to induce IL-10 production, favouring Treg cell differentiation (224). In germ-free mice, 17 strains of bacteria from clusters IV, XIVa and XVIII of Clostridia stimulated a TGF-β rich environment. This anti-inflammatory response induces IL-10 and Treg cells and was found to alleviate colitis, arthritis and asthma (225). IL-10 also inhibits macrophage activation, proliferation and autophagy, thus maintaining macrophages and DC in an inactive state resulting in homeostasis (226, 227). In addition, colonic macrophages maintain tissue integrity through this microbes-IL-10 interaction (228).

1.4.2.1.4 Vascular and Extra-intestinal Influence of the GI Microbiota

Intestinal microbes also stimulate angiogenesis, as demonstrated in germ-free mice when the capillary network formation resumed following introduction of microbiota from conventionally-reared mice (229).
The influence of intestinal microbiota extends beyond the GI tract. A possible mechanism of extra-intestinal influence may be mediated via the translocation of microbiota-derived peptidoglycan from the intestine to the bone marrow, modulating immune responses by bone-marrow-derived neutrophils. Its beneficial effects were demonstrated when depletion of Gram-positive bacteria by oral Neomycin resulted in impaired immune responses against respiratory influenza virus infection (193). In addition, defective splenic and lymph node structure and development have been observed in germ-free mice (230). There is also accumulating evidence for GI microbial involvement in the pathogenesis of inflammatory conditions such as atopic dermatitis (205), and diarrhoea (231).

1.4.2.2 Microbial involvement in Host Metabolism

Our symbiotic relationship with the intestinal bacteria extends to the metabolism of carbohydrates, proteins, vitamins and other compounds such as oxalate, which are converted into calcium oxalate stones in the absence of Oxalobacter formigenes (232-234).

1.4.2.2.1 Carbohydrate

Colonic bacteria rely on the supply of indigestible carbohydrates from the small bowel. In return for this energy source, colonic commensal bacteria ferments these carbohydrates to produce metabolites such as SCFA and gases. SCFA are a major source of energy for colonocytes and are involved in several physiological and immunological functions. In the absence or restricted carbohydrates, alternative energy sources may be utilised, resulting in potentially toxic metabolites.

1.4.2.2.2 Protein

Colonic microbiota is also capable of metabolising ingested dietary and endogenous proteins from host enzymes, mucin, and sloughed off intestinal cells. These are converted into shorter peptides, amino acids and derivatives, short and branched-chain fatty acids. Gases are concurrently produced, namely NH₃, H₂, CO₂, and H₂S (235). Macfarlane and Cummings identified Bacteroides and Propionibacterium species as the
predominant proteolytic species but also *Clostridia*, *Streptococci*, *Staphylococci*, and *Bacillus* species (235, 236). More recently, other proteolytic organisms, including several species of *Bacteroides*, *Eubacterium hallii*, and *Clostridium barletti*, have been identified (237).

Several factors affect colonic proteolytic activity. It varies depending on the concentration of different amino acids and colonic pH, with a preference for higher pH. Carbohydrates are preferentially fermented, resulting in 60% reduction of proteolysis when fermentable carbohydrates are present (238). Fermentation is mostly saccharolytic in the proximal colon, whilst proteolysis, as does the pH, increases distally in the colon (239). Products of this fermentation include branched-chain fatty acids, phenol, and indole derivatives of amino-acid fermentation and ammonia (239).

### 1.4.2.2.3 Vitamin synthesis

The human host relies on commensal intestinal microbiota to synthesise certain vitamins, notably vitamin K, and B group vitamins including thiamine (B₁), riboflavin (B₂), niacin (B₃), pantothenic acid (B₅), pyridoxine (B₆), biotin (B₇), folate (B₉) and cobalamin (B₁₂) (240). It is estimated that these microbes contribute over a quarter of the recommended dietary intake for 4 vitamins, pyridoxine (B₆) (86%), folate (B₉) (37%) cobalamin (B₁₂) (31%), and niacin (B₃) (27%) (241). Their significant role was demonstrated by a study on adults on low vitamin K diets for 3–4 weeks. They did not develop vitamin deficiency, but developed significant decreased plasma prothrombin levels when treated with broad-spectrum antibiotics (242).

Most bacterial phyla contribute to the synthesis of the 8 B vitamins. All Fusobacteria and 10-50% of the other 4 phyla, Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria, produce vitamin B₁₂. Approximately 90% of Bacteroidetes produce the remaining B vitamins. Riboflavin, the most commonly synthesised vitamin, and biotin are produced by virtually all microbes of the phyla Bacteroidetes, Fusobacteria and Proteobacteria, and some Firmicutes and Actinobacteria.

### 1.4.2.2.4 Bile Acids

The majority of bile acids are absorbed in the distal ileum but 1-5% enter the colon. Here, bile acids exert their antimicrobial properties such as detergent effects on bacterial cell
membranes. They also induce DNA damage and disruption of protein structures (243-245), thereby influencing microbiota composition.

Intestinal microbiota can modulate and reduce bile acid toxicity. This is achieved by utilising bile salt hydrolase expressed by bacterial genera *Bacteroides, Bifidobacterium, Clostridium, Lactobacillus, and Listeria* (246). In addition, cholic acid is converted to the more potent deoxycholic acid by microbes (247). Thus, a mutually beneficial interaction exist between the intestinal microbiota and the human enterohepatic circulation.

1.4.2.2.5 Polyphenols

Polyphenols are naturally present in food such as fruit, vegetables, tea, coffee, legumes and cereals. They include phenolic acids, flavonoids (flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavonols), stilbenes, lignans, and secoiridoids (248). Majority escape small intestinal absorption and enter the colon, where many are metabolised and converted for absorption, after degraded by colonic microbes such as *Bacteroides distasonis, Bacteroides uniformis, Bacteroides ovatus, Enterococcus casseliflavus, Eubacterium cellulosolvens, Lachnospiraceae CG19-1, and Eubacterium ramulus* (248, 249). Polyphenols confer benefits to humans as antioxidants, protecting against free radical damage and defence against UV radiation and pathogens (250).

1.4.3 Microbial Dysbiosis

The loss of healthy balance in the microbial composition is known as dysbiosis. This occurs when the proportions of the main phyla are altered such as increase Firmicutes:Bacteroidetes ratio as seen in obesity (251), or increased Proteobacteria in IL-10 knock-out (KO) model of IBD (252). Microbes with major roles in health may be reduced. Alternatively or consequently, pathogens or pathobionts may be increased.

Dysbiosis could also be due to reduced diversity, making it easier for particular populations to dominate. Dominant pathogenic microbes can increase disease susceptibility. Similarly, decreased abundance or richness of the microbial population may compromise host function, thus affecting health. In disease, there is often distorted diversity and abundance, in addition to an imbalance between pathogenic or pathobiont versus protective microbes, resulting in dysbiosis (253, 254). It seems that inflammation
itself can lead to dysbiosis as was seen before and after the onset of colitis in IL-10 KO mice (255).

Reduced diversity and abundance are often associated with single-species dominance. Single commensal species such as *E. faecalis*, *E. coli*, *Shigella*, *Alistipes* and *Helicobacter* can trigger inflammation (256-259). There have been several studies on the intestinal microbiota examining the effects of isolated organisms (260). The human GI tract, however, contains a complex community of microbiota within it. It is increasingly appreciated that for several beneficial and pathogenic processes to occur, a combination of microbes are required. An example reported was *H. hepaticus* requiring the presence of other members of the microbiota to induce colitis in an IL-10 KO murine model (261). Infection with nematode *Trichuris muris* (262) also required gut microflora. This study also demonstrated that the alteration of the microbiota with antibiotics affected disease phenotype. In the presence of several different microbial communities, colitis induction and progression was possible (263). It is also possible that once induced, inflammation can alter the microbiota to reduce diversity and richness as demonstrated by Butchler *et al.* (264).

Interactions between bacterial communities must occur in the process of colonisation and intestinal homeostasis. Competition for substrate and ecological niche encourages diversity of bacterial communities. Reduced diversity and richness (or abundance) of commensal microbiota predisposes to inflammation as demonstrated in IL-10 KO murine model by Bassett *et al.* who found a strong negative correlation between caecal diversity and inflammation score (256). Their ability to recover following disruption of homeostasis is impaired, and could lead to exposure to pathogens and other environmental triggers. A well-known example of this is the expansion of *C. difficile* and *Salmonella enterica serovar Typhimurium* by removing competition from commensal bacteria for host-derived sugars such as sialic acid with the use of antibiotics (265). Some commensal bacteria can, however, become pathogenic under certain circumstances, thus known as “pathobiosis”.

Commensal bacteria control colonisation and growth of exogenous pathogens, a process known as colonisation resistance. In addition to competition, they alter the host microenvironment such as pH, through mechanisms that include its production of metabolites e.g. SCFA (266-268). Microbial metabolites are also known to down-regulate microbial pathogenicity by down-regulating virulence gene expression (266). Commensal bacteria thus has the advantage of creating a suitable environment for itself whilst making it hostile for others, thereby excluding potentially pathogenic organisms.
Microbial abundance and diversity is thus a significant factor in maintaining health. Deviation from the norm appears to predispose individuals to diseases. Their influence is not limited to the GI tract but extends to affect health in other organs such as lungs, skin and the nervous system.

1.4.3.1 Dysbiosis in Inflammatory Bowel Disease

It is now well-accepted that the gut microbiome of IBD patients differs from that of healthy individuals. *Firmicutes* and *Bacteroides* dominate in health but in IBD, there is dysbiosis with reduced bacterial diversity and abundance(269, 270). This is discussed further in Section 3.1.

Whether it is the inflammation that causes microbial alterations or the pathogenic microbial profile that leads to IBD remains unclear. Knights *et al.* suggested that the gut microbiome is inherited(48). Supporting this hypothesis, healthy family members of IBD patients share some similarities in their intestinal microbiome composition with the patient. As family members share the same diet and environment, perhaps this is not surprising (271). However, there are twin studies that report differences in microbial profiles between twins with and without IBD (272, 273).

Potential shifts in microbial population with disease activity in IBD have been reported (Section 1.3). Better understanding of the variations in IBD should enable prediction of potential relapses and disease severity. The suitability of step-up (i.e. increasing immunosuppression to achieve remission) or step-down approach for individual patients has been much debated. The ability to predict/anticipate disease severity may guide clinicians in treatment decisions and enable more personalised treatment.

1.4.3.2 Restoration of Symbiosis as a Treatment Strategy

As dysbiosis causes disease, it is thus tempting to hypothesise that manipulation of the microbiota can restore symbiosis and therefore health. IBD treatment appears to favourably alter the intestinal microbiota. Whether this causes disease improvement is unclear. EEN in CD has been widely studied to better understand these mechanisms.
In 1907, the Russian Ilya Ilyich Mechnikov suggested that microbial ingestion improved host health. Indeed, he hypothesized that the consumption of lactic-acid-producing bacteria (LAB) strains found in yogurt might enhance longevity (274). LAB is present in the human GI tract but can also be ingested. In 1953, Werner Kollath referred to these microbes as “probiotics” (275).

Probiotics are defined as ‘live microorganisms which, when administered in adequate amounts confer a health benefit to the host’ by WHO and Food and Agriculture Organization of the United (276). Probiotics can consist of dead bacteria or bacterial components (277). The beneficial effects of probiotics have been known for some time but its use in treating disease continues to be evaluated. Progress with this have been hampered by the use of different probiotics of varying compositions and quantitates thus making analysis challenging.

Prebiotics, on the other hand, are nutritional supplements, typically non-digestible fibre, which promotes bacterial growth and functions. Prebiotics increase SCFA production and have the potential to modify immunity and development (278, 279).

Synbiotics are a combination of pre- and probiotics designed to confer health benefits of both components. There have been encouraging results reported on the use of synbiotics in eczema but there is insufficient evidence to widely recommend its use (280, 281).

Another strategy being widely investigated currently is faecal microbial transplantation (FMT). This essentially replaces the intestinal microbiota of an individual with that of a healthy donor (discussed further in Section 3.1).

1.4.4 The Virome in IBD

The diverse intestinal microbiome also includes the virome. The number of human cells are estimated at $10^{13}$, with the number of bacteria thought to be 10-fold of human cells and viruses to be 100-fold (282). Commensal virome increases in infancy (283) then evolves and decreases in richness and diversity over time, their composition shifting with alterations in the bacterial profile (284). Similar to the microbiota, the virome is greatly influenced by environmental factors such as diet, antibiotics, nutritional status, health, geographic location, age, season and medications (285-289).
The intestinal virome consists predominantly of bacteriophages (prevalent families Caudovirales and Microviridae) and eukaryotic viruses (prevalent genus Picornavirus and Anellovirus)\(^{(283, 289-293)}\). Anelloviridae and Siphoviridae are prevalent in children under 2 years but then gradually decrease while Microviridae increases with age \(^{(284)}\). There are high inter-individual variations but high intra-personal stability in the human viromes \(^{(290)}\).

Bacteriophages infect bacteria, and in the intestine, most phages are associated with the phyla Bacteroidetes and Firmicutes \(^{(290)}\). Antibiotics therefore affect the virome indirectly by modulating the composition of its bacterial hosts \(^{(294)}\). Activated prophages may cause lysis of their bacterial host, releasing virions and resulting in inflammatory responses \(^{(286, 295)}\). Phages can also be beneficial to the human host. Within the mucosa, it is thought to limit mucosal bacteria and protect the host from bacterial infection \(^{(296)}\).

The virome appears to have a significant role in GI inflammation. ATG16L1 mutation in humans predisposes to CD but appears to be inconsequential in mice until infected by Norovirus, which resulted in intestinal inflammation \(^{(297)}\). In addition, mice administered anti-viral drugs following administration of DSS had more severe colitis than mice with intact virome \(^{(298)}\). The protective effect appears to be via simultaneous stimulation of TLR3 and TLR7, with TLR3/TLR7-deficient mice being more sensitive to colitis. Indeed, IBD patients carrying mutations of both TLR3 and TLR7 had higher hospitalisation rates compared to IBD patients without the mutations \(^{(298)}\).

In IBD, the primary virome alteration is the amplification of Caudovirales \(^{(299, 300)}\) with its associated changes in bacterial diversity, specifically *Lactococcus*, *Lactobacillus*, *Clostridium*, *Enterococcus* and *Streptococcus* \(^{(286)}\). Caudovirales was inversely correlated with *Bacteroidaceae*, and positively correlated with *Enterobacteriaceae*, *Pasteurellaceae* and *Prevotellaceae* in CD. No correlations were found in UC samples \(^{(286, 301)}\).

Alterations were, however, observed in UC. Manrique *et al.* \(^{(302)}\) reported perturbed faecal virome in UC (n=66) and CD (n=36) patients. There was also significant reduction in the percentage of the 23 core phages in CD (30%) and UC (37%) compared to the average 62% in HC. Analysing colonic biopsies, CD patients were found to have significantly more phages than HC \(^{(300, 303)}\). In addition, the ulcerated mucosa had significantly fewer viral-like particles (VLPs) than non-ulcerated mucosa. In another
study involving 10 IBD (6 CD, 4 UC) patients, colonic tissues were compared with those of 5 HC. Adenoviridiae which was present in IBD was absent in HC (304).

Patients on corticosteroids and/or immunosuppression had lower viral diversity than untreated patients. Those only on immunosuppression had lower diversity than those on combination therapy or steroids only (286). This study also found increased phage-associated richness of Caudovirales and Microviridae in the stools of CD and UC patients compared to HC (286).

Despite this, specific bacteriophages have been suggested as a potential treatment (305). These bacteriophages successfully replicated in murine ileal, caecal and colonic sections as well as homogenised murine stool. A single administration of these 3 bacteriophages reduced adherent intestinal *E. coli* (AIEC) in the stool and intestinal sections. The increased AIEC in ileal mucosa of CD patients have been associated with inflammation. Galtier *et al.* also demonstrated that these bacteriophages reduce the severity of DSS-induced colitis in conventional mice colonised with AIEC. Thus, bacteriophages may be a treatment options for some CD patients.

The success of FMT, now a treatment for refractory *C. difficile* infections, is also influenced by the donor virome diversity, with success more likely if donors had higher Caudovirales diversity than recipients. In addition, over half of recipients experience re-infection if donor Caudovirales diversity was less (306).

There is increasing awareness of the importance of the intestinal virome. Our understanding of their roles and significance is still limited due to the challenges in analysing the samples and interpreting the results. The only reliable molecular method to analyse viral composition is by metagenomics. This, however, should not detract us from investing and pursuing better understanding of its interactions with the rest of the microbiome and human host.

### 1.4.5 The Mycobiome in IBD

Thus far, there has been little interest in this small constituent of the intestinal microbiome. Nevertheless, the mycobiome appears to play a significant role in health and disease.
Humans are colonised with numerous fungal species, namely *Candida* (*C*), *Aspergillus*, *Fusarium* and *Cryptococcus* (307). Within the GI tract, the fungi community consists predominantly of *C. albicans* and *C. parapsilosis* (308-310). Hoffman *et al.* demonstrated that *Saccharomyces* were the most prevalent genus (present in over 89% of samples), followed by *Candida* (57%) and *Cladosporium* (42%). Similarly, Dollive *et al.* (311) reported *Candida*, *Aspergillus*, *Penicillium*, *Pneumocysttii*, *Cryptococcus* and *Saccharomycetaceae* yeasts in stool.

Like the virome, fungi appear to collaborate with commensal bacteria to improve their survival and virulence. The fungal phyla Ascomycota and Basidiomycota were inversely correlated, and there was negative association between *Candida* and Bacteroidetes (312). *Candida* and *Saccharomyces* were found to be associated with *Bacteroides, Parabacteroides*, *Lachnospiraceae*, *Ruminococca-ceae* and *Faecalibacterium* (166, 312). In addition, the *Prevotella/Bacteroides* ratio positively correlated with fungal abundance (312). The mechanism of the mutually beneficial relationship is unknown.

*Candida* was positively correlated with carbohydrates perhaps as it is capable of breaking down starch, which can then serve as raw material for bacterial fermentation by organisms such as *Prevotella* and *Ruminicoccus* (313, 314). In contrast, *C. albicans* and *Ruminococcus gnavus* have been shown to produce mucolytic enzymes (37, 38 Hager) that can disrupt the protective intestinal mucus layer. Also, *Candida* negatively correlated with total saturated fatty acids, and *Aspergillus* negatively correlated with SCFA (312).

On the other hand, fungal infections are a major cause of morbidity in IBD patients (291), with immunosuppressed patients being at particular risk of these opportunistic infections (312). *Pneumocystis jirovecii* (*P. jirovecii*) infection, histoplasmosis, cryptococcosis and *Aspergillosis* infections have been reported in immunosuppressed IBD patients (315, 316).

Similar to the microbiota and virome, fungal dysbiosis occurs in IBD (317-319). *C. albicans* in a murine colitis model was demonstrated to aggravate intestinal inflammation, which was then reversed with antifungal treatment (320). In CD patients, there was increased fungal diversity and prevalence of *C. albicans*, *Aspergillus clavatus* and *Cryptococcus neoformans* reported in stool (321), whilst most *Candida* species was detected in the stool and mucosal biopsies of UC patients (320, 322).
In a separate study involving 235 IBD patients and 38 HC, the dysbiosis was described as a ratio. The *Basidiomycota/Ascomycota* ratio was increased in IBD patients, and there was decreased proportion of *Saccharomyces cerevisiae* and increased proportion of *C. albicans* compared with HC (323). Interestingly, a positive correlation was also observed between abundance of *E. coli*, *Serratia marcescens* and *C. tropicalis* in CD intestine compared to their healthy relatives (324). In UC, mucosal *Candida* colony counts were higher than in non-UC controls. *C. tropicalis* was also increased in CD and positively correlated with anti-*Saccharomyces cerevisiae* antibodies (ASCA) in these patients (324). *C. tropicalis*, *E. coli* and *S. marcescens* together form thicker robust biofilms. Biofilms are known to maintain stability providing these microbes with protection from other microbes and host defence. Therefore, a symbiotic balance is clearly needed for health. Currently, more studies are required to help us better understand the mycobiome within us.

### 1.5 Microbial Metabolites

The resident intestinal microbiota relies on the host for substrates, producing waste products or metabolites from these substrates. These metabolites provide insight into the metabolic pathways that take place within us. Bacterial metabolites include aryl hydrocarbon ligands (AHR), B vitamins and polyamines (325).

In addition, fermentation of plant fibres results in the production of SCFA. These plant fibres are indigestible by the host but the SCFA produced from these benefit the host in several ways (see Section 4.1). The survival of intestinal microbiota, its function and ability to produce beneficial metabolites are dependent on host dietary intake, thus linking nutrition, intestinal microbiota, SCFA production and host health.

There has recently been renewed interest in the role of the diet in modulating 21st century diseases including obesity, diabetes mellitus and cardiovascular diseases (CVD). The Diabetes Remission Clinical Trial (DiRECT) Study has reported encouraging preliminary results (326). Overweight patients with Type 2 Diabetes were enrolled in the programme. Half of the patients had their daily calorie intake restricted, initially with nutritionally-complete soups or shakes then supported with the introduction of healthy, balanced diet, resulting in weight loss, remission and improvement of other conditions and quality of life in many of the patients.
Atherosclerosis, a major factor in CVD, has been linked with intestinal metabolism of dietary lipid phosphatidylcholine, found in eggs, soya and sunflower oil (327, 328). One of the metabolites, trimethylamine oxide (TMAO), was found to upregulate receptors associated with atherosclerosis. Red meat is known to contain high levels of carnitine, which is also converted into TMAO (329, 330).

In IBD, high dietary total fats, polyunsaturated fatty acids, omega-6 fatty acids and meat are associated with increased risk of CD and UC, whilst high fibre and fruit intake reduces CD risk, and high vegetable intake reduces UC risk (331). In addition, increased faecal amino acids are seen in CD and UC, supported by high protein catabolism reported in IBD (332, 333).

These studies begin to explore the complex interactions between the host, their diet and resident microbiota. Some provide explanations to what is already known and some studies suggest therapeutic options for many diseases with increasing prevalence. However, research funding in this area continues to be an issue in the UK.

### 1.6 Immunity in Inflammatory Bowel Disease

As discussed in Section 1.2.2.1, genetic mutations are one of the predisposing factors in developing IBD. Mutations identified include those causing impaired barrier function and bacterial handling.

In addition, IBD patients have been demonstrated to have exaggerated immune responses to pathogens. The dysregulated immune defence and responses, coupled with predisposing environmental factors (discussed in Section 1.2.3) create an environment of increased risk of IBD. A trigger sufficiently significant then leads to disease onset.

There is increasing evidence linking early childhood factors to IBD e.g. breastfeeding and antibiotic exposure. Many of these factors are now recognised to influence intestinal microbiota and early bacterial colonisation. As the immune system is also undergoing development and “education” during this critical period, these early factors help shape individual immunity. Thus factors that lead to aberrant microbial development during the early years also affect the risk of immune diseases in later years. This aspect is discussed further in Chapter 5.
1.7 Aims and Objectives of the Study

There has been major advances in our understanding of the intestinal microbiota, their roles and influence on human health. Over the last decade, research in intestinal microbiota has evolved from reporting its associations with diseases to demonstrating its causative effect. Advances in technology, and these being more accessible, are increasingly enabling us to identify microbial genera and species, and understanding the functional consequences of dysbiosis at a molecular level.

In 2019, the quest for personalised medicine continues. IBD, a disease encompassing different pathogenesis, involves a variety of factors and a wide spectrum of disease severity, thus a personalised approach to the treatment and management would certainly benefit patients. This study was initiated to allow better understanding of how the intestinal microbiota and its metabolites influence host immunity. Children with IBD, a group relatively less studied compared to adult IBD patients, were selected to explore how these factors respond to specific treatments and with disease activity.

The following chapters focus on a different aspect of the study with its own introduction, methods, results and discussion, and the final chapter discusses these different aspects in combination. This study was conducted to test the following hypotheses:

1. The intestinal microbiota composition and function (metabolome) is a key determinant of IBD pathogenesis.
2. Shifts in microbial metabolome are associated with systemic inflammatory responses in the host with IBD.

The specific aims of the study were to:

1. Define the intestinal (luminal and mucosal) microbiota composition in PIBD at diagnosis and in response to treatment
2. Determine the stool SCFA profile of PIBD at diagnosis and in response to treatment
3. Define the systemic cytokine and vascular profiles of PIBD at diagnosis and in response to treatment
2. Chapter 2

Selection, Recruitment and Demographics of Study Populations

2.1 Introduction

In designing the study, it was important to consider patient groups to be recruited to specifically address the research questions, as stated in Section 1.7. The population of interest was newly-diagnosed IBD patients and those starting treatment. Other patient populations were recruited as comparison to and controls for these patients. In this chapter, the selection of patients, their demographic characteristics and clinical histories including nutritional statuses were determined.

2.2 Methods

2.2.1 Ethical Approval

For ethical approval of the study, the application, consent forms and information leaflets for parents and children of varying ages were prepared. Considerations for the patients, particularly control patients were discussed and justified.

Ethical approval was granted by the National Research Ethics Service (NRES) Committee London - Central. (REC reference 14/LO/0364). Patients with very early-onset IBD (VEOIBD) and those undergoing HSCT were recruited by Dr Kammermeier under the Patients with Early-onset Intestinal inflammation (PETIT) Study (REC reference 12/LO/1965). Blood samples from healthy adolescents were obtained by Dr
James Bonner (REC reference 14/LO/0644). Patients were recruited as per the approved study protocol from the Gastroenterology Departments in Great Ormond Street Hospital for Children NHS Foundation Trust (GOSH) and University College Hospital (UCH), London. Research samples were collected opportunistically as permitted under the ethical approval.

2.2.2 Patient Recruitment

2.2.2.1 Patients with Inflammatory Bowel Disease

Patients referred with signs and symptoms suggestive of IBD were identified prior to their endoscopic mucosal assessment (Figure 2.1). The clinical team caring for the patient gained consent at an appropriate time for a researcher to approach. When the parents agreed, they were approached with information on the study. Recognising the stress that they were under, written consent to participate in the study was sought after at least 24 hours. Patients with histological evidence of IBD were followed up prospectively. Patients with no histological evidence of inflammation were recruited as non-inflammatory control (NIC) patients and one-off samples of blood, stool, lavages and biopsies were collected.

Similarly, patients advised to start Infliximab were identified and approached as the newly-diagnosed patients were. Those with severe IBD were often transferred to GOSH from local hospitals. Patients undergoing HSCT were admitted in advance for planned pre-HSCT treatment and recruited under the PETIT Study.

Recruitment took place between October 2014 and January 2017. IBD patients were followed up prospectively with subsequent samples collected opportunistically alongside samples for clinical assessments.

2.2.2.2 Patients with Cryopyrin-Associated Periodic Syndrome (CAPS)

As one of the study aims was to investigate the effects of treatment in IBD, the opportunity to study patients with mutations in the IL-1β pathway arose. CAPS patients starting treatment with Canakinumab were identified by Professor Paul Brogan, Rheumatology Department at GOSH, then approached and recruited into the study (Figure 2.1). These patients were subsequently followed up and stool samples were collected prospectively.
2.2.2.3 Allergic and Healthy Infants

Another comparison group of inflammatory GI conditions were infants with cow’s milk protein allergy (CMPA). Similar to CAPS patients, these patients were recruited opportunistically. Allergic infants under the care of GOSH, were recruited to the study. As a control group, healthy infants (HI) were approached at Hertfordshire community centres and recruited. One-off stool samples were obtained after gaining informed consent. Relevant clinical information was collected at the time of recruitment.
2.2.2.4 Healthy Children

Initial efforts to recruit suitable healthy children (HC) from GOSH were unsuccessful. Following a substantial amendment of the ethical approval, HC aged over 4 years from the community were recruited for one-off stool samples and clinical information. These children were matched for age and sex to IBD patients. In addition, blood samples were collected from healthy adolescents for immune and vascular profiling.

2.2.2.5 Sample Collection

Collection protocol for stool, blood, intestinal mucosal biopsies and duodenal lavage are discussed in the relevant chapters. Preliminary experiments included validation studies to determine the most practical and optimal methodologies required for obtaining robust data. These details are discussed in the appropriate chapters.

2.2.2.6 Collection of Clinical Data

Personal information on HC and patients were obtained during interviews, aided by a Clinical History and Dietetic form (Appendix 2A). Clinical and dietetic information, updates and results were obtained from the GOSH computer results systems. These included disease activity scores, which were PUCAI and wPCDAI scores (Tables 1.3 and 1.4, Chapter 1), and inflammatory markers, more specifically ESR, CRP and stool calprotectin.

Current nutritional intakes were recorded based on 24-hour recall of usual meals. Dietetic restrictions and supplements were also recorded. Macronutrient and micronutrient intakes were kindly assessed by Mrs Rita Shergill-Bonner, Principal Dietician in IBD, GOSH.

Identifiable confidential information was stored securely on Data Safe Haven, UCL. Hard copies of confidential clinical information and linked laboratory codes were stored in a locked drawer in a locked office in the ICH.
2.2.3 Statistics

The sample sizes required for statistical significance were estimated based on previous studies comparing IBD patients with HC. These studies utilised *F. prausnitzii* as a marker of disease activity (334-336). Assuming power of 0.80, 25-31 CD and 36 UC patients would have been required to detect statistical significance. Given that GOSH accepted an average of 5 newly-diagnosed children with IBD per month, with 48 new patients in the January-June 2014 period, this was felt to be achievable. In addition, there was the opportunity to recruit 20 adolescents presenting to University College Hospital a year. There were 2-5 patients with IBD undergoing surgery or HSCT per year. A patient with IL-10 pathway mutation was diagnosed every 12-18 months with 6 already known to GOSH.

Comparison between HC and IBD groups was made using MedCalc the "N-1" Chi-squared test, 95% confidence interval, as recommended by Campbell (337) and Richardson (338). The confidence interval was calculated according to the recommended method given by Altman et al. (339).

2.3 Clinical Characteristics and Nutritional Data of Study Population

To truly appreciate the differences between these groups, clinical and dietetic histories were recorded for comparison and to enable correlation with laboratory findings. The details of the populations and their clinical and nutritional data are presented below.

2.3.1 Demographics of Study Populations

A total of 60 individuals (HC and patients) (age 5.5 months to 17 years) were recruited to the study (Table 2.1). The control groups included 6 healthy infants (HI) [age 5.5-9 (median 8) months, 3 girls], 11 healthy children (HC) [age 4-17 (median 12) years, 6 girls], and 16 non-inflammatory control (NIC) patients [age 4-11 (median 11) years, 8 girls].

A total of 19 IBD patients were recruited, including those who were newly-diagnosed (n=9), those with severe disease (n=4) and those commencing Infliximab (n=6). The newly-diagnosed IBD patients were diagnosed with CD (n=4, age 9-13 years, 1 girl), UC
In addition, there was a patient who was diagnosed with CD, and subsequently was also diagnosed with Granulomatosis polyangiitis (age 11 years, girl). Patients with severe disease were known to have CD (n=2, age 15-16 years, boys), UC (n=1, age 12 years, girl) and VEOIBD (n=1, age 4 years, girl). The patients commencing Infliximab had diagnoses of CD (n=4, age 9-15 years, 4 girls), UC (n=1, age 15 years, girl) and IBDU (n=1, age 16 years, girl).

Limited samples were collected from a patient with IL-10RA mutation (age 3 years, boy), a patient with IL-10RB mutation (age 15 years, girl) and a patient with infantile IBD (unidentified genetic mutation, age 6 years, boy) under the PETIT study (total n=3). In addition, patients with Cryopyrin-Associated Periodic Syndrome (CAPS) (n=2, age 2 and 6 years, 2 girls) and infants with CMPA [n=3, age 7-16 (median 12) months, 3 girls] were recruited. Thus a total of 60 patients were recruited and analysed in 9 groups.

Most of the HC (80%), 56% of the NIC and 39% of the IBD patients were White British (Table 2.1). Other ethnicities include Indian (2 IBD, 1 NIC), Pakistani (3 IBD, 2 NIC, 1 HC), White European (1 IBD, 2 HC, 1 Infantile IBD), Black Caribbean (1 CAPS), Black African (1 IBD) and Black British (1 IBD). There were 2 patients with unknown ethnicity, 2 unclassified, 1 of unspecified Asian and 1 of unspecified White descent. In addition, there were patients of mixed ethnicities (1 White and Black Caribbean, and 2 White and Asian).

Samples were collected from patients opportunistically alongside clinical samples at varying intervals depending on their clinical status (Tables 2.2 and 2.3). They were more likely to visit the hospital when unwell thus most samples were collected during active disease. Stool samples were collected for paired analysis of microbiota and SCFA of the same sample (n=83). In addition, 18 stool samples were collected for microbiota analysis only.

Intestinal mucosal biopsies (n=39 sets) and duodenal lavages (n=27) were obtained during endoscopic investigations performed when clinically indicated. These samples were collected prior to diagnosis and opportunistically after (n=4). A set of mucosal biopsies generally consisted of duodenal, terminal ileal, right colonic and left colonic mucosal biopsies (maximum of 6 biopsies per patient) but this varied depending on patient anatomy. Duodenal lavages were performed by flushing 10 ml sterile water into the duodenum via the endoscope, which was then suctioned into a mucus extractor (Vygon, UK) connected to the suction port of the endoscopy stack system (Figure 2.2).
The suctioned water was then transferred into a sterile universal bottle using a sterile needle and syringe.

Blood samples (n=71) were collected from patients during their admissions and/or hospital visits. Similarly, samples were collected prior to diagnosis or treatment, then opportunistically thereafter. For ethical reasons, collection of blood samples was not possible for HC and HI recruited to the study.
**Figure 2.2: Mucus Extractor connection to the Endoscopy Stack System**

The mucus extractor (Vygon, UK) (A), with the lid closed, was connected to the suction port of the endoscopy stack system (Olympus, UK) via a connector (B). The suction tube of the mucus extractor was removed and replaced by the larger tube attached to the waste collector and pump (C).
<table>
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<th>Patient Group</th>
<th>Number of Patients</th>
<th>Diagnosis (n=)</th>
<th>Age (median) (years/months)</th>
<th>Sex (Female)</th>
<th>Ethinicity (n=)</th>
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<td>Number of Mucosal Microbiota Sets (Number of Patients)</td>
<td>Number of Duodenal Lavage Microbiota Samples (Number of Patients)</td>
<td>Number of Stool Short Chain Fatty Acids Samples (Number of Patients)</td>
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<td>NA</td>
<td>5 (2)</td>
</tr>
<tr>
<td>Allergic Infants</td>
<td>3</td>
<td>3 (3)</td>
<td>NA</td>
<td>NA</td>
<td>2 (2)</td>
</tr>
</tbody>
</table>
Table 2.3: Samples Collected Prospectively from Patients
2.3.2 Healthy Children

The HC [n=11, age 4-17 (median 12) years, 6 girls] were generally healthy at the time of sample collection. Some had medical past histories of minor GI symptoms and/or atopy but for the purpose of the study, these patients will be named HC. Despite this, these children are representative of healthy paediatric population. None of the HC had received antibiotics at least 2 years prior to stool sample collection. HC were matched for sex and age to IBD patients.

2.3.3 Patients with Inflammatory Bowel Disease

The 19 IBD patients [age 4-16 (median 12 years), 10 girls] were phenotypically classified using the Paris Classification for CD and UC (Table 2.4, Appendix 2B). IBDU and VEOIBD patients were classified using the UC Paris Classification. The treatments received alluded to their clinical course; some patients with complicated courses required escalation of treatment. Three patients who were refractory to medical treatment required colectomy, 1 of whom underwent surgery during the study period.

There were 3 patients with atypical IBD, one with collagenous colitis, one with CD complicated by Granulomatosis polyangiitis and another with VEOIBD. In addition, 3 patients with infantile IBD received HSCT for severe intractable IBD. Most patients received vitamin D supplementation and corticosteroids were administered with a proton pump inhibitor.

When known, potential disease triggers were also noted (n=13) (Table 2.4). Four patients started having symptoms following an episode of gastroenteritis. In addition, 4 patients were reported to develop symptoms of IBD after an episode of gastroenteritis during a stressful period such as parental divorce, moving countries, changing childcare provider, moving home, family bereavement and changing schools. Of these 8 patients, 3 developed symptoms after traveling abroad to visit relatives. The remaining 4 acquired gastroenteritis in the UK.

In addition, 2 patient reported a stressful event preceding symptoms and another developed IBD symptoms following a complicated appendicitis. The patient with infantile IBD was reported to have an episode of pyrexia with diarrhoea and vomiting prior to developing IBD. Following introduction of full fat unmodified cow’s milk, the patient with
IL-10Rβ mutation developed mucousy, bloody diarrhoea which progressed to severe intractable IBD.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Phenotype</th>
<th>Treatment</th>
<th>Possible disease trigger</th>
</tr>
</thead>
<tbody>
<tr>
<td>A14</td>
<td>CD</td>
<td>A1b, L3, L4, B1, G1</td>
<td>EEN, Azathioprine, Infliximab</td>
<td>Unknown</td>
</tr>
<tr>
<td>A15</td>
<td>CD</td>
<td>A1a, L3, L4a?, B1, G1</td>
<td>EEN, Azathioprine, Infliximab</td>
<td>Unknown</td>
</tr>
<tr>
<td>A17</td>
<td>CD</td>
<td>A1a, L3, G1</td>
<td>EEN, Azathioprine</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>A25</td>
<td>Fistulising CD</td>
<td>A1b, L3, L4a, B3, P, G1</td>
<td>EEN, Azathioprine, Metronidazole, Ciprofloxacin, Infliximab, Triple therapy for H. pylori</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>C2</td>
<td>CD</td>
<td>A1a, L3, L4a, B1, G1</td>
<td>Prednisolone, Azathioprine, Mesalazine, Methotrexate, Folic acid, Infliximab, Sirolimus, Thalidomide, Basiliximab, PN, Ustekinumab, Surgical resection</td>
<td>Unknown</td>
</tr>
<tr>
<td>D3</td>
<td>CD, OGM</td>
<td>A1b, L3, L4a, B3, G0</td>
<td>Prednisolone, Azathioprine, Cinnamon/benzoate/dairy-free diet, Infliximab, Adalimumab</td>
<td>Unknown</td>
</tr>
<tr>
<td>C5</td>
<td>CD</td>
<td>A1b, L2, B1, G0</td>
<td>Hydrocortisone, Prednisolone, Ciprofloxacin, Mesalazine, PN, Azathioprine, Infliximab</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>D2</td>
<td>CD, Coeliac</td>
<td>A1a, L2, L4a, B1, G1</td>
<td>Gluten-free, Azathioprine, Budesonide, Pentasa, Prednisolone, Adalimumab, Infliximab, Methotrexate, Folic acid</td>
<td>Unknown</td>
</tr>
<tr>
<td>D4</td>
<td>CD</td>
<td>A1b, L2, L4a, B1, G1</td>
<td>Azathioprine, Allopurinol, Prednisolone, Infliximab</td>
<td>Gastroenteritis, Stress</td>
</tr>
<tr>
<td>D6</td>
<td>CD</td>
<td>A1b, L3, L4, B2, G1</td>
<td>EEN, Azathioprine, Mesalazine, Infliximab, Prednisolone, Budesonide, Doxycycline, Surgical resection</td>
<td>Gastroenteritis, Stress</td>
</tr>
<tr>
<td>A8</td>
<td>UC</td>
<td>E4, S1</td>
<td>Prednisolone, Azathioprine, Infliximab, Pentasa</td>
<td>Unknown</td>
</tr>
<tr>
<td>A21</td>
<td>UC</td>
<td>E4, S1</td>
<td>Azathioprine, Prednisolone, Mesalazine</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>C1</td>
<td>UC</td>
<td>E4, S1</td>
<td>Hydrocortisone, Methylprednisolone, Azathioprine, Infliximab, PN, Infliximab, Sirolimus, Adalimumab, Mesalazine, Ceftriazone, Metronidazole (before diagnostic scopes)</td>
<td>Unknown</td>
</tr>
<tr>
<td>D5</td>
<td>UC</td>
<td>E4, S1</td>
<td>Prednisolone, Azathioprine, Mesalazine, Methotrexate, Folic acid, Infliximab</td>
<td>Gastroenteritis/Stress</td>
</tr>
<tr>
<td>Patient</td>
<td>Diagnosis</td>
<td>Phenotype</td>
<td>Treatment</td>
<td>Possible disease trigger</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>A2</td>
<td>Collagenous colitis</td>
<td>NA</td>
<td>Mebeverine</td>
<td>Stress</td>
</tr>
<tr>
<td>A9</td>
<td>IBDU</td>
<td>E3, S1</td>
<td>EEN, Prednisolone, Azathioprine, Mesalazine, Infliximab</td>
<td>Unknown</td>
</tr>
<tr>
<td>A11</td>
<td>CD with Granulomatosis polyangiitis</td>
<td>A1b, L3, L4, B1, G1 Vasculitis involvement of skin, liver, pancreas, eyes</td>
<td>EEN, Prednisolone, Azathioprine, Methotrexate, Folic acid, Infliximab, Adalimumab, Cyclophosphamide, Rituximab</td>
<td>Stress</td>
</tr>
<tr>
<td>D7</td>
<td>IBDU</td>
<td>E4, S1</td>
<td>EEN, Azathioprine, Mesalazine, Hydrocortisone, Infliximab, Adalimumab, Ganciclovir</td>
<td>Appendicitis</td>
</tr>
<tr>
<td>C3</td>
<td>VEOIBD</td>
<td>E4, S1</td>
<td>EEN, Neocate, Prednisolone, Azathioprine, Methylprednisolone, Ciprofloxacin, Metronidazole, Infliximab, Surgical resection</td>
<td>Gastroenteritis, Stress</td>
</tr>
<tr>
<td>B1</td>
<td>Infantile IBD</td>
<td>NA</td>
<td>Prednisolone, Azathioprine, Mesalazine, Infliximab, Adalimumab, Sirolimus, HSCT</td>
<td>Pyrexia, diarrhoea, vomiting at age 5 months</td>
</tr>
<tr>
<td>B2</td>
<td>IL-10RA mutation</td>
<td>NA</td>
<td>Prednisolone, Azathioprine, EEN, PN, Infliximab, Adalimumab, topical steroids, HSCT</td>
<td>Unknown</td>
</tr>
<tr>
<td>B3</td>
<td>IL-10RB mutation</td>
<td>NA</td>
<td>Corticosteroids, Azathioprine, Cyclosporin, Mesalazine, Sulfasalazine, Courses of antibiotics, Betamethasone, Prednisolone, PN, Nursoy, Nutramegen, Loperamide, Surgical resection, HSCT</td>
<td>Full fat unmodified cow's milk at 3 months</td>
</tr>
</tbody>
</table>

**Table 2.4: Clinical Phenotype by Paris Classification (Appendix 2B), Treatment and Possible Disease Trigger in IBD Patients**
2.3.3.1 Medical Histories of HC and IBD Patients

Background and medical histories of HC and IBD patients were obtained during interviews and compared (Table 2.5). Despite the small numbers, there were some striking differences seen.

All HC and IBD patients were conceived naturally. Antenatal complications [HC n=4 (36%), IBD n=3 (16%) and antenatal antibiotics [HC n=0, IBD n=3 (16%) did not reveal any significant differences. Mode of delivery at birth was also analysed but no significant differences were seen with vaginal delivery [HC n=9 (82%), IBD n=14 (74%)] nor Caesarean section [HC n=2 (18%), IBD n=5 (26%)]. There were 2 HC and 2 IBD patient who were admitted to Special Care Baby Unit (SCBU) after birth. There were 2 children in each group who received antibiotics soon after birth.

A history of GI problems was reported by 6 (55%) HC and 11 (58%) IBD patients. There were no significant differences seen with gastroesophageal reflux [HC n=1 (9%), IBD n=4 (21%)], loose stools [HC n=3 (27%), IBD n=1 (5%)] and constipation [HC n=1 (9%), IBD n=4 (21%)]. IBD patients were, however, more likely to report a history of infantile colic [HC n=0, IBD n=5 (26%), NS].

Most children in each group (10 HC, 17 IBD) received antibiotics but IBD patients were more likely to report a history of recurrent infections requiring antibiotics (Table 2.6). One (9%) HC and 5 (26%) IBD patients received treatment for urinary tract infections. One child in each group received prophylactic Trimephoprim (HC for few months, IBD patient for 5 years). Respiratory infections requiring antibiotics were mostly reported by IBD patients [HC n=1 (9%), IBD n=7 (37%), p=0.1034], who were perhaps more likely to report asthma/wheeze [HC n=1 (9%) vs. IBD n=6 (32%), NS]. In addition, more IBD patients reported hayfever [HC n=1 (9%) vs. IBD n=9 (47%), p=0.0351] and eczema [HC n=4 (27%) vs. IBD n=11 (58%), NS]. Two IBD patients were found to have nonspecifically raised IgE levels.

Commonly reported problems such as tonsillitis [HC n=2 (18%), IBD n=3 (16%)] and otitis media [HC n=2 (18%), IBD n=6 (32%)] were also reported by children of both groups. More IBD patients reported headaches [n=9 (47%)] compared to HC [n=2 (18%), p=0.0351]. Behavioural issues [n=2 (11%)] and dyslexia [n=1 (6%)] were also reported by IBD patients. There was an IBD patient with learning difficulties with a detected genetic mutation of unknown significance.
Interestingly, more patients with IBD \[ n = 6 \ (32\%), \ p = 0.0405 \] reported joint aches and/or pains than HC \( n = 0 \). There was a HC reporting joint hypermobility.

There were 3 HC who lived in rural areas, with the remaining HC and all IBD patients reported to live in urban areas. Both HC and IBD patients had siblings [HC \( n = 11 \ (100\%) \) vs. IBD \( n = 15 \ (89\%) \)] and pets [HC \( n = 5 \ (45\%) \) vs. IBD \( n = 4 \ (21\%) \)]. Unsurprisingly, more IBD patients reported a family history of IBD \[ n = 7 \ (41\%) \] than HC \[ n = 1 \ (9\%), \ NS \]. Both groups reported smokers within the immediate family [HC \( n = 3 \ (27\%) \), IBD \( n = 5 \ (26\%) \)].

### 2.3.3.2 Medications Received by HC and IBD Patients

IBD patients received several treatments as detailed in Table 2.4. This included proton-pump inhibitors alongside corticosteroids. In addition, regular use of anti-pyretic/analgesic agents was also assessed. Most children from both groups reported receiving Paracetamol and Ibuprofen with no significant differences seen between the groups (Table 2.5).

Antibiotics received by children from both groups were assessed (Tables 2.5 and 2.6). One (9\%) HC and 2 (11\%) IBD patients did not receive any antibiotics. The ages of first encounter with antibiotics were similar in each group with more IBD children receiving antibiotics at age 3-4 years. IBD children were less likely to receive only 1 course of antibiotics in their lives [HC \( n = 9 \ (90\%) \) vs. IBD \( n = 2 \ (11\%) \), \( p = 0.0001 \)] and more likely to receive 2 or more antibiotic courses [HC \( n = 1 \ vs. \ IBD \ n = 13 \ (68\%), \ p = 0.002 \], with 5 children receiving more than 10 courses (26\%, \( p = 0.0669 \)).
<table>
<thead>
<tr>
<th>Medications</th>
<th>Healthy Children (n=11)</th>
<th>IBD Patients (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n  (%)</td>
<td>n  (%)</td>
</tr>
<tr>
<td>Antibiotics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1  9</td>
<td>2  11</td>
</tr>
<tr>
<td>Age at First Antibiotic Course</td>
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<td></td>
</tr>
<tr>
<td>After birth</td>
<td>3  27</td>
<td>1  5</td>
</tr>
<tr>
<td>1 week</td>
<td>0  -</td>
<td>1  5</td>
</tr>
<tr>
<td>1 year</td>
<td>3  27</td>
<td>1  5</td>
</tr>
<tr>
<td>2 years</td>
<td>1  9</td>
<td>3  16</td>
</tr>
<tr>
<td>3 years</td>
<td>2  18</td>
<td>5  26</td>
</tr>
<tr>
<td>4 years</td>
<td>0  -</td>
<td>4  21</td>
</tr>
<tr>
<td>5 years</td>
<td>0  -</td>
<td>1  5</td>
</tr>
<tr>
<td>&gt;5 years</td>
<td>1  9</td>
<td>1  5</td>
</tr>
<tr>
<td>Number of Courses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Course</td>
<td>9  90</td>
<td>2  11***</td>
</tr>
<tr>
<td>&gt;1 course</td>
<td>1  -</td>
<td>13  68***</td>
</tr>
<tr>
<td>2 Courses</td>
<td>0  -</td>
<td>3  16</td>
</tr>
<tr>
<td>3 Courses</td>
<td>0  -</td>
<td>1  5</td>
</tr>
<tr>
<td>4 Courses</td>
<td>0  -</td>
<td>2  11</td>
</tr>
<tr>
<td>5 Courses</td>
<td>1  10</td>
<td>4  21</td>
</tr>
<tr>
<td>&gt;10 Courses</td>
<td>0  -</td>
<td>5  26</td>
</tr>
<tr>
<td>Prophylactic Trimethoprim</td>
<td>1  10</td>
<td>1  5</td>
</tr>
<tr>
<td>Paracetamol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2  18</td>
<td>2  11</td>
</tr>
<tr>
<td>Few/ week</td>
<td>0  -</td>
<td>1  5</td>
</tr>
<tr>
<td>Few/ month</td>
<td>2  18</td>
<td>4  21</td>
</tr>
<tr>
<td>Few/ year</td>
<td>5  45</td>
<td>11  58</td>
</tr>
<tr>
<td>Rarely</td>
<td>2  18</td>
<td>1  5</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3  27</td>
<td>7  37</td>
</tr>
<tr>
<td>Few/ month</td>
<td>3  27</td>
<td>2  11</td>
</tr>
<tr>
<td>Few/ year</td>
<td>5  45</td>
<td>5  26</td>
</tr>
<tr>
<td>Rarely</td>
<td>0  -</td>
<td>5  26</td>
</tr>
</tbody>
</table>

*Table 2.5: Medications Received by HC and IBD Patients (*** - p<0.005)*
<table>
<thead>
<tr>
<th>Complications</th>
<th>Healthy Children (n=11)</th>
<th>IBD Patients (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>(%)</td>
</tr>
<tr>
<td>Complications</td>
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<td>36</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Birth</th>
<th>Healthy Children (n=11)</th>
<th>IBD Patients (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>(%)</td>
</tr>
<tr>
<td>Vaginal Deliver</td>
<td>9</td>
<td>82</td>
</tr>
<tr>
<td>Caesarean Section</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Admission to SCBU</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>2</td>
<td>18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gastroesophageal reflux</th>
<th>Healthy Children (n=11)</th>
<th>IBD Patients (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>(%)</td>
</tr>
<tr>
<td>Gastroesophageal reflux</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Colic</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Loose stools</td>
<td>3</td>
<td>27</td>
</tr>
<tr>
<td>Constipation</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Urinary Tract Infections</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Respiratory Infections</td>
<td>1</td>
<td>9</td>
</tr>
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</table>

<table>
<thead>
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<th>Atopy</th>
<th>Healthy Children (n=11)</th>
<th>IBD Patients (n=19)</th>
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<tr>
<td>Eczema</td>
<td>4</td>
<td>36</td>
</tr>
<tr>
<td>Hay fever</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Asthma/Wheeze</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Raised IgE</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th>ENT</th>
<th>Healthy Children (n=11)</th>
<th>IBD Patients (n=19)</th>
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</thead>
<tbody>
<tr>
<td>Tonsilitis</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Otitis Media</td>
<td>2</td>
<td>18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Joints</th>
<th>Healthy Children (n=11)</th>
<th>IBD Patients (n=19)</th>
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</thead>
<tbody>
<tr>
<td>Hypermobility</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Aches/pains</td>
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<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Family</th>
<th>Healthy Children (n=11)</th>
<th>IBD Patients (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rural home</td>
<td>3</td>
<td>27</td>
</tr>
<tr>
<td>Siblings</td>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td>Pets</td>
<td>5</td>
<td>45</td>
</tr>
<tr>
<td>Family history of IBD</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Smoker</td>
<td>3</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 2.6: Medical Histories of HC and IBD Patients (* - p<0.05)
2.3.3.3 Assessing Early and Current Nutritional Intakes in HC and IBD Patients

As early nutrition is a known factor for developing IBD, nutritional assessments were included in the clinical history. In addition, current intakes of energy, protein, micronutrients and fibre were recorded to assist with interpretation of laboratory results.

The initial milk and weaning food received by children in both groups were assessed (Table 2.7). HC were breastfed from 1 week to 9 months of age whereas IBD patients were fed longer – 2 were fed for more than 18 month. The number of children breastfed for 9 months was significantly different between the two groups [HC n=3 (27%), IBD n=1 (6%), p=0.0280]. Four IBD (24%) patients received formula feed from birth and 3 (18%) received formula in the first week of life. In comparison, only one (9%) HC received formula in the first week and 4 (36%) HC received formula in the first month (vs. IBD n=0, p=0.0116). There were, however, more IBD patients who were breastfed for longer [>12 months, n=4 (24%)] but there were more HC who were exclusively breastfed [n=3 (27%), IBD n=1 (6%)].

There were some differences seen in weaning practices. Three (18%) of the IBD patients were weaned early at 3 months and all had started solids by 6 months of age while HC were weaned at ages 4-7 months. HC [n=10 (91%)] were more likely to have received home-cooked puree food than IBD patients [n=9 (47%)]. In addition, more IBD patients reported allergies or intolerances to food [HC n=4 (36%) vs. IBD n=13 (71%), p=0.0656], and more IBD patients were reported to be fussy with their food [HC n=0 vs. IBD n=9 (47%), p=0.0094].

Nutritional intake was assessed based on a 24-hour recall of dietary intake in most IBD patients (n=17), some NIC patients (n=7) and the HC (n=11). Daily energy intake was assessed based on the Estimated Average Requirement (EAR) and protein intake was assessed based on the Reference Nutrient Intake (RNI), Dietary Reference Values 2011 (Figure 2.3A, 2.2B). Four (36%) HC [total n=11, median 80 (65-130)%] received adequate intake (>90% EAR) compared to only 3 (19%) IBD patients [total n=17, median 65 (30-90)%, p=0.0045] and none of the NIC patients [total n=7, median 75 (60-80)%].

In general, most HC [n=11, median 100 (50-150)%] met their protein requirements while less NIC patients [total n=7, median 83 (50-120)%, NS] and IBD patients [total n=17, median 75 (30-110)%, NS] received adequate protein.
### Table 2.7: Nutrition of HC and IBD Patients (* - p<0.05, ** - p<0.01)

<table>
<thead>
<tr>
<th>Breastfeeding Duration (up to)</th>
<th>Healthy Children (n=11)</th>
<th>IBD Patients (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0 (-)</td>
<td>3 (16)</td>
</tr>
<tr>
<td>1-2 weeks</td>
<td>1 (9)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>3 months</td>
<td>4 (36)</td>
<td>4 (21)</td>
</tr>
<tr>
<td>6 months</td>
<td>3 (27)</td>
<td>5 (26)</td>
</tr>
<tr>
<td>9 months</td>
<td>3 (27)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>12 months</td>
<td>0 (-)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>15 months</td>
<td>0 (-)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>&gt;18 months</td>
<td>0 (-)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>Age when Formula Milk Introduced</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3 (27)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>At birth</td>
<td>0 (-)</td>
<td>4 (21)</td>
</tr>
<tr>
<td>First week</td>
<td>1 (9)</td>
<td>3 (16)</td>
</tr>
<tr>
<td>First month</td>
<td>4 (36)</td>
<td>0 (**)</td>
</tr>
<tr>
<td>3 months</td>
<td>2 (18)</td>
<td>6 (32)</td>
</tr>
<tr>
<td>6 months</td>
<td>1 (9)</td>
<td>5 (26)</td>
</tr>
<tr>
<td>Age Weaned</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td>0 (-)</td>
<td>3 (16)</td>
</tr>
<tr>
<td>4 months</td>
<td>4 (36)</td>
<td>3 (16)</td>
</tr>
<tr>
<td>5 months</td>
<td>2 (18)</td>
<td>7 (37)</td>
</tr>
<tr>
<td>6 months</td>
<td>3 (27)</td>
<td>4 (21)</td>
</tr>
<tr>
<td>7 months</td>
<td>2 (18)</td>
<td>0 (-)</td>
</tr>
<tr>
<td>9 months</td>
<td>0 (-)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>After 1 year</td>
<td>0 (-)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Weaning</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Home-cooked</td>
<td>10 (91)</td>
<td>9 (47*)</td>
</tr>
<tr>
<td>Jars</td>
<td>1 (9)</td>
<td>3 (18)</td>
</tr>
<tr>
<td>Mixed Jars and Home-cooked</td>
<td>0 (-)</td>
<td>6 (29*)</td>
</tr>
<tr>
<td>Food allergies/Intolerances</td>
<td>4 (36)</td>
<td>13 (71)</td>
</tr>
<tr>
<td>Fussy</td>
<td>0 (-)</td>
<td>9 (47**)</td>
</tr>
</tbody>
</table>

Table 2.7: Nutrition of HC and IBD Patients (* - p<0.05, ** - p<0.01)
A.  
B.  

Figure 2.3: Energy and Protein Intake of HC, IBD and NIC Patients

The energy (A) and protein (B) intakes of HC (n=7/8), IBD (n=16) and NIC (n=7) patients were assessed based on the Estimated Average Requirement (EAR) and the Reference Nutrient Intake (RNI), Dietary Reference Values 2011. HC received more energy compared to IBD patients (p=0.0045) but there were no significant differences in protein intake compared to NIC and IBD patients.

Comparison of weight and height percentiles was not possible due to limited information available for the HC (n=2) (Figure 2.4A, 2.4B). They were all well-grown and proportionate but many had not had their measurements (heights and weights) taken recently. Information was, however, available for NIC [n=7, median weight 30 (2-96)%, median height 58 (2-99.6)]% and IBD [n=16, median weight 22 (0.4-87)%, median height 16 (1-74)]% patients but no significant differences were seen between the two groups.
Figure 2.4: Weight and Height Percentiles in HC, IBD and NIC Patients

Available weight (A) and height (B) percentiles for HC (n=2), NIC (n=7) and IBD patients (n=16) were compared. Due to the small numbers of HC, comparison was only made between the NIC and IBD patients. No significant differences were seen.

Micronutrients and fibre intakes of patients and HC were also assessed (Figure 2.5A, 2.5B, 2.5C). More HC (n=5, 63%) had adequate calcium intake compared to NIC (n=1, 14%, p=0.0620) and IBD (n=6, 38%, p=0.2570) patients. Very few, if any, received adequate vitamin D in their diets [HC n=2 (25%), NIC n=0, IBD n=2 (13%)]. There were differences seen in fruit and vegetable intake with HC (n=6, 75%) having more fruit and vegetable intake, compared to NIC (n=3, 43%) and IBD (n=4, 27%, p=0.0306) patients. Thus most HC (n=9, 82%) were having moderate and with some having high (n=2, 18%) amounts of fibre. More NIC patients were receiving low fibre diet (n=3, 43%) compared to moderate (n=2, 29%) and high (n=2, 29%) fibre diets. Of the 15 IBD patients, 10 (67%) received moderate, 3 (20%) received low and 2 (13%) received high amounts of fibre in their diets.

A. B. C.
Figure 2.5: Micronutrient and Plant Fibre Intake of HC, IBD and NIC Patients

The micronutrient (A,B) and fibre (C) intake of HC (n=11), NIC (n=7) and IBD (n=15, 16) were assessed based on their dietetic assessments and dietetic questionnaire. HC received more calcium, fruit and vegetable, thus fibre, in their diets compared to NIC and IBD patients. All patients and most HC received inadequate vitamin D in their diets. More NIC patients had adequate fruit and vegetable intake compared to IBD patients but there were more NIC patients having low fibre diets.

2.3.4 Non-Inflammatory Control Patients

Patients with non-inflammatory gastrointestinal (GI) conditions were recruited as a comparison group to the IBD patients (Table 2.8). Their GI mucosal biopsies and duodenal lavages were used as control samples as it was not possible to collect these samples from HC due to ethical constraints. These patients presented with symptoms suggestive of IBD thus underwent endoscopic investigations. One-off samples of blood, stool (for paired microbiota and SCFA analysis), intestinal mucosal biopsies and duodenal lavages were collected.

These patients were found to have normal mucosal biopsies or minimal non-specific changes inconsistent with a diagnosis of IBD. Eight of these patients were thus diagnosed with Irritable Bowel Syndrome (IBS) and managed accordingly. One patient was diagnosed with food allergy, and another 3 patients’ symptoms resolved with no diagnosis made. Four patients were referred by the Rheumatology Department: 2 patients with JIA, 1 with Chronic Sterile Neutrophilic Folliculitis and another with Leukocytoclastic Vasculitis. Some of these patients were on medications during sample collection, including Patient A24 (on Methotrexate) and Patient A7 (Colchicine and Prednisolone).

In addition, 2 patients with CAPS were recruited to the study, Patient E1 prior to the second dose and Patient E2 prior to starting Canakinumab. Patient E1 was also on Azithromycin and Cyclopentolate at the time of the first sample collection. As the CAPS patients were not undergoing endoscopic investigations, only stool samples were available for analysis.
2.3.5 Cow’s Milk Protein Allergic and Healthy Infants

A total of 9 infants were recruited (Table 2.9). Six healthy infants (HI) were recruited from the community, 3 of whom were girls, and aged 5.5 – 9 months (median age 8 months, 4 White British, 1 mixed White and Asian, 1 White European). Four of the infants were born by normal vaginal delivery whilst the other 2 infants were born by Caesarean section. Four infants were exclusively breastfed, 1 infant received a bottle a day and another was breastfed twice a day. Aptamil formula milk was started at birth and 1 week for these 2 infants. The HI were weaned onto solids at 4.5-6 months of age (median 5.3 months).
In contrast, the 3 allergic infants recruited were all girls aged 7-16 months (median age 12 months, 2 White British, 1 Indian) with family histories of atopy. Two infants were born by normal vaginal delivery and 1 was born by Caesarean section. All of the infants were previously exposed to the usual medications for gastrooesophageal reflux, namely Infacol, Colic Ease, Paracetamol, Gaviscon, Ranitidine and Omeprazole. In addition, one infant was receiving Ketotifen at the time of sample collection. It was recognised that these commonly-used medications may influence the study findings.

All infants were initially exclusively breastfed. Three infants were then given Neocate, with the infants starting the amino acid-based formula at 5 weeks, 4 months and 6 months of age. They were all then weaned onto solids at 6 months of age.
<table>
<thead>
<tr>
<th>Infant</th>
<th>Status</th>
<th>Sex</th>
<th>Age (months)</th>
<th>Ethnicity</th>
<th>Delivery</th>
<th>Breast-feeding</th>
<th>Feed</th>
<th>When started feed</th>
<th>Weaning Age (mths)</th>
<th>Previous Medications</th>
<th>Current Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HC</td>
<td>M</td>
<td>7</td>
<td>WB</td>
<td>NVD</td>
<td>100</td>
<td>Feed</td>
<td>100</td>
<td>Birth</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>HC</td>
<td>F</td>
<td>5.5</td>
<td>WB</td>
<td>NVD</td>
<td>mostly, 1 bottle/day</td>
<td>Aptamil</td>
<td>Birth</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>HC</td>
<td>F</td>
<td>9</td>
<td>White and Asian</td>
<td>LSCS</td>
<td>2x/day</td>
<td>Aptamil</td>
<td>Birth</td>
<td>5.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>HC</td>
<td>F</td>
<td>7</td>
<td>WB</td>
<td>NVD</td>
<td>100</td>
<td>Feed</td>
<td>100</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>HC</td>
<td>M</td>
<td>9</td>
<td>WB</td>
<td>NVD</td>
<td>100</td>
<td>Feed</td>
<td>4.5</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>All</td>
<td>F</td>
<td>16</td>
<td>WB</td>
<td>EI LSCS</td>
<td>100 birth to 5wks</td>
<td>Neocate</td>
<td>5 wks</td>
<td>6</td>
<td>Infacol, Colic ease</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>All</td>
<td>M</td>
<td>9</td>
<td>White European</td>
<td>EI LSCS</td>
<td>100</td>
<td>Feed</td>
<td>6</td>
<td>6</td>
<td>Paracetamol</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>All</td>
<td>F</td>
<td>7</td>
<td>WB</td>
<td>NVD</td>
<td>100</td>
<td>Neocate</td>
<td>6</td>
<td>6</td>
<td>Gaviscon, Ranitidine, Omeprazole</td>
<td>Ketotifen</td>
</tr>
<tr>
<td>9</td>
<td>All</td>
<td>M</td>
<td>12</td>
<td>Indian</td>
<td>NVD</td>
<td>100 birth to 4mths</td>
<td>Neocate</td>
<td>4 mths</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3.5 Summary of Results

Healthy Children (HC)
- HC ranged from 4 to 17 years of age (median 12 years, 6 girls). Of the 11 children, 8 (73%) were White British and 2 were White European.
- There was a history of atopy (n=6) and minor GI symptoms (n=5) in early childhood in a few of the children.
- None of the children had received antibiotics for at least 6 months.

Non-inflammatory Control (NIC) Patients
- NIC patients were aged 4 to 15 years (median 11 years, 8 girls). Of the 16 patients, 9 were White British (56%), 1 unspecified White, 1 mixed White and Black Caribbean, 2 Pakistanis and 1 Indian.
- Although NIC patients had normal or minimal histological changes on intestinal mucosal biopsies, they had GI symptoms significant enough to warrant endoscopic investigations.
- Eight patients were diagnosed with IBS, 1 with food allergy, 4 had coexisting Rheumatological conditions, 2 with CAPS and 3 patients with self-resolving symptoms remained undiagnosed.
- Some patients were on treatment at the time of sample collection, which may have influenced laboratory findings.

IBD Patients
- IBD patients ranged from 4 to 16 years of age (median 12 years, 10 girls). Of the 19 patients, 39% were White British.
- Of the 4 UC patients, 2 were of Asian and another of mixed White and Asian descent.
- Two patients diagnosed with CD also had Granulomatosis Polyangiitis and another Coeliac Disease.
- Three patients refractory to medical therapy required surgical resection (Patients C2, C3, D2).
- Commonest possible triggers for IBD were gastroenteritis and emotional stress.

Healthy Children (HC) versus IBD Patients
The medical histories, medications and early nutrition of HC (n=11) and IBD patients (n=19) were compared. Despite the small numbers, there were some differences apparent;
• More IBD patients reported hay fever and headaches
• Joint aches/pains were only reported in IBD patients
• Most HC received 1 course of antibiotic whilst 68% of IBD patients received ≥2 courses, 5 of whom received ≥10 courses
• Most HC received home-cooked weaning food while IBD patients were more likely to receive jar foods. Approximately half of the IBD patients were reported to be fussy eaters.
• HC had a greater intake of fruit and vegetable compared to NIC and IBD (p=0.0306) patients, with most HC having moderate amount of fibre in their diets

Patients receiving HSCT for Infantile IBD
• Of the 3 patients recruited, 2 were identified to have IL-10 receptor mutations (IL-10RA and IL-10RB).
• Patients were aged 3 years (boy, unspecified Asian), 6 years (boy, White European) and 15 years (girl, Indian)
• These patients presented in infancy with severe IBD, refractory to medical and surgical therapies, thus underwent HSCT.
• In the IL-10 patients, an infection and exposure to unmodified cow’s milk may have triggered the inflammatory process

Cow’s Milk Protein Allergic and Healthy Infants (HI)
• Of the 6 HI [age median 8 (5.5 – 9) months; 3 girls; 4 White British, 1 mixed White and Asian, 1 White European], 4 were born by vaginal delivery and 4 were exclusively breast-fed
• The 3 infants allergic to cow’s milk protein were older than the HI group [age median 12 (7-16) months; all girls; 2 White British, 1 Indian], 2 of the infants were born by vaginal delivery.
• The allergic infants had received medications for gastroesophageal reflux disease and colic. At the time of sample collection, 1 infant was on Ketotifen.


2.4 Discussion

Each child is unique, with their own background, medical histories and environmental factors that shape their clinical status. Although these patients and children are discussed as groups, it is important to understand the person behind the research samples. In-depth analysis of the patient groups highlighted environmental differences that influenced their clinical statuses.

This study was primarily focused on paediatric IBD. As opportunities arose, other patient groups were included for comparison with IBD patients. Conducting the study at GOSH allowed the opportunity to recruit unique patients such as patients with IL-10 receptor mutations. In addition, CAPS patients commencing Canakinumab were also recruited as another comparison group. Being rare diseases, patient numbers for these groups were small, but despite the small patient numbers, there were clear trends seen.

2.4.1 Control Patients

To assess true divergence from the norm, it was important to compare the IBD patients to healthy children. Non-inflammatory Control (NIC) patients [n=16, median age 11 (4-15) years, 8 girls], although did not have IBD, did exhibit GI symptoms. In fact, Irritable Bowel Syndrome (IBS), seen in 8 NIC patients (Table 2.8), is associated with microbiota dysbiosis (340-343). In addition, 6 of the NIC patients were also known to the Rheumatology department with conditions such as Juvenile Idiopathic Arthritis (JIA) which has been associated with microbial dysbiosis (344-346). The NIC patients were therefore not an ideal comparison group. It was, however, possible to obtain blood samples and non-inflamed intestinal mucosal biopsies from NIC patients, unlike the healthy children (HC), which provided good comparators with IBD patients.

HC [n=11, median age 12 (4-17) years, 6 girls] were thus recruited for collection of a single stool sample, which was relatively easier to obtain in a non-invasive manner. A few of the HC had atopy and a few had minor GI symptoms in early childhood, which are common. The HC was thus fairly representative of healthy children in the community. Despite the inclusion criteria detailed above (Section 2.2.4), it was possible to match the HC for age and sex with IBD patients, but not ethnicity (27% ethnic minorities in HC vs. 63% in IBD patients). This is likely to influence their diet and environmental exposures.
2.4.2 Inflammatory Bowel Disease Patients

The cohort of IBD patients for this study was similar to those previously reported (18) except for the higher percentage of ethnic minorities, reflecting the greater diversity of the Greater London population. UC patients were mainly of Asian ethnicity, reflecting their genetic risk and/or dietary influence (347).

Parents were asked how their children’s symptoms began and if there were any possible triggers. Of the 19 IBD patients, potential triggers were identified in 11 patients. Seven patients reported symptoms of gastroenteritis prior to developing IBD symptoms. Of these patients, 3 children, who were likely to be second or third generation immigrants, acquired gastroenteritis whilst abroad visiting relatives. The environmental and dietary differences between the UK and countries such as India and Pakistan are likely to influence the gut microbiota (166), which in turn may make visiting British children more susceptible to gastroenteritis leading to dysbiosis. Dysbiosis may have also been a contributing factor in a patient with complicated appendicitis and in another who had repeated course of antibiotics for infected eczema for 2-3 years preceding IBD symptoms.

Of the 7 children with gastroenteritis, 4 patients were reported to develop gastroenteritis during a stressful period such as parental divorce, moving countries and changing childcare provider. An additional patient reported a stressful event prior to IBD symptoms. Emotional stress has been recognised as a factor triggering and exacerbating IBD (348-350), with patients receiving CBT reporting reduced anxiety and stress, and improved mental quality of life at 6 months (351). Surprisingly, no differences were seen between this group and the control group at 2 years but this could be related to the addition of online CBT to address the high attrition rate thus resulting in less personalised face-to-face therapy (351). Increasingly, intestinal microbiota is linked to psychological and psychiatric conditions via the gut-brain axis (352). In rats, chronic stress has been found to impair mucosal barrier and initiate mucosal inflammation (350, 353). Whether the emotional and physical stresses in IBD patients are associated with intestinal dysbiosis has yet to be determined. It will admittedly be challenging to differentiate dysbiosis due to stress from the varying dysbiosis of IBD patients with disease status and treatments.

Patients with severe disease were recruited to understand the potential differences with patients having mild to moderate disease. In addition, some patients responded well to treatment but many required treatment escalation (Table 2.4). During the study period,
1 patient with severe intractable disease required surgical intervention. There were 2 other patients who subsequently required colectomy for refractory disease after recruitment and sample collection for the study ceased. In addition, 3 patients presented with severe intractable IBD from infancy. Genetic mutations were identified for 2 of these patients (IL-10RA and IL-10RB), but all 3 patients received HSCT.

In patients with Infantile IBD, bloody diarrhoea followed an infective episode at 5 months and in another, following the introduction of full-fat, unmodified cow’s milk. These episodes may have led to unregulated GI inflammation which was too severe for conventional IBD treatments. Infantile IBD is associated with underlying immune dysregulation thus why many of these children respond well to HSCT (354, 355). The immune dysregulation may also influence intestinal colonisation thus the gut microbiome in these patients.

Two patients recruited had coexisting disease, Granulomatosis Polyangiitis and Coeliac Disease. Coeliac Disease is known to be associated with microbial dysbiosis which differs from IBD but it was not clear which profile would dominate in this patient and if the SCFA profile would differ from the other IBD patients. At present, the microbiota of patients with Granulomatosis Polyangiitis or small vessel vasculitides is unknown but this would need to be taken into consideration when analysing the study results (356).

2.4.3 Comparison of IBD Patients with Healthy Children

Despite the small number of children in both groups, there were trends and a few significant differences seen between the HC and IBD patients. When reviewing their past histories, IBD patients were more likely to report infantile colic. They were also more likely to report gastroesophageal reflux, constipation, eczema, hayfever and wheeze. This finding was consistent with previous reports of increased atopy in IBD patients (357-360).

Many of the IBD patients received proton-pump inhibitor (PPI) as part of their treatment, usually alongside corticosteroids. PPI reduces gastric acidity thus influences gut microbiota (361, 362). In contrast, none of the HC had been exposed to a PPI.

IBD patients were also more likely to report benign joint aches and pains. Indeed, arthritis and arthralgia are well-recognised in IBD patients (363, 364), which may be related to the intestinal dysbiosis reported in these patients (344, 346, 365).
2.4.3.1 IBD Patients received more Antibiotic Courses than Healthy Children

Only 1 child in each group had never received antibiotics (Table 2.5). In both groups, children received antibiotics in early childhood, as young as infancy. The 10 HC who received antibiotics had only 1 course of antibiotics, compared to 13 IBD patients \((p=0.0001)\) who had received 2 or more courses of antibiotics for pneumonia, urinary infections, tonsillitis, infected eczema, complicated appendicitis and otitis media. There were 5 IBD patients who reported to receive more than 10 courses of antibiotics.

Only IBD patients received antibiotics for respiratory infections, which may have been related to underlying asthma, and more IBD patients received antibiotics for urinary tract infections compared to the HC group. A child in each group had received prophylactic Trimethoprim for urinary tract infections \((HC \text{ for few months, IBD for } \sim 5 \text{ years})\).

This illustrates the high usage of antibiotics in early childhood, potentially influencing the health of children with genetic susceptibility to particular diseases. Repeated courses of antibiotics are recognised as a risk factor for IBD \((52, 53, 366-368)\) but not widely appreciated. As we increasing understand the impact of the gut microbiota in early childhood, antibiotic prescribing for the various conditions ought to be reviewed. Prophylactic Trimethoprim has been a widely accepted practice but may need reassessment to identify ways to minimise antibiotic exposure in young children.

2.4.3.2 Nutrition in IBD Patients

Breast milk has been postulated to have a protective effect on IBD development \((369)\). All HC and most IBD patients were breastfed (Table 2.7). Interestingly, more IBD patients were breastfed for longer than 12 months, with 2 patients being breastfed for longer than 18 months, confirming that other factors are at play in the development of IBD. In addition, more HC had received some formula feed in the first month of life, although 7 IBD patients had received formula feed earlier.

Despite this, breastfeeding was found to be the only nutritional intervention to decrease the risk of IBD, with this effect being duration-dependent, thus should not be underestimated \((369-372)\). Although the available evidence is conflicting, it has been recognised that the lactoferrin in breast milk, which is absent in formula feed, may have
antibacterial and antiviral properties which offers protection to the growing infant (373-375).

There were small differences in weaning practices seen between HC and IBD patients. Although an insignificant finding, there were 3 IBD patients who were weaned early at 3 months. Most children were weaned between 4-6 months of age with 2 HC weaned at 7 months and an IBD patient weaned at 9 months of age. In the development of Type 1 Diabetes Mellitus, infants weaned early (before 4 months) and late (after 6 months) were at increased risk (161). It is unclear whether this is related to the colonisation of the infant gut or reduced breast milk with more intake of solid food, or other factors. In addition, more HC in this study received home-cooked purees than IBD patients, again alluding to nutritional influences on the developing infant intestinal microbiota.

Food allergies and/or intolerances were frequently reported by IBD patients (n=11), which may explain why significantly more of them were reported to be fussy with their food. Dairy allergy specifically has been associated with UC but as yet, not CD (376). The pathogenesis of this remains unexplored but the allergic inflammation in the colon may impair mucosal barrier and influence intestinal microbiota, exacerbating the IBD.

Nutritional assessment of HC (n=11), NIC (n=7) and IBD (n=17) patients were also compared. There were significant differences in energy intake between HC and IBD patients (p=0.0187). HC also had significantly more protein intake compared to NIC (p=0.0307) and IBD (p=0.0118) patients. Calcium intake was significantly greater in HC compared to NIC and IBD patients. Although most children had inadequate vitamin D intake, HC had more. These findings are likely to be a reflection of the reduced intake in IBD patients during periods of active disease. Additionally, they may intentionally reduce their fibre intake to control their GI symptoms such as abdominal pain and diarrhoea. They were thus found to have significantly less fruit and vegetable, resulting in less fibre intake. Although this is understandable, it should be discouraged and patients appropriately educated on good macro- and micronutrients in their diets to help maintain health and well-being (377, 378).
2.4.4 Impact of Medications and Nutrition on Healthy and Allergic Infants

Four of the 6 HI were born by normal vaginal delivery, and 4 were exclusively breast-fed; both are significant factors known to influence the gut microbiota (379-383). It was essential that these HI had no exposure to antibiotics, nor that antibiotics were not given to their mothers during pregnancy or birth. Although challenging to recruit, it was also important that these infants had no family history of atopy. These infants were however, younger than the allergic infants [median 8 (5.5-9) months vs. median 12 (7-16) months] thus arguably not an ideal cohort for comparison with the allergic infants. Many allergic infants, however, develop symptoms after a few months, for example after weaning, thus presenting later.

The HI group was weaned onto solids earlier than the allergic infants [median 5.25 (4.5-6) months vs. 6 months] but all were having solids during the time of sample collection. Their progress with solids would of course differ, with the older infants having more varied and solid foods.

In addition, the allergic infants were exposed to medications, such as antacids which can influence the gut microbiota. These would need to be taken into consideration when analysing the results.

2.4.5 Study Limitations

The number of recruited patients was smaller than anticipated. After the study commenced, the Gastroenterology Department at GOSH was closed to new patients except those requiring services funded by the National Commissioning Group. The patient groups were then reconsidered and patients commencing Infliximab were then recruited. The Gastroenterology team at UCLH was approached, resulting in recruitment of teenage patients.

This was the main limitation for this study but the smaller numbers enabled in depth analysis of each individual patient, taking into account their disease status and treatment. As a result, there was greater understanding of the impact of the various factors affecting the patients.

This study was also limited by the imperfectly matched HC for comparison with the IBD patients due to the differences in ethnicities. Likewise, the HI were younger compared to
the allergic infants. These factors will need to be considered when analysing the laboratory results.

2.5 Conclusion

In any clinical study, it is imperative that the clinical factors of patients are recorded for correlation with laboratory findings. The treatments received and other additional factors that may influence the gut microbiota, SCFA and cytokine production were recorded for consideration when interpreting laboratory results. How these influence the microbial profiles is discussed further in Chapter 3.

Although this was a small study, as a result, it was possible to analyse individual patients, taking their various clinical factors into consideration. In addition, the various patient cohorts enable cross-analysis between the different groups.
3. Chapter 3

Intestinal Microbiota in Inflammatory Bowel Disease

3.1 Introduction

The commensal intestinal microbiota and its functions in health were discussed in Chapter 1. Although details are lacking, it is now well established that perturbations in this symbiotic relationship occur in disease states. Here, dysbiosis and its potential impact in IBD are discussed.

3.1.1 Dysbiosis in IBD

Arumugam et al. attempted to classify the human intestinal microbiota into 3 different enterotypes (Table 3.1) (384), however, reports of varying microbial profiles prove that this may be too simplistic a concept. Gevers et al. reported on the Microbial Dysbiosis Index (MD-index), a calculation which included selected families and orders with no obvious biological relationship (Figure 3.1, Appendix 3A), which discriminated between CD and HC (56). Subsequently, Halfvarson et al. demonstrated a “healthy plane”, with the dysbiotic IBD (stool) profiles deviating from this (270).
In IBD, luminal dysbiosis is associated with reduced bacterial diversity, and reduction of beneficial bacteria such as *F. prausnitzii*, with increased pathogenic or opportunistic organisms such as *Enterobacteriaceae*. There are several studies reporting various organisms implicated in the dysbiosis but we have yet to commit to a more descriptive or precise definition of dysbiosis in IBD.

Although there are similarities in the dysbiosis in CD and UC, differences have also been observed. Halfvarson *et al.* found that the deviation from the “healthy plane” was greatest in the stool of patients with ileal CD (270). This was supported by Pascal *et al.* who also reported greater dysbiosis in CD than UC (385), with more altered stool microbial composition, more unstable community over time and lower microbial diversity compared to UC. In addition, the dysbiosis in CD predominantly involved loss of beneficial organisms than a gain in pathogenic species. Pascal *et al.* also reported on 8 groups of organisms that differentiated CD from non-CD. Lower abundance of *Faecalibacterium* (genus of Firmicutes), an unknown *Peptostreptococcaceae* (family of Firmicutes), *Anaerostipes* (genus of Firmicutes), *Methanobrevibacter* (genus of the archea Euryarchaeota), an unknown *Christensenellaceae* (family of Firmicutes) and *Collinsella* (genus of Actinobacteria), while higher abundance of *Fusobacterium* and *Escherichia* (genus of Proteobacteria) were observed in CD. Interestingly, there was also a difference between CD colitis and ileal CD, with *Enterococcus faecalis* being more abundant in ileal CD (385).

Dysbiosis may precede clinical symptoms. CD patients with lower rates of *F. prausnitzii* (species of Firmicutes) and *Bacteroides* (genus of Bacteroidetes) after withdrawal of Infliximab subsequently relapsed (334). In UC, presence of *Ruminococcus gnavus*

<table>
<thead>
<tr>
<th>Enterotypes</th>
<th>Dominant Genera</th>
<th>Co-occurring Genera</th>
<th>Energy Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacteroides</td>
<td>Parabacteroides</td>
<td>Fermentation of carbohydrates and proteins</td>
</tr>
<tr>
<td>2</td>
<td>Prevotella</td>
<td>Desulfovibrio</td>
<td>Degradation of mucin</td>
</tr>
<tr>
<td>3</td>
<td>Ruminococcus</td>
<td>Akkermansia</td>
<td>Degradation of mucin</td>
</tr>
</tbody>
</table>

*Table 3. 1: Human Intestinal Enterotypes (384)*

**Microbial Dysbiosis Index** = \[ \log_{10} \left( \frac{\text{total abundance of organisms increased in CD}}{\text{total abundance of organisms decreased in CD}} \right) \]

*Figure 3. 1: Microbial Dysbiosis Index (56)*
(genus of Firmicutes), *Bacteroides vulgatus* (species of Bacteroidetes) and *Clostridium perfringens* (species of Firmicutes), and the absence of *Blautia* and *Roseburia* (both genus of Firmicutes) before surgery was associated with increased risk of pouchitis (386).

Microbial diversity in health was discussed in Section 1.4.3. A more diverse intestinal microbiota has greater stability and ability to resist disturbances in the microbial community. In IBD, there is lower species richness associated with the dysbiosis when compared to HC (269, 270, 387).

**Dysbiosis in Severe IBD**

Intestinal microbial composition may assist in predicting treatment response and disease severity. Several studies have reported alterations of various bacterial taxa with disease activity. In general, higher pre-treatment α-diversity was associated with subsequent remission. In addition, CD patients with higher abundance of *Roseburia inulinivorans* (species of Firmicutes), Clostridiales (order of Firmicutes) and a Burkholderiales species (of Proteobacteria) were more likely to achieve remission (388). Zhou *et al.* suggested incorporating microbiota analysis to predict treatment response to Infliximab. The families with greatest impact on the predictive model were *Lachnospiraceae* (family of Firmicutes) and *Bacteroidaceae* (family of Bacteroidetes). The predictive model was enhanced by utilising the CDAI and calprotectin concentrations (389).

Severe CD phenotype includes stricturing, penetrating and fistulising disease. Specific microbes have been associated with these phenotypes. *Ruminococcus* (genus of Firmicutes), *Enterobacteriaceae* and *Pseudomonadaceae* (both families of Proteobacteria) were linked to stricturing disease while *Veillonella* (genus of Firmicutes) and *Aeromonadaceae* (family of Proteobacteria) were associated with penetrating disease (389, 390). Furthermore, increased *Enterococcaceae* and *Pseudomonadaceae* (both families of Proteobacteria) were observed in fistulising disease.

At diagnosis, those with severe UC were found to harbour increased *Aggregatibacter* (genus of Proteobacteria), *Fusobacterium* (genus of Fusobacteria), *Veillonellaceae* (family of Firmicutes), *Enterobacteriaceae* (family of Proteobacteria), *Neisseriaceae* (family of Proteobacteria) and Haemophilus *parainfluenzae* (species of Proteobacteria). In addition, increased abundance of mucin-degrading *R. gnavus* and *R. torques* (both
species of Firmicutes) were also observed. Clostridiales (class of Firmicutes), Veillonella dispar, H. parainfluenzae and Campylobacter (genus of Proteobacteria) were increased while Ruminococcaceae, Lachnospiraceae (family of Firmicutes), Blautia, F. Prausnitzii were decreased in those who subsequently had colectomy due to refractory disease. Patients with severe disease also had decreased Clostridiales, incorporating 24 Lachnospiraceae and 18 Ruminococcaceae, many of which are known SCFA-producing bacteria (391, 392). Depletion of these taxa were recorded following antibiotic administration (391).

In severe, active UC, increase in Bacteroides was observed. Interestingly, Brandon et al. found that this increase was associated with greater expansion of C. albicans and Debaryomyces species. Prior to treatment with corticosteroid, those with increased abundances of 9 Clostridia and 2 Erysipelotrichaceae (classes of Firmicutes) were more likely to achieve remission. Those in remission 4 weeks after corticosteroid had increased Actinomyces (genus of Actinobacteria), which remained low in those with ongoing active disease (391, 392).

Thus, in summary, increased alpha diversity and SCFA-producing bacteria such as Clostridiales are associated with subsequent remission. In severe UC, greater decrease in butyrate-producing taxa were observed. In addition, expansion of Proteobacteria correlated with severe disease, especially in CD. Together, these studies suggest greater dysbiosis with more severe disease, with higher likelihood of clinical improvement associated with decreased dysbiosis in less severe disease.

### 3.1.2 Microbes involved in Dysbiosis in IBD

In the GI tract, bacteria can exist in planktonic forms and/or in biofilms, which consists of single or multiple species of bacteria. They produce exo-polysaccharides (EPS) which form a matrix that stabilises the biofilm. In addition, the biofilms are composed of bacterial cells and up to 97% water (393). Within these communities, metabolically different bacteria rely on each other for substrates and to overcome environmental stresses.

Bacteroidetes and Firmicutes are dominant members of a healthy human GI tract, but in IBD shifts in the microbial profiles can occur at phyla to species level. The abundance of Proteobacteria is low in health, but it is increased and appears to have a significant role in the pathogenesis of IBD.
3.1.2.1 Bacteroidetes

Bacteroidetes are gram-negative, rod-shaped bacteria that can be anaerobic or aerobic. There are 3 main classes of this phylum, Bacteroidia, Flavobacteriia and Sphingobacteria. Most of its species are symbiotic commensals in the GI tract but some can be opportunistic pathogens e.g. B. fragilis. Their functions include degradation of plant fibres, complex sugars for the host, immune modulation and regulation of the gut-brain axis (394).

**Bacteroides, Genus of Bacteroidetes**

*Bacteroides* are obligate anaerobes that make up a significant proportion of the intestinal microbial profile, but is reduced in IBD patients compared to HC (395). *B. fragilis* induces Treg differentiation, and when introduced into germ-free (GF) mice, restored TGF-β2 and IL-10 production, thus promoting tolerance (189). In contrast, *B. vulgatus* and *B. thetaiotaomicron* mediate colitis in mice with defective TGF-β and IL-10 signalling but not if these signalling mechanisms are intact, suggesting that TGF-β and IL-10 suppress the pro-inflammatory effects of Bacteroides (396).

3.1.2.2 Firmicutes

Firmicutes are gram-positive organisms that are either round (cocci) or rod-like (bacillus). Many produce endospores which are highly resistant to extreme conditions. This phylum can be divided into the obligate anaerobic Clostridia and the obligate or facultative anaerobic Bacilli. It also includes the parasitic Mollicutes, an order of Firmicutes.

**Clostridium, Genus of Firmicutes**

Clostridium is a major genus which includes several commensal taxa. *Clostridium* cluster IV and XIV are less abundant in IBD compared to HC (397). In another study, *Clostridium butyricum* (cluster I), used as a probiotic, increased Treg differentiation through TGF-β1 produced by DC (398). This is supported by another study where transplantation of *Clostridium* cluster IV and XIV into GF mice induced Treg differentiation and TGF-β production (224, 399).

**Faecalibacterium prausnitzii, species of Firmicutes from the order of Clostridiales and family of Ruminococcaceae**

*F. prausnitzii* is a gram-positive, rod-shaped anaerobic commensal of the human GI tract. It ferments indigestible plant fibres in the colon, producing SCFA such as butyrate. Its
decreased abundance in CD and UC (400, 401) has been widely-reported, however, increased proportions have also been reported in newly-diagnosed paediatric CD (402). This contradicting finding suggests its more complex involvement in CD pathogenesis.

3.1.2.3 Proteobacteria

Proteobacteria is a phylum of gram-negative bacteria that includes many pathogens. These include Brucella and Rickettsia, Bordetella and Neisseria, Escherichia, Shigella, Salmonella, and Yersinia and Helicobacter (403). It is not a dominant intestinal bacterial phylum in health.

The mechanisms resulting in increased Proteobacteria are unknown; it is however postulated that increased luminal oxygen concentrations, referred to as the Oxygen Hypothesis, is a critical factor (403). In health, the colonocytes utilise oxygen through beta-oxidation processes, creating an anaerobic environment (404). With inflammation, there is reduced capacity for beta-oxidation thus increasing luminal oxygen levels. This facilitates the growth of Proteobacteria, promoting dysbiosis (405, 406). In addition, nitrate produced by the host during inflammation allows Enterobacteriaceae to grow and contribute to dysbiosis (407).

Enterobacteriaceae

Enterobacteriaceae is a minor component of the healthy GI commensal flora. In IBD patients and in most animal models of colitis, increased Enterobacteriaceae is observed. It is associated with inflammation and increased oxidative stress. Its growth is controlled by TGF-β produced by DC. In addition, its relative abundance in intestinal biopsies has been associated with the NOD2 risk allele count (48).

Adherent-invasive Escheria coli

Unlike opportunistic pathogens, pathobionts influence the host indirectly by manipulating the host immune system (408). Adherent-invasive Escheria coli (AIEC) is a specific pathogenic group of E. coli characterised by its ability to adhere and invade IEC (409, 410), survive and replicate within macrophages without inducing host cell death (411). It has no other pathogenic invasive abilities (412) or conventional pathogenicity. It is increased in CD, especially ileal CD, being a prevalent organism in the ileal biopsies (412). This highlights the defective barrier function and mucosal immunity in CD.
3.1.3 Small Bowel Microbiota in IBD

In contrast to the colon, which teems with $10^{12}$ microorganisms/gram of stool, the duodenum and jejunum are home to $10^{3-4}$ microorganism/ml of content. The more distal ileum harbours $10^7$ bacteria/g of content (413).

The main functions of the small intestine are digestion and absorption of proteins, lipids and simple sugars for energy and nutrients. It is responsible for 90% of energy absorption through the large mucosal surface area which is also a barrier between the human host and the luminal microbiota (173).

The duodenal intestinal microbiota differs from that of the distal GI tract. Firmicutes and Proteobacteria dominates while obligate anaerobes are reduced or absent (127, 414). In a comparison of luminal duodenal microbiota of 5 obese adults with 5 HC, Angelakis et al. reported dominance of Firmicutes and Actinobacteria with the obligate anaerobic Bacteroidetes being largely absent. Less abundant phyla included Proteobacteria, Fusobacteria, TM7, Bacteroidetes and Tenericutes. There were non-significant differences in Firmicutes and Proteobacteria between the groups but the presence of Rubrobacter (genus of Actinobacteria) in HC was a significant finding (415). In another study investigating 8 children with UC, the microbial richness of Firmicutes (genera Bacillus, Lactobacillus), Bacteroidetes and Actinobacteria (genus Collinsella) in duodenal fluid were reduced despite inflammation affecting the colon. In the same study, 5 children with CD had reduced microbial abundance but greater variability was observed (416).

The microbiota of ileal effluent includes Bacteroidetes (Bacteroides, Prevotella), Proteobacteria and Actinobacteria as well as Firmicutes (Lachnospiraceae, Bacillus, Streptococcus, Faecalibacterium) (417-419). The bacterial composition is reportedly similar to the jejunal and proximal ileal profiles (417). Streptococcus and Veillonella species are often present in higher abundances in the ileum, with Streptococcus reported to support transport and metabolism of simple carbohydrate substrates (417, 420). In contrast, R. gnarus, R. obeum and B. plebeius are less abundant in the ileum (421).

3.1.4 Alterations in Intestinal Microbiota with IBD Treatment

The aim of IBD treatment is induction of remission and maintenance of remission with mucosal healing, whether it is at diagnosis or during relapse. In the process of controlling the exaggerated immune responses and healing the inflamed mucosa, there are
intestinal microbial shifts within the lumen and on the mucosal surface, as discussed below. It is not entirely clear whether these microbial alterations are caused by the treatment or are consequences of the immune and mucosal changes occurring. In addition, combined therapies in a heterogenous age-group make it challenging to identify the causative factors. Understanding the mechanisms involved may bring us a step closer to clarifying the pathogenesis of CD and UC.

3.1.4.1 Exclusive Enteral Nutrition (EEN)

The European Society of Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) and British Society of Paediatric Gastroenterology, Hepatology, and Nutrition (BSPGHAN) recommend EEN as the first-line therapy for induction of remission for CD patients. These guidelines recommend 6-8 weeks of treatment but prolonged courses may offer benefits, particularly for those with faltering growth and pubertal delay (67, 422-425).

Partial EN (PEN), supplementing a regular diet, has been used for induction of remission but with less success and mucosal healing than EEN (426, 427). In a randomised control trial involving 50 children, clinical remission rate with PEN (50% of calories provided by EN) was lower than EEN (15% vs. 42%, p=0.035) (Johnson Gut 2006). Interestingly, the bacterial composition of children receiving PEN did not alter, suggesting that it is the food restriction rather than introduction of formula that modulates the microbiota (428). On the other hand, encouraging remissions rates were observed in a study of 47 patients (34 children and 13 adults) receiving PEN plus a strict diet excluding animal fat, high sugar intake, gliadin, emulsifiers and maltodextrin (70% in children, 69% in adults) (429, 430).

The type of formula (elemental, semi-elemental or polymeric) used varies from centre to centre but this appears to be of little consequence. A pooled analysis of 4 RCTs also suggested no differences in remission rates achieved with EEN and corticosteroids (431), however, mucosal healing was more likely with EEN (430).

Several studies have reported reduced Firmicutes associated with increased Proteobacteria in IBD (30, 432, 433). EEN reverses this pattern to increase the relative abundance of Firmicutes and decrease the relative abundance of the Escherichia, Shigella and Sutterella (genera of Proteobacteria) (434, 435) (Table 3.2). In another study, EEN resulted in decreased relative abundance of Bacteroidetes (including the
families Bacteroidaceae, Porphyromonadaceae and Rikenellaceae) and increased the relative abundance of Firmicutes (particularly the family Christensenellaceae) (435).

The microbial composition can alter as soon as 1 week but generally after 2-3 weeks of EEN, with associated improvements in clinical parameters e.g. ESR, CRP, neutrophils, albumin and haemoglobin. This appears to be the case despite incomplete mucosal healing (435, 436). Following a course of EEN, butyric acid decreased while luminal pH and total sulphide increased but these revert to baseline levels after completing the EEN course (437).

A retrospective study evaluated patient-specific genetic polymorphisms associated with response to EEN. Those with NOD2 genotypes were more likely to have poorer sustained remission with higher relapse rates at 1 year post-EEN (438). Patients with significantly altered microbial composition and reduced species diversity/richness after EEN were found to have sustained remission (439, 440). Their microbial profiles were similar to HC, including dominance of Firmicutes (387, 441). Interestingly, those who relapsed had less significant alteration of their microbial composition compared to controls (387). Remission was associated with increased Akkermansia (genus of Verrucomicrobia), Alistipes and Bacteroides (both genera of Bacteroidetes) (387), and decreased Proteobacteria (E. coli, Shigella and Suterella) and Firmicutes (Dialister invisus, Blautia, unclassified Ruminococcaceae and Coprococcus) (387, 439, 440, 442).

Currently, the mechanism by which EEN induces remission remains unexplained. The changes in microbial composition can be due to the absence of dietary fibre, reduced carbohydrate for fermentation and/or increased intestinal transit time (443). Cuiv et al. suggested that restricted bacterial growth and pathogenic metabolic activity are responsible for the effects observed (444). In addition, EEN may enhance mechanisms of autophagy for mucosal repair (444). The microbial alterations with EEN are also associated with reduced inflammatory responses and increased anti-inflammatory responses of PBMC and Treg subset (435). The reduced fermentation with EEN may explain the significant decrease in toxic metabolites 1-propanol, p-cresol, phenol, 1-butanol, dimethyl disulphide and fatty acid ethyl esters (436), which were not present in HC (445).

IBD patients are known to have higher percentages of stool microbes coated with IgG, IgA and IgM compared to HC and IBS patients (446, 447). The number of Ig-coated microbes were significantly reduced after 2 weeks of EEN, suggesting reduced immune responses associated with reduced bacterial abundance (436).
The intestinal microbiota often reverts to its pre-treatment composition after completion of EEN course (437). Despite the successes of EEN, approximately 60–70% of patients relapse within 12 months of EEN cessation (448, 449). Compared to pre-EEN profiles, Leach et al. reported 15-38% similarity to the pre-EEN profile after EEN and 31-41% similarity 4 months after EEN (440). Thus, a continuing challenge is maintaining an improved intestinal microbial profile that promotes sustained remission.

One of the biggest challenges with EEN therapy is treatment compliance. Svolos et al. looked at an alternative to EEN (450). A prescriptive and personalized diet, named CD-TREAT, which recreates EEN by the exclusion of certain dietary components, have been trialled with success in 5 children with CD. Its palatability was a major advantage.

<table>
<thead>
<tr>
<th>Increased with EEN</th>
<th>Decreased with EEN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Firmicutes</strong></td>
<td></td>
</tr>
<tr>
<td>Relative abundance of Firmicutes (p=0.227) (435)</td>
<td>Levels of <em>F. prausnitzii</em> (Subgroup A2-165 p=0.0046, Subgroup M21/2 p 0.61) (451)</td>
</tr>
<tr>
<td></td>
<td>Concentration of <em>F. prausnitzii</em> (p=0.002) (437)</td>
</tr>
<tr>
<td>Relative abundance of <em>Lactococcus</em> (p=0.017) (452)</td>
<td>Relative abundance of <em>Faecalibacterium</em> (p=0.068) (452)</td>
</tr>
<tr>
<td></td>
<td>Relative abundance of <em>Dialister</em> (p=0.04) [30] Relative abundance of <em>Ruminococcaceae</em> (p=0.04) (452)</td>
</tr>
<tr>
<td>Relative abundance of <em>Christensenellaceae</em> (p=0.0237) (435)</td>
<td>Relative abundance of <em>Subdoligranulum</em> (p=0.023) (452)</td>
</tr>
<tr>
<td></td>
<td>Relative abundance of <em>Clostridiales, Roseburia, Coprococcus, Ruminococcaceae</em> (453)</td>
</tr>
<tr>
<td></td>
<td>Relative abundance of <em>Akkermansia muciniphila, Bacteroides</em> (including <em>B. fragilis</em> and *B. ovatus), <em>Lachnospiraceae, Ruminococcaceae</em> (454)</td>
</tr>
<tr>
<td><strong>Bacteroidetes</strong></td>
<td></td>
</tr>
<tr>
<td>Concentration of <em>Bacteroides</em> (n=1, p not reported) (455)</td>
<td>Concentration of <em>Bacteroides fragilis</em> (p=0.034) (456)</td>
</tr>
<tr>
<td>Abundance of <em>Alistipes</em> (p&lt;0.05) (434)</td>
<td>Concentration of <em>Bacteroides/Prevotella</em> (p=0.053) (437)</td>
</tr>
<tr>
<td></td>
<td>Concentration of <em>Prevotella</em> (p=0.27) (452)</td>
</tr>
<tr>
<td></td>
<td>Relative abundance of Bacteroidetes (p=0.039) (435)</td>
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<tr>
<td>--------------------------</td>
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<tr>
<td><strong>Proteobacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Relative abundance of</td>
<td>Concentration of Proteobacteria (n=1, p not reported) (455)</td>
</tr>
<tr>
<td>Enterobacteriaceae (453)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Concentration of Sutterella (p&lt;0.05) (434)</td>
</tr>
<tr>
<td><strong>Actinobacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Abundance of Bifidobacterium (p&lt;0.1) (434)</td>
<td>Abundance of Bifidobacteriaceae (p=0.005) (452)</td>
</tr>
<tr>
<td></td>
<td>Concentration of Bifidobacteria (p=0.003) (437)</td>
</tr>
</tbody>
</table>

*Table 3.2: Microbial Alterations with EEN (adapted from Gatti et al. and MacLellan et al.) (442, 457)*

### 3.1.4.2 Corticosteroids

High-dose corticosteroids are the first-line of therapy to induce remission at presentation and during relapses in UC. It is also prescribed in CD, especially if EEN fails to induce remission.

Corticosteroids have been associated with alterations of the intestinal microbiota. Following treatment with Dexamethasone, *Bifidobacterium* (genus of Actinobacteria) and *Lactobacillus* (genus of Firmicutes) are increased while *Mucispirillum* (genus of Deferribacteres), a colonic mucin degrader, was absent (458). In addition, corticosteroids restored *F. prausnitzii* to high levels within days in adults with IBD (459).

A proton-pump inhibitor (PPI) is often prescribed alongside corticosteroids to minimise the risk of peptic ulceration. PPIs are able to alter the pH of intestinal environment, and therefore modulate microbial composition. A meta-analysis demonstrated PPI as a potential risk for *C. difficile* (species of Firmicutes) infections, small intestinal bacterial overgrowth, spontaneous bacterial peritonitis, community-acquired pneumonia and hepatic encephalopathy (460). In addition, PPI is associated with worse outcomes in IBD, perhaps as it increases *Streptococcaceae* (family of Firmicutes) and *Enterococcaceae*, but also as it increases the risk for *C. difficile* infection, and decreases *Faecalibacterium* (genus of Firmicutes). This is support by Shah et al., who reported increased risk of IBD-related hospitalisation and surgery in IBD patients on PPIs and histamine2-receptor antagonists (461).
3.1.4.3 Infliximab

Anti-TNF agents like Infliximab, Adalimumab, Golimumab and Certolizumab pegol are increasing being used in moderate to severe IBD. Infliximab is effective in inducing remission, treating fistulas in CD and maintaining remission in CD and UC. They are often administered in combination with Azathioprine, resulting in improved outcomes in comparison to either of these as monotherapy (462).

Infliximab influences the intestinal microbiota. In a study of 72 CD, 51 UC and 73 healthy adults, 16 CD patients receiving Infliximab were analysed for stool microbial profiles (389). Increased Actinobacteria and Proteobacteria (Enterobacteriaceae), while decreased Firmicutes (Clostridiales) strongly correlated with IBD severity. Those who responded to Infliximab had increased microbial diversity and relative abundance of Clostridiales compared to those who relapsed. In addition, Clostridiales abundance predicted treatment efficacy with 86.5% accuracy alone and 93.8% accuracy in combination with calprotectin levels and Crohn’s disease activity index (CDAI).

In a longitudinal study involving 33 adult CD patients, Rajca et al. found that the microbial profile predicted relapse after withdrawal of Infliximab. Those who relapsed had lower abundance of Firmicutes compared to those who maintained remission. In addition, decreased F. prausnitzii and Bacteroides were reported to predict relapse (334).

Wang et al. compared 11 children with CD to 16 HC. CD patients were found to have increased Enterococcus and reduced SCFA-producing bacteria including Anaerostipes, Blautia, Coprococcus, Faecalibacterium, Lachnospira, Roseburia, Ruminococcus (all genera of Firmicutes), Odoribacter (genus of Bacteroidetes) and Sutterella (genus of Proteobacteria). With Infliximab, the microbial composition shifted towards that of the HC. Enterobacteriaceae, Enterococcaceae, Planococcaceae and Streptococcaceae were reduced while Blautia, Faecalibacterium, Odoribacter and Sutterella increased. These alterations were maintained during Infliximab therapy and were associated with increased biodiversity and functional capacity more similar to the HC, but several SCFA-producing taxa were not re-established. Overall, patients with greater abundance of SCFA-producing bacteria had sustained response to Infliximab (463).

Furthermore, a meta-analysis of adult CD patients found that those on Infliximab and specialised enteral nutrition were more likely to achieve and maintain remission compared to Infliximab monotherapy. This suggests that their otherwise usual diet of
high animal protein and low-fibre may have promoted intestinal inflammation, thus counteracting the effects of Infliximab treatment (464, 465).

3.1.4.4 Sirolimus (Rapamycin)

Sirolimus (Rapamycin) is a macrocyclic antibiotic isolated from *Streptomyces hygroscopicus*. It acts by inhibiting mammalian target of rapamycin (mTOR), a protein kinase with an important role in cell division and proliferation, and is infrequently used in severe IBD (466). In addition to its immunosuppressive and antineoplastic properties, it is also an antifungal and found to increase the lifespan in animal models, thus was investigated as an anti-aging agent. In mice, microbial changes associated with Sirolimus were reportedly similar to those on a high-fat diet resulting in impaired glucose tolerance and worsening intestinal inflammation. As yet, its effects on humans has not been evaluated (467).

3.1.4.5 Haematopoietic Stem Cell Transplantation (HSCT)

HSCT is a recognised treatment for monogenic infantile IBD or VEOIBD. These patients present with severe pan-enteric inflammation that is refractory to medical treatment. Some of these patients have had colonic resection in a bid to control the inflammation before diagnosis. When diagnosed, HSCT is often considered. HSCT involves intestinal decontamination, a period of parenteral nutrition and long-term treatment with immunosuppression and antimicrobials. It “re-programmes” the immune system, enabling normal GI function including normal interactions and functions with the colonising microbiome.

3.1.4.6 Faecal Microbiota Transplantation

Faecal microbiota transplantation (FMT) attempts to reset a dysbiotic intestinal microbiota. It has been highly successful in treating resistant *C. difficile* infection as discussed in Section 1.1.1. In IBD, however, the results have been mixed.

Rectal FMT in UC patients have been demonstrated to induce steroid-free clinical and endoscopic remission (468, 469). Amoxicillin, Fosfomycin and Metronidazole prior to
FMT achieved similar results (470). Those who achieved sustained remission harboured increased butyrate-producers while those who relapsed had increased Bacteroidetes and Proteobacteria. Failed FMT was associated with *R. gnavus* in the donor stool and a post-FMT profile rich in Bacteroidetes and Proteobacteria but low in *Clostridium* clusters IV and XIVa (471).

At present, there is no standard practice of conducting FMT, including administering the treatment. A systematic review was conducted, consisting of 29 studies with 514 FMT-treated IBD patients who were followed up for 4 weeks to 3 years (472). There appeared to be worsening disease after FMT with the rates worse after lower GI FMT delivery compared to upper GI delivery. It is unclear if it was the FMT that caused the deterioration or whether other factors were at play in these small studies. However, this highlights that a better understanding of the role of the microbiota in UC pathogenesis is needed before FMT is adopted as a recommended treatment.

### 3.1.4.7 Probiotics, Prebiotics and Synbiotics

Probiotics modulate intestinal microbial composition and in so doing, confer beneficial effects to the host, including improving mucosal protection against pathogens by inducing protective immune responses through immunization, and modifying gut-associated lymphoid cells (473).

A meta-analysis of 27 RCT, including 9 articles with CD and 18 with UC, analysed the effects of probiotics in achieving remission (474). It concluded that probiotics have significant beneficial effects in IBD, especially mixed *Lactobacillus* and VSL#3 probiotics. No significant effects were seen in CD patients unless probiotic combinations of *S. boulardii*, *Lactobacillus* and VSL#3 probiotics were used. Most beneficial effects were seen in UC patients with VSL#3 (containing *S. thermophiles*, *B. breve*, *B. longum*, *B. infantis*, *L. paracasei*, *L. delbrueckii* subsp. *Bulgarcus*, *L. acidophilus* and *L. plantarum*), which was more effective at achieving remission than a combination of *Lactobacillus* and prebiotics.

*Lactobacillus rhamnosus* GG is a gram-positive heterofermenter anaerobe that withstands acid and bile, and colonises the epithelial cells, preventing pathogenic bacterial access to the mucosa. In addition, it increases the expression of MUC2 and MUC3, which inhibit pathogenic bacterial adherence (475).
Prebiotics are indigestible plant fibres that promote the growth of beneficial microbes such as *Bifidobacterium* and *Lactobacillus* species (476-478). In animal models of IBD, plant fibres reduce pro-inflammatory cytokines and intestinal inflammation by modulating intestinal microbiota and enabling increased SCFA production (479-481).

More recently, combinations of probiotics and prebiotics, called synbiotics, have been developed. Synergy 1, a prebiotic and *B. longum*, was found to improve sigmoidoscopy scores and decreased β-defensin, TNF-α, and IL-1α in biopsies of UC patients (482-485). These are encouraging results worth further investigating.

### 3.1.4.8 Dietary Manipulations in Modulating Intestinal Microbiota

The intestinal microbiota in IBD alters with disease activity, favouring increased bacterial diversity and abundance in health. The enterotypes, however, appear to be largely stable and is associated with the diet (330). Despite being stable, it was possible to alter these enterotypes, thus it is tempting to manipulate the diet to favourably alter the microbiota.

There is sound evidence for the role of diet in modulating intestinal microbiota. This begins in infancy, with breastfeeding protecting against IBD (137, 369). Breastfed infants have less diverse intestinal microbiota (383) but includes increased Actinobacteria, which is also present in the skin. On the other hand, formula-fed infants have higher levels of Gammaproteobacteria (16, 486). The subsequent introduction of food during this critical time of immune "education" is also crucial. Reduction or cessation of breastmilk with the introduction of solids promotes increased Firmicutes and Bacteroidetes, which are more characteristic of the adult microbiota (139, 487, 488).

The modern Western diet is generally high in saturated fat, animal protein, salt and refined sugar, with low food variability. All of these are associated with a significant reduction in dietary fibre. At a molecular level, the microbiota appears to consume the mucus layer when there is inadequate fibre (489), resulting in reduced epithelial cell proliferation (490, 491). Together, these enable pathogen invasion through the thinner mucus layer and mucosa. Supplementation with fermentable fibre has been reported to protect against pathogen invasion, thought to be mediated by the induction of IL-22, the levels of which positively correlated with fibre (490, 491). Fibre has been reported to promote *Prevotella* while high protein and fat consumption promoted *Bacteroides* dominance (166, 330, 492, 493).
The Mediterranean diet has led to improvement of several diseases (494, 495). In IBD, it has been shown to modulate gene expression and normalise intestinal microbiota (496). Vegetarian or semi-vegetarian diets have also been shown to reduce the risk of relapse in IBD patients (497, 498). On the other hand, a diet with higher fatty acids, refined sugars and protein, while being low in vegetable and fruit, is associated with increased risk of developing IBD (499-503). In addition, daily fast food intake and high caffeine intake was associated with severe disease and increased risk of surgery in CD and UC patients (504).

In addition, high dietary fat content, specifically a high ratio of omega-6 polyunsaturated fatty acids (PUFA) (pro-inflammatory) to omega-3 PUFA (anti-inflammatory) has been associated with increased incidence of UC (499, 505). Omega-3 PUFA appear to modulate the intestinal bacterial composition by correcting dysbiosis, increasing production of anti-inflammatory compounds, promoting intestinal barrier and influencing the gut-brain axis (506).

Dairy has been implicated in the aetiology of IBD. A European study with over 400,000 participants found that milk consumption prior to diagnosis significantly reduced the odds of CD development and non-significantly reduced the odds of UC development. This however, did not take into consideration that those with low dairy intake may in fact have underlying allergy or intolerance to dairy. Interestingly, lactose restriction has been shown to reduce the clinical symptoms of IBD (507).

### 3.1.4.8.1 Food Additives

Processed food containing additives such as maltodextrin (MDX), emulsifying agents or thickeners, such as carboxymethyl cellulose (CMC), carrageenan (often combined with polysorbate-80), and xanthan gum, can affect intestinal homeostasis (508).

Maltodextrin is a highly-processed powder made from corn, rice, wheat or potato starch. Alpha-amylase from bacteria is used in the making of it. Maltodextrin has been demonstrated to encourage intracellular *Salmonella* viability and ileal *E. coli* expansion, perhaps through impaired anti-bacterial functions of macrophages in animal models (509, 510). In CD patients, maltodextrin also promoted colonisation and increased survival of mucosal *E. coli*.
CMC and xantham gum are natural products, while polysorbate-80 is a synthetic product. These are emulsifiers that decrease mucus viscosity and cause thinning of mucosal layer, thus impairing mucosal barrier. It can also alter the bacterial composition by promoting bacterial overgrowth and enabling bacterial translocation. These emulsifiers have been found to induce or worsen inflammation in mice models (511-513).

**3.1.4.8.2 Dietary Restrictions**

Dietary restriction has been reported to improve clinical symptoms in IBD. In a small cohort of adults with active CD, those who excluded trigger foods after a course of elemental diet had prolonged clinical remission and improvement in serum inflammatory markers compared with those who consumed a regular diet (514).

There are, in fact, several restricted diets for managing IBD, namely the specific carbohydrate diet (SCD), fermentable oligo-, di-, mono-saccharides and polyols (FODMAP), CD exclusion diet (CDED) and IBD–anti-inflammatory diet (IBD-AID). The main principles of these are exclusion of food that promote inflammation and inclusion of those with anti-inflammatory effects.

Specific carbohydrate diet (SCD) eliminates disaccharide and polysaccharide found in all grains, sweeteners except for honey, most processed foods, and all milk products except for hard cheeses and yogurt fermented longer than 24 hours (515). These foods are thought to be poorly absorbed, thus promote bacterial and yeast overgrowth that could lead to mucosal damage (508, 516). Restricting carbohydrates to monosaccharide glucose, fructose, and galactose has been demonstrated to improve disease activity scores and maintain remission in children with IBD (20 CD, 6 UC) (517). In a larger study of 417 IBD patients (47% CD, 43% UC), a third reported clinical remission after 2 months, and a further 42% at 6 and 12 months. In addition, laboratory results also improved in almost half of the patients (518).

Cohen et al. reported clinical and mucosal improvements in 7 children on SCD for 52 weeks (519). In another study, Suskind et al. reported 7 children with active CD who achieved clinical remission with exclusive SCD without any additional medications (516). This was supported by the findings of a multicentre study involving 12 children with mild to moderate CD or UC (age 10-17 years), 8 of whom achieved clinical remission on SCD, with clinical and laboratory improvements associated with significant alterations in intestinal microbiota (520).
The FODMAP diet recommends restricting short chain carbohydrates that are poorly absorbed by the small bowel. It is more restrictive than SCD, limiting some fruit and vegetables. There were 2 small studies reporting clinical improvement with the diet in IBD (521, 522).

The CD exclusion diet (CDED) recommends elimination of processed foods, specifically excluding gluten, dairy, gluten-free baked goods, animal fat, processed meats, products containing emulsifiers, and all canned and processed foods (515). In a prospective study of 34 children and 13 adults with mild to moderate CD, a diet consisting of 50% partial EN and CDED achieved remission in >70% of patients (429). A subgroup of this study had exclusive CDED which resulted in clinical remission in 6 of the 7 patients.

An extension of the CDED diet, the IBD–anti-inflammatory diet (IBD-AID), restricts foods with complex carbohydrates and certain starches but also includes prebiotics and probiotics. Softer food textures are also recommended during active inflammation (523). Four weeks of this diet in 11 adults with IBD resulted in clinical improvement and discontinuation of at least one IBD medication (523).

These restricted diets can potentially deprive a child of necessary macronutrients and micronutrients. Several micronutrients have immune functions that are affected when deficiencies occur. These micronutrients include vitamins A, C, D and E, folic acid, beta carotene and trace elements such as zinc, selenium, manganese and iron. It is thus imperative that a specialist paediatric dietician monitors a child on a restricted diet to ensure adequate intake for growth and development. Clear and consistent guidance and support are also needed, particularly as there were several websites recommending various diets and restrictions. Monitoring involve reviewing the nutritional status, including body weight, height and body mass index plotted on appropriate growth charts, at all clinical contacts (70, 524).

The diet has been shown to influence epigenetic changes. Beneficial epigenetic changes can also occur e.g. with plant metabolites, such as polyphenols that improve barrier function, and curcumin, which has anti-inflammatory properties (525, 526). With poor dietary intake, these epigenetic changes can be encoded into the human intestine and genetic make-up, which could then be transferred to the offspring (527, 528), thus having long-lasting effects.
3.1.4.9 Other IBD Treatments

3.1.4.9.1 Bowel Preparation

Prior to endoscopic mucosal assessment, patients are prescribed strong laxatives to prepare the bowel. The effects of bowel preparation in children were investigated and found to cause significant shifts in microbial communities (529). This transient effect has also been observed by others (56).

3.1.4.9.2 5-Aminosalicylates

5-Aminosalicylates (5-ASAs) are effective treatments for inducing and maintaining remission in UC patients. The suggested mechanism of actions include reduced prostaglandin synthesis via cyclooxygenase inhibition, reduced production of pro-inflammatory cytokines and oxygen-free radicals, blockade of neutrophil chemotaxis and mast cell activation (393).

Mesalazine, a commonly-used 5-ASA, works on the luminal side of the inflamed colonic mucosa and effectively decreases inflammation. It has been shown to decrease mucosal-adherent Bacteroidales in colonic biopsies of children with IBD (530).

3.1.4.9.3 Vedolizumab

In a prospective study involving 85 IBD (43 UC, 42 CD) patients, intestinal microbiota was analysed before and during treatment with Vedolizumab, an anti-integrin therapy. Patients who achieved remission at 14 weeks were those who had higher α-diversity as well as *Roseburia inulinivorans* and a *Burkholderiales* species at baseline (388). This implies that remission was more likely in those with less dysbiotic microbial profiles.

3.1.5 Intestinal Microbiota in Other Inflammatory Intestinal Diseases

3.1.5.1 Irritable Bowel Syndrome

Irritable Bowel Syndrome (IBS) is defined according to the Rome IV criteria as recurrent abdominal pain for at least 6 months, occurring at least once weekly over the preceding 3 month period, associated with two or more of the following: 1) defecation, 2) a change in stool frequency, and 3) a change in stool consistency (531). It is estimated to affect
10-15% of the general population in the industrialised world (532). These patients present with chronic abdominal pain and altered bowel habits, in addition to other symptoms such as abdominal distension, bloating, flatulence, straining and urgency. These symptoms can significantly impact their health, well-being and quality of life (533). Depending on the altered bowel habit, IBS can be classified as IBS with constipation (C-IBS), IBS with diarrhoea (D-IBS), alternate IBS (A-IBS) with both constipation and diarrhoea as well as unclassified IBS with neither constipation nor diarrhoea (534, 535).

IBS is increasingly recognised as an inflammatory condition, albeit milder than IBD. Increased systemic and mucosal TNF-α, IL-1β, IL-6, IL-8 and IL-12, while decreased IL-10 have been reported in IBS patients (536-538). In addition, mast cells activated through TLR lead to the release of inflammatory mediators such as histamine and tryptase, which are increased in IBS (539). There are also studies reporting increased mucosal intraepithelial lymphocytes, mast cells and 5-hydroxytryptamine-secreting enterochromaffin cells (540-544). These can influence intestinal microbial composition through its effects on intestinal motility, secretions, barrier and permeability (545).

Intestinal microbiota has strongly been implicated in the development of IBS. Host genetics is known to influence intestinal microbial composition; IBS can affect members of the same family. This could also be due to environmental influences such as diet. Food intolerances were reported in up to 70% of IBS patients (546-548). In addition, a course of antibiotics increased the risk of developing IBS three-folds in subsequent months (549). Furthermore, 10% of IBS patients reported onset of symptoms following an episode of gastroenteritis (550-552), which is known to disrupt microbial communities, leading to dysbiosis.

In the healthy duodenum and jejunum, *Lactobacillus spp.*, *E. coli* and *Enterococci* are abundant (413). In IBS, there is dysbiosis with contradicting reports of the microbial profiles in this heterogeneous disorder, however, increased abundance of Proteobacteria and decreased alpha diversity are consistent findings (553-556). Saulnier et al. reported increased Gammaproteobacteria in a study comparing 22 children with IBS to 22 HC (553). A major component of this Proteobacteria class was *H. parainfluenzae*. In addition, several taxa from the genus *Alistipes* correlated with increased pain frequency.

In addition, there are increased mucin-degrading organisms *R. torques* and *R. gnarus* (557-560). In contrast, reduced butyrate-producing organisms have also been reported (561, 562). Butyrate, is produced from intestinal bacterial fermentation of
indigestible plant fibres. It has several beneficial functions promoting barrier function (discussed in Section 4.1).

### 3.1.5.2 Coeliac Disease

Approximately 20-50% of those with the genetic susceptibility develop Coeliac Disease (115, 563), suggesting the role of environmental factors in disease onset. Most of the risk factors for Coeliac Disease are similar to those of IBD and food allergies, many of which affect the intestinal microbiota thus influencing immunity.

The HLA-DQ2/8 status appears to influence early intestinal colonisation. Infants with HLA-DQ2 had greater bacterial diversity with several genera of Proteobacteria (Klebsiella, Escherichia, Shigella) and Firmicutes (Clostridium, Veillonella and Lactobacillus), and absence of Bacteroides and Blautia (564). The evidence thus suggests that genetic mutation and early life risk factors select early intestinal microbiota composition that leads to the characteristic dysbiosis seen in these children. Generally, there are more abundant Proteobacteria and decreased abundance of Firmicutes and Actinobacteria within a microbiota of higher diversity in the stool and duodenal biopsies compared to healthy controls (564-567). Decreased Lactobacillus abundance is another consistently reported finding in adults and children with Coeliac Disease (568-571). In addition, the abundance of Bifidobacterium and Neisseria were higher (565) whilst Lactobacillus and Bacillus cereus, which are bacteria involved in gluten metabolism, were not detected in active Coeliac Disease (566).

It appears that dietary manipulation can affect disease manifestation. In the 1980’s, Sweden delayed the introduction of gluten from 4 to 6 months of age. Concurrently, breastfeeding was stopped and gluten in industrially produced food was increased in children under 2 years (132, 159). Subsequently, the incidence of Coeliac Disease in children increased, called the “Swedish epidemic”, pointing towards the importance of weaning food and the critical timing of it. Delaying gluten introduction to 12 months of age for genetically susceptible infants resulted in decreased immune response to gluten and lower incidence of Coeliac Disease (160).

As the breast milk of mothers with Coeliac Disease has reduced Bifidobacteria and immunoprotective compounds TGF-β1 and sIgA (564), children supplemented with B. longum alongside a gluten-free diet had improved clinical outcomes associated with
reduced *B. fragilis* group and faecal slgA (572). In support of this, in a murine model of gliadin-induced enteropathy, *B. longum* was found to attenuate TNF-α and increase IL-10 in the small bowel (573).

The microbiota alters with disease activity. Active disease was associated with significantly higher abundance and proportions of gram-negative bacteria (568). There were higher abundances of mucosal Proteobacteria (*Enterobacteriaceae*), *Staphylococcaceae*, *Bacteroides*, *E. coli* and opportunistic pathogens *Candida*, *Saccharomyces* sp. and *Staph. epidermidis*, while Firmicutes and specifically *Streptococcaceae* were absent (568). After long-term gluten-free diet, the dysbiosis improved but was only partially restored (120, 574, 575). Interestingly, levels of stool IgA-coated bacteria were significantly lower in untreated and treated Coeliac Disease patients than in healthy controls.

The altered microbial population in Coeliac Disease inevitably leads to microbial metabolite production that deviates from the norm. In fact, lactate and butyrate have been hypothesised to play a role in the pathogenesis of Coeliac Disease (160, 576).

### 3.1.5.3 Food Allergies

Allergic diseases often present in early childhood with eczema. In some, disease progresses to include food allergies, then asthma followed by rhinitis. This systematic progression has been named the allergic march (577).

The commonest food allergens are cow’s milk, eggs, peanuts, tree nuts, wheat, soy, fish and shellfish (578). Cow’s milk protein (CMP) are often the first of these antigens to be introduced into the infant diet, thus CMP allergy (CMPA) generally presents in the first year of life. The cause of food allergy remains undefined but family history is a known risk factor. Over recent decades, the incidence has increased at a pace that is greater than our genetic evolution, thus pointing to environmental factors contributing to disease onset and progression.

Canani *et al.* showed that the intestinal microbiota of infants allergic to cow’s milk is significantly more diverse than that of healthy age-matched controls when obtained at diagnosis at 4–5 months of age (579). Thompson *et al.* also reported higher microbial diversity with significantly higher proportions of the *C. coccoides* group (580). Contrary
to this, other studies have demonstrated decreased bacterial diversity associated with allergy development (581-584).

At 1 year, Estonian children (low allergy prevalence) had more Lactobacilli and Eubacteria, and were less likely to be colonised with C. difficile than Swedish children (high allergy prevalence)(585). In addition, allergic infants had lower Bifidobacteria abundance. Environmental factors such as diet may have influenced the findings. The Estonian diet mostly consist of locally produced foods including lactic acid fermented products whilst Swedish children had mostly industrialised foods. In addition, increased exposure to microorganisms and infections may also contribute to an immune shift towards a Th1-like immunity.

The Canadian Healthy Infant Longitudinal Development (CHILD) group reported the association of food sensitisation with low microbial richness and higher Enterobacteriaceae/Bacteroidaceae ratio in early infancy. This was supported by another study reporting reduced Bacteroidetes in food-sensitised children (586, 587). On the other hand, at 2 months of age, raised faecal calprotectin was associated with low abundance of E. coli, and predicted the development of asthma and eczema by 6 years of age (588).

In a separate study of 226 children, Bunyavanich et al. found that the 128 children whose milk allergy resolved by 8 years of age had greater abundance of Firmicutes specifically Clostridia in their stool at 3-6 months of age (589). Fujimura et al. studied 298 infants aged 1-11 months and found that infants at highest risk of CMPA showed lower relative abundance of Bifidobacterium, Akkermansia and Faecalibacterium, and higher relative abundance of fungi Candida and Rhodotorula (590). Associated with these were higher concentrations and percentages of butyric acid seen in CMPA infants (580).
The Role of IgA

The development of allergy has been associated with low mucosal total IgA levels (591-594). IgA deficiency is, however, common and not always associated with disease (595), perhaps due to compensatory secretion of IgM (596). However, it does appear to influence the development and composition of intestinal microbiota (219, 596).

IgA-coating of microbes hampers their access to the epithelium by slowing their movement in the mucus layer, thereby reducing the chances of contact and entry (597, 598). Childhood allergic diseases, particularly asthma was associated with a reduced proportion of IgA bound to faecal bacteria at 12 months (599). There were also differences in the IgA bacterial targets and IgA recognition patterns between the 2 groups (20 allergic vs 28 HC). *Faecalibacterium* and *Bacteroides* were mainly IgA-free in allergic children at 1 and 12 months of age but were predominantly IgA-coated in healthy children, especially at 12 months (599).

Allergy development has been associated with decreased seroreactivity to gut microbial antigens (600). Children with CMPA-related eczema had lower IL-10 concentrations than HC, with the levels being lower still in severe disease. Total IgE concentrations negatively correlated with IL-10 concentrations (601). In another study, 20 children with IgE-associated eczema had lower abundance of *Ruminococcaceae* at 1 week of age, and this was inversely associated with TLR-2 induced IL-6 and TNF-α. Similarly, the abundance of Proteobacteria was inversely associated with TLR-4 induced TNF-α (602).

Manipulation of Intestinal Microbiota in the Treatment of Allergic Diseases

Manipulation of intestinal microbiota appears to improve atopy. A questionnaire of more than 21 000 (89% response rate) Japanese teenagers found that regular intake of lactic acid bacteria was associated with remission of their eczema (603). In a study of 21 infants with early-onset atopic eczema, total IgE concentrations in the highly-sensitised group (HSG) correlated with *E. coli* counts and *Bacteroides*. These infants were intolerant to extensively hydrolysed formula (EHF) and had greater Lactobacilli/Enterococci ratio than the sensitised-group tolerant of EHF. Seven infants received *B. lactis* Bb-12 alongside EHF, resulting in decreased *E. coli* and enhanced protection against increasing *Bacteroides* during weaning (604). Subsequently, Canani et al. reported on 110 children on EHF and 100 children on EHF supplemented with *Lactobacillus GG*. Over 3 years, supplementation with LGG reduced the incidence of
other allergic manifestations and hastened oral tolerance in children with IgE-mediated CMPA (579).

As with Coeliac Disease, the cumulative evidence suggests that the combination of genetic and environmental factors influence and shape the intestinal microbiota in early childhood, at a time when critical immune development occurs. Impaired mucosal immune function, associated with relatively low abundance of beneficial immune-modulating microbiota, enables greater colonisation of pathogenic microbes that lead to pro-inflammatory responses that increase disease susceptibility.
3.1.5.4 Mutations in the IL-10 Pathway

IL-10 KO murine models have been utilised to investigate the role of microbiota in IBD. IL-10 KO mice maintained in germ-free (GF) conditions did not develop intestinal inflammation until colonised with conventional or specific pathogen free (SPF) microbiota, with the intestinal inflammation occurring as early as 1 week after colonization (605). In human, intestinal colonisation occurs rapidly after birth, influenced by several environmental factors already discussed (Section 1.4). An intact immune function is required for symbiotic microbial colonisation to occur. We now appreciate the role of IL-10 in shaping and maintaining homeostatic intestinal environment. Commensal microbiota induces IL-10 production mostly by colonic macrophages, but also Treg cells, thus negatively regulating inflammatory responses to allow its colonisation. IL-10 has also been implicated in the development, programming and function of host immunity, with the intestinal microbiota playing an essential role as discussed above.

IL-10 KO mice have been reported to develop dysbiotic gut flora. Maharshak et al. demonstrated these changes comparing formerly germ-free (GF) IL-10 KO mice with wild type mice (252). Following colonisation with specific pathogen-free (SPF) microbiota, intestinal microbial diversity and richness decreased over time (4 weeks) in the formerly GF IL-10 KO mice. Significantly decreased Bacteroidetes, Verrucomicrobia, Actinobacteria and Firmicutes, and increased Proteobacteria and Tenericutes occurred. E. coli accounted for the increased Proteobacteria seen. The dysbiosis demonstrated in IL-10 KO mice models have yet to be reported in IL-10 deficient patients.

A question that remains unanswered is whether dysbiosis precedes chronic intestinal inflammation, or whether it occurs because of inflammation. Here the authors conclude that decreased microbiota richness and diversity occurred after the onset of colonic inflammation thus dysbiosis is secondary to the inflammatory responses but may be involved in perpetuating and amplifying intestinal inflammation.

3.1.5.5 Juvenile Idiopathic Arthritis (JIA)

In his thesis “Toxemic Factor Hypothesis” in 1909, Carl Warden suggested that the noxious substances produced by the overabundant intestinal gram-negative anaerobes and absorbed by the GI tract are responsible for the development of Rheumatoid Arthritis
More recently, the gut microbiota has been implicated in the pathogenesis of various JIA subtypes.

JIA is diagnosed in a child of less than 16 years when arthritis of unknown origin persists for more than 6 weeks. JIA is the most common childhood rheumatic disease with a prevalence of 16–150 cases per 100,000 children (607, 608). There are several different types of JIA including oligoarticular, polyarticular, systemic JIA and enthesitis-related arthritis (ERA). The JIA susceptibility genes identified are broadly subdivided in HLA and non-HLA genes, and differ between the JIA subtypes.

In all subtypes of JIA, increased intestinal permeability has been reported (609). As with IBD, intestinal microbiota has been implicated in some of the risk factors for the development of JIA, namely mode of delivery, breastfeeding and early exposure to antibiotics (140, 175, 345, 610, 611). Dysbiosis with reduced microbial diversity is also associated with JIA (612), RA and psoriatic arthritis (365, 613, 614). Interestingly, antiviral nor antifungal agents have been associated with JIA (175).

The dysbiosis seen in active JIA patients appears to be more pronounced than in patients in remission, with an intermediate dysbiotic composition observed in patients in remission compared to HC. It is unclear whether this intermediate dysbiosis is treatment or inflammatory-related. Zhang et al. reported partial normalisation of the gut microbiota after treatment in newly-diagnosed RA patients (615), suggesting that treatment influences the microbiota.

The dysbiosis in JIA has been quantified as Firmicutes:Bacteroidetes ratio. Grevich et al. compared 10 newly-diagnosed, treatment-naïve JIA patients (4 oligoarticular, 2 polyarticular, 3 ERA, and 1 psoriatic) with their mothers. JIA patients had reduced abundance of Firmicutes, increased abundance of Bacteroidetes or decreased Firmicutes as well as increased Bacteroidetes. The mean Firmicutes:Bacteroidetes ratios were lowest in oligo JIA, followed by poly JIA and ERA, and in those positive for antinuclear antibodies (ANA) (616).

3.1.5.6 Granulomatosis with Polyangiitis

Microbes have long been speculated to play a role in the pathogenesis of GPA. To date, there is no study reporting intestinal dysbiosis in patients with GPA but murine models of glomerulonephritis have intestinal dysbiosis with increased abundance of *E. coli* or
Citrobacter rodentium. This is thought to be mediated by intestinal-derived Th17 lymphocytes, which migrate to the kidneys (617).

3.1.5.7 Cryopyrin-Associated Periodic Syndromes (CAPS)

Thus far, there are no reports of intestinal microbiota composition in patients with CAPS.

3.1.6 Aims of the Study

In summary, the intestinal microbiota has a significant role in IBD pathogenesis. Dysbiosis occurs within the lumen and on the mucosal surfaces, with disease observed to be more severe with increasing dysbiosis. Shifts in the microbes from the 3 main phyla occur in IBD with disease activity and with treatment. Health is restored as the microbial profiles become less dysbiotic, shifting towards profiles of HC in remission or clinical improvement. Dysbiosis also occurs in other diseases such as IBS, Coeliac Disease, food allergies and JIA, with variations observed between these diseases.

Resources are being poured into developing personalised medicine. Incorporating microbial profiles in determining disease management is an attractive option that appears to be more sensitive than the biomarkers currently used in the clinical setting. Another attractive option is modulating intestinal microbiota to correct dysbiosis and restore health. Although this research field has been significantly explored and extended, deeper understanding of disease-specific alterations in the microbial profiles are needed to further this field and incorporate it into clinical practice. With this in mind, the aims of this study were:

1. To investigate the luminal and mucosal microbial profiles of control and disease groups;
2. To investigate alterations in the microbial composition with treatment in children with IBD; and
3. To identify potential associations between bacterial phyla and disease activity.
3.2 Validation of Methods

3.2.1 Introduction

Advancing technology has made it possible to identify not just specific organisms but the content of the whole complex microbial composition within the gut. However, in the absence of standardised methods, microbiome analysis has been conducted by many groups using varying methods and technology, resulting in variations in reported outcomes.

Variations in storage temperature (storage at 4°C, -20°C and -80°C) and duration also exist. This has the potential to alter the microbes detected due to processes such as bacterial degradation, which is known to be exacerbated by increased freeze-thawing (618). The Human Microbiome Project used the MOBio Powersoil DNA extraction kit for stool samples. Since then, other DNA extractions kits have been developed with better yields reported (619, 620).

Subsequent downstream analysis of the DNA can be done by quantitative PCR, FISH, 16S rDNA sequencing or metagenomics. In addition, the primers used as well as the protocol for library preparation can alter the final results. Finally, many bioinformatics pipelines are currently available, thus providing choice, ease for users and differences in results. Given that there are no standardised protocol for analysing the results, research groups may opt for different standards for meaningful results, for example a lower threshold than 97% for read similarity.

Ease of sample collection is an important consideration as patients are more likely to bring a stool sample to the hospital. Recognising the challenges in obtaining stool samples has led to the development of several user-friendly kits for sample collection (www.fecescatcher.com, Easy Sampler Faecal Collection Kit, Alpha laboratories, UK). This has to be balanced with sterility and a method that can be standardised for the study.

To obtain the best possible results from the samples obtained, a series of experiments were thus conducted to determine the optimal methods. Every stage, i.e. patient recruitment, sample collection, storage and analysis were considered (Figure 3.2).
Figure 3.2: Journey of the sample considered and optimised
3.2.2 Microbiota Analysis of Stool

For the study of stool microbiota, the journey of the stool sample from passage to analysis was considered (Figure 3.3). Potential variations and challenges at each step were identified and measures were taken to minimise these.

3.2.2.1 Stool Sample Collection

Various methods for stool collection were considered. In hospital, a bedpan placed in the toilet can be used to collect a sample. At home, collection can be more challenging. There are a number of aids available to assist with this.
One such aid is the FecesCatcher (Tag Hemi, The Netherlands). The folded piece of paper is unfolded and each end stuck to the toilet seat. The stool sample is caught and can be transferred into a stool collection bottle using the spatula attached to the bottle lid. The FecesCatcher can then be detached and flushed away. It has the added benefit of being easy and cheap to post. For patients with diarrhoea, the FecesCatcher became very wet and fell away thus foil bowls were preferred.

The OMNIgene collection tubes were considered but the solution contained within it was not guaranteed to be sterile. As IBD patients often have loose or diarrhoeal stools, collection of these samples would be more challenging using the OMNIgene.

In conclusion, both FecesCatcher and foil bowls were offered to study participants for ease of stool collection. The stool samples were then transferred into standard stool collection bottles widely available in the National Health Service (NHS). As the stool did not enter the toilet bowl, contamination was minimised.

The FecesCatcher and foil bowls were thus included in a Stool Collection Kit containing a padded envelope, gloves, stool collection bottles, an ice pack and instructions.

### 3.2.2.2 Transport of Stool Sample to Laboratory

Collected samples should be kept chilled. To maintain the temperature during transfer of samples from home to hospital, various options were considered. To supply each patient with an insulated bag would be costly thus a more cost-effective solution was considered.

Using a padded A4 envelope, a frozen icepack and thermometer, the temperature within the enveloped was monitored and found to be maintained between 1-6°C over 7 hours. Patients were thus provided with an A4 padded envelope to transport the stool sample to hospital.

Samples produced in hospital were stored at 4°C before being transferred within a few hours to the laboratory in an insulated bag kept chilled with ice packs.
3.2.2.3 Stool DNA Extraction Kit, Sample Storage Temperature and Duration

To determine the effects of temperature and duration of storage on the stool samples, the same stool sample was spiked with *Streptococcus pneumoniae* at $10^2$ and $10^6$ CFU/ml. Aliquots of this sample was prepared and extracted immediately (fresh) or stored at different temperatures for varying durations. Bacterial DNA was extracted after 1 day of storage at 4°C and -80°C and after 1 week of storage at 4°C and -80°C. Duplicate samples were prepared and DNA extraction was performed using 2 DNA extraction kits, FastDNA SPIN Kit for Spoo (MPBio, UK) and QIAamp Mini DNA (Qiagen, The Netherlands). Concurrently, the 2 different extraction kits were compared to determine optimal extraction conditions.

3.2.2.3.1 Preparation of *Streptococcus pneumoniae*

Firstly, *S. pneumoniae* was cultured. Todd-Hewitt broth was prepared by adding 11 g Todd-Hewitt (36.5 g in 1L) (Oxoid, UK) to 300 ml water and 1.5 g yeast. Of this solution, 73 g was added to 3 g agar (Fluka, Switzerland), 1 g yeast and 200 ml water. The solution was sterilised using an autoclave and poured into sterile petri dishes while still warm. Once cooled and firm, *S. pneumoniae* was streaked and spread on the agar, then incubated overnight.

The bacteria were then quantified. Bacterial culture was suspended in 250 μl PBS in 15 ml falcons. The solution was then diluted 1:10 with PBS in a 1.5 ml Eppendorf tubes (Eppendorf, Germany) then transferred into a cuvette (10mm path length, Fisher Scientific, USA). A control of PBS only was prepared in another cuvette. Both were placed in a spectrophotometer (Jenway 6300 Spectrophotometer; Jenway, Essex) and bacterial optical density (OD) quantified at 600nm. The OD$_{600}$ was 1.18 x $10^8$ CFU/ml (OD of 1.0 = $10^8$ CFU/ml).

Next, the colony-forming Unit (CFU) was counted. CFU counting was performed to determine absolute number of bacteria. Agar plates were prepared and divided in 2 with a permanent pen. Serial dilutions by 10-fold were prepared from $10^{-1}$ to $10^{-8}$. Four to 5 aliquots of 20 μl of each dilution were spotted on one side of a plate. Once dried, the plates were incubated forr 48 hours. Colonies of the lowest dilution that could possibly be counted were counted and based on an average, the CFU was calculated. Subsequently, 100 μl aliquots of $10^4$ and $10^8$ CFU/ml were prepared and stored at -80°C.
for later use. Adding 10 μl of \textit{S. pneumoniae} at these concentrations would give an end concentration of 10^2 and 10^6 CFU/ml respectively.

3.2.2.3.2 DNA Extraction Kits

When compared to the MOBio extraction kit used by the Human Microbiome Genome Project, both the FastDNA SPIN for Soil (MPBio, UK) and QIAamp Mini Kit (Qiagen, The Netherlands) produced better yields. At the time, there were no published data comparing these 2 kits with each other.

In order to determine the optimal storage temperature and duration, together with the extraction efficacy of bacterial DNA present at high and low concentrations, the same stool sample was transferred into sterile 2 ml Eppendorf tubes weighing approximately 300 mg then spiked with \textit{S. pneumoniae} at 10^2 and 10^6 CFU/ml. \textit{S. pneumoniae} was used as it is a microorganism that is not present in the normal gastrointestinal tract (Ronnachit, 2017). Control samples of unspiked stool and kit reagents containing 10^2 and 10^6 CFU/ml \textit{S. pneumoniae} were also prepared.

The protocol used for the FastDNA SPIN Kit for Spoil (MPBio, UK) was as described in Section 3.3.3.1. This kit was compared with the QIAamp Mini DNA (Qiagen, The Netherlands), which were used as per manufacturers’ recommendations with the following amendments:

- Lysing matrix E (MPBio, UK) were added prior to tissue lysing to aid cell wall destruction, particularly of Gram-positive organisms
- Supernatant, AL buffer and Proteinase K were incubated at 56°C in a dry bath (Fisher Scientific, USA) for 10 minutes to improve yield

3.2.2.3.3 Detection of \textit{Streptococcus pneumoniae} by real-time Polymerase Chain Reaction (PCR)

The DNA extracted above were analysed with real-time polymerase chain reaction (PCR) using QuantiTect Multiplex PCR Kit (Qiagen, The Netherlands) and Taqman (7300 System SDS Software). The master mix was prepared as follows;
<table>
<thead>
<tr>
<th>Master Mix Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x Quantitect</td>
<td>20</td>
</tr>
<tr>
<td>PCR grade water</td>
<td>12.2</td>
</tr>
<tr>
<td>Primer 1, LytA-F (10 pmol/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Primer 2, LytA-R (10 pmol/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Probe, LytA-P (10 pmol/µl)</td>
<td>0.8</td>
</tr>
<tr>
<td>Sample</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>40</strong></td>
</tr>
</tbody>
</table>

LytA-F sequence 5’-ACG CAA TCT AGC AGA TGA AGC-3’
LytA-R sequence 5’-TGT TTG GTT GGT TAT TCG TGC-3’
LytA Probe sequence FAM 5’-TTT GCC GAA AAC GCT TGA TAC AGG G-3’ TAMRA

Samples were then loaded on the Taqman with the following conditions:

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 sec</td>
<td>40</td>
</tr>
<tr>
<td>Annealing and extension</td>
<td>60</td>
<td>1 min</td>
<td>40</td>
</tr>
<tr>
<td>Dissociation</td>
<td>95</td>
<td>15 sec</td>
<td>1</td>
</tr>
<tr>
<td>Dissociation</td>
<td>60</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Dissociation</td>
<td>95</td>
<td>15 sec</td>
<td>1</td>
</tr>
</tbody>
</table>

3.2.2.3.4 Results of Experiments

Comparing FastDNA SPIN Kit for Soil (MPBio, UK) with QIAamp Mini Kit (Qiagen, The Netherlands), stool samples spiked with $10^2$ and $10^6$ CFU/ml *S. pneumoniae* were measured with cycle threshold (CT) values by real-time PCR. CT is the number of cycles required for the fluorescence to exceed a given threshold. Comparing yield from fresh stool samples extracted immediately, similar CT values were obtained from both kits (Figure 3.4). These were compared to the control unspiked stool samples (stool only) and spiked buffers. Samples with $10^2$ CFU/ml *S. pneumoniae* were undetectable using both kits.

When comparing the DNA extraction kits using samples stored at 4°C for 1 day and 1 week, the CT values were lower in the samples extracted using FastDNA compared to QIAamp (Figure 3.5). At -80°C, CT values were similar using both kits. Comparison was
also made between control unspiked stool samples, which were appropriately negative, and freshly extracted stool samples. No CT value was obtained for the QIAamp sample stored at -80°C for 1 day. Generally, CT values were lower for samples stored at 4°C and extracted using FASTDNA SPIN Kit for Soil (MPBio).

![Bar chart showing PCR CT values for different samples: FastDNA 10^6 Unspiked Stool, FastDNA Spiked Stool, QIAGEN 10^6 Unspiked Stool, QIAamp Spiked Stool.](chart)

**Figure 3. 4: Comparison of Stool DNA Extraction Kits, FastDNA SPIN kit for Soil (MPBio) and QIAamp Mini Kit (Qiagen), using Fresh Stool Samples**

Fresh stool samples were spiked with *S. pneumoniae* (10^6 CFU/ml) and extracted using FastDNA SPIN Kit for Soil (MPBio) or QIAamp Mini Kit (Qiagen). These were compared to unspiked stool samples (stool only) and control spiked buffers. *S. pneumoniae* DNA was quantified using real-time PCR. Values are shown.
Figure 3.5: Comparison of FastDNA and QIAamp DNA Extraction Kits on Stored Stool Samples

Stool samples were spiked with S. pneumoniae (10⁶ CFU/ml) and DNA extracted using either FastDNA (A and B) or QIAamp (C and D). Samples were stored at 4°C (A and C) or -80°C (B and D) for 0 days (fresh), 1 day or 1 week. Unspiked stool samples were used as negative controls. S. pneumoniae DNA was then quantified using real-time PCR. CT values are shown.
3.2.2.3.5 Determination of Optimal Sample Weight

The FastDNA SPIN Kit for Soil recommended using samples weighing less than 500 mg. To determine the optimal sample weight, the same stool sample was transferred into sterile 2 ml Eppendorf tubes in aliquots of 230-500 mg. The samples were then spiked with *S. pneumoniae* at $10^6$ CFU/ml prior to DNA extraction and detection of bacterium were conducted by real-time PCR. There were small differences in CT values for stool samples weighing 230-488 mg, with slightly lower CT values for 299-396 mg (Figure 3.6). This may be due to the slightly smaller sample having more room within the tube for the mechanical breakdown of sample in the Tissue Lyser (TissueLyser LT, Qiagen, The Netherlands).

In conclusion, the FastDNA SPIN Kit (MPBio, UK) was selected for this study as the yield from stool DNA extraction was greater compared to the QIAamp Mini Kit (Qiagen, The Netherlands). Samples weighing 250-350 mg were stored at 4°C for short periods and at -80°C for longer term storage.

![Figure 3.6: Determination of Optimal Stool Weight for Extraction with FastDNA SPIN Kit for Soil (MPBio, UK)](image)

*Figure 3.6: Determination of Optimal Stool Weight for Extraction with FastDNA SPIN Kit for Soil (MPBio, UK)*

*Stool samples weighing 200-500 mg and spiked with $10^6$ CFU/ml *S. pneumoniae* were extracted. *S. pneumoniae* DNA was quantified using real-time PCR. CT values are shown.*
3.2.2.4 Bacterial DNA Quantification of Stool Samples

Absolute quantification of bacterial load was performed first to identify samples that may contain much lower or higher DNA load thus may result in template inhibition. This was done using Power SYBR Green to amplify the V5 to V6 regions of the 16S rDNA. Initial 16S rDNA sequencing produced no results. This is likely due to carry-over of proteins causing inhibition. Subsequently, real-time PCR performed on samples demonstrated detectable DNA but in concentrations of 1:10, 1:100 or 1:1000 diluted in nuclease-free water (nf-H₂O), except DNA extracted from ileostomy samples (Figure 3.7). Using a fluorometer (Qubit 2.0, Invitrogen Life Technologies, USA) 5 µl of control samples had appropriately undetectable DNA. Depending on the concentrations as determined using real-time PCR quantification, samples were prepared at the appropriate dilutions.

Figure 3.7: Bacterial Quantification of Extracted Stool DNA by real-time PCR

Stool DNA from study patients (n=7) were extracted using the FastDNA SPIN kit for Soil (MPBio, UK). DNA was then diluted 1:10, 1:100 and 1:1000 with AE buffer and then analysed for bacterial DNA using a 16S real-time SYBR Green PCR reaction. Data is presented as CT values.
3.2.3 Microbiota Analysis of Mucosal Biopsies

The protocol for DNA extraction from mucosal biopsies using QIAamp Mini Kit (Qiagen, The Netherlands) was optimised to determine the best conditions for detection of high and low concentrations of Gram-positive and Gram-negative microorganisms. This extraction kit has been used by other teams producing reliable results (621). Intestinal mucosal biopsies collected were limited thus were only to be used for the actual experiment. As other animal mucosal biopsies were not readily available, chicken meat pieces the size of biopsies, i.e. between 30-100mg, were spiked with combinations of $10^2$ and $10^6$ CFU/ml of *S. pneumoniae* and *Pseudomonas aeruginosa*.

### 3.2.3.1 Preparation of Pseudomonas aeruginosa

Firstly, *P. aeruginosa* was cultured. Plates were prepared by mixing 8.75g of LB agar (35 g in 1 L) (Sigma, Germany) and 250 ml of water. The solution was sterilised using an autoclave and poured into sterile petri dishes. Once cooled and firm, *P. aeruginosa* was streaked onto the agar and incubated overnight.

The OD was calculated as per *S. pneumoniae* and CFU counted as described above (Section 3.2.2.3.1). An OD$_{600}$ of 0.84 x$10^9$ CFU/ml was obtained (Davidson *et al.* reported OD$_{595} = 1 \times 10^9$ CFU/ml). As with *S. pneumoniae*, 100 µl aliquots of $10^4$ and $10^8$ CFU/ml were prepared and stored at -80°C.

Chicken pieces weighing 173-319 mg were spiked with either 10 µl of *S. Pneumoniae* or *P. aeruginosa* at $10^2$ or $10^6$ CFU/ml. In addition, chicken pieces were also spiked with combinations of $10^2$ or $10^6$ CFU/ml of both organisms. DNA extraction was then performed.

### 3.2.3.2 DNA Extraction from Chicken Pieces

The QIAamp Mini Kit was used as per the manufacturer’s instructions with the following alterations:

- ATL buffer was added to the frozen meat samples and allowed to equilibrate to room temperature. The Lysing Matrix B (MPBio) was then added.
- An additional 10 µl (thus total of 30 µl) of Proteinase K (Qiagen kit) was added for the initial lysis period as per Thomas et al. PLOSone 2011 (622).
- Samples were first incubated with Proteinase K, ATL and AE buffers in a dry bath (Fisher Scientific, USA) at 56°C.

Extracted DNA was stored at -80°C.

### 3.2.3.3 Detection of Streptococcus pneumoniae and Pseudomonas aeruginosa by real-time PCR

DNA extracted above were analysed with real-time PCR using QuantiTect Multiplex PCR Kit (Qiagen, The Netherlands) and Taqman (7300 System SDS Software). The total volume of 40 µl master mix was prepared as follows;

<table>
<thead>
<tr>
<th>Master Mix Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x Quantitect</td>
<td>20</td>
</tr>
<tr>
<td>Water</td>
<td>10.6</td>
</tr>
<tr>
<td>Primer LytA-F (0.1 µM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Primer LytA-R (0.1 µM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Probe LytA-P (0.1 µM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Primer PA23-F (0.3 µM)</td>
<td>1.2</td>
</tr>
<tr>
<td>Primer PA23-R (0.3 µM)</td>
<td>1.2</td>
</tr>
<tr>
<td>Probe PA23 (0.2 µM)</td>
<td>0.8</td>
</tr>
<tr>
<td>Sample</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>40</strong></td>
</tr>
</tbody>
</table>

LytA-F sequence 5’-ACG CAA TCT AGC AGA TGA AGC-3’
LytA-R sequence 5’-TGT TTG GGT GGT TAT TCG TGC-3’
LytA Probe sequence FAM 5’-TTT GCC GAA AAC GCT TGA TAC AGG G-3’ TAM
PA23-F sequence 5’-TCCAAGTTTAAGGTGGTAGGCTG-3’
PA23-R sequence 5’-CTTTTCTTGGAAAGCATGGCATC-3’
PA23 Probe sequence JOE 5’-AGG TAA ATC CGG GGT TTC AAG GCC-3’ TAM
Serial dilutions of $10^2$ – $10^8$ *S. pneumoniae* and *P. aeruginosa* as well as negative controls were prepared. Samples were then loaded on the Taqman with the following conditions:

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 sec</td>
<td>40</td>
</tr>
<tr>
<td>Annealing and extension</td>
<td>60</td>
<td>1 min</td>
<td>40</td>
</tr>
<tr>
<td>Dissociation</td>
<td>95</td>
<td>15 sec</td>
<td>1</td>
</tr>
<tr>
<td>Dissociation</td>
<td>60</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Dissociation</td>
<td>95</td>
<td>15 sec</td>
<td>1</td>
</tr>
</tbody>
</table>

The chicken pieces spiked with *S. pneumoniae* and *P. aeruginosa* at $10^2$ or $10^6$ CFU/ml, and in combination were measured with real-time PCR CT values (Figure 3.8). *S. pneumoniae* at $10^6$ CFU/ml in isolation and in combination with *P. aeruginosa* at $10^2$ or $10^6$ CFU/ml were detected. *P. aeruginosa* at $10^2$ or $10^6$ CFU/ml only were also detected in the spiked chicken pieces. *S. pneumoniae* at $10^2$ CFU/ml in buffer and chicken pieces were undetectable.

Serial dilutions of *S. pneumoniae* and *P. aeruginosa* at $10^3$, $10^5$ and $10^7$ CFU/ml were also quantified. *P. aeruginosa* at $10^3$ CFU/ml was undetectable but the other samples had CT values appropriately higher for more dilute samples. The PCR negative control and DNA extraction control were appropriately negative but the control chicken biopsy was most likely contaminated with *P. aeruginosa*. 
Figure 3.8: Detection of *S. pneumoniae* and *P. aeruginosa* at 10^2 or 10^6 CFU/ml in Chicken Pieces

Chicken pieces were spiked with *S. pneumoniae* and *P. aeruginosa* at 10^2 or 10^6 CFU/ml as well as combinations of the 2 organisms at 10^2 or 10^6 CFU/ml. These were compared with negative controls (chicken biopsy only, extraction control and real-time PCR negative controls) and dilutions of *S. pneumoniae* and *P. aeruginosa* at 10^3, 10^5 and 10^7 CFU/ml. DNA was extracted using the QIAamp Mini Kit (Qiagen, The Netherlands) and quantified using real-time PCR. CT values are shown.

3.2.3.4 Digestion of Mucosal Biopsies with Proteinase K and ATL Buffer

The DNA extraction method with the QIAamp Mini Kit was altered by Thomas *et al.* to include a 17-hour incubation period at 56°C with Proteinase K and ATL Buffer (622). The method used by the GOSH laboratory included mincing the sample on dry ice and a 2-hour incubation period at 56°C. To assess the best method of digesting the mucosal biopsies, adult mouse colon was spiked with *S. pneumoniae* and *P. aeruginosa*.

Stool was removed from the mouse colon and the resected length irrigated with sterile water thrice, mimicking bowel preparation prior to endoscopic assessment. The colon was cut into smaller pieces using a sterile scalpel and spiked with 10 µl of *S. pneumoniae*
and *P. aeruginosa* at 10^6 CFU/ml. The pieces were weighed in 1.5 ml tubes then stored at -80°C. Duplicate samples and negative control samples were also prepared.

Samples incubated for 17 hours were subsequently extracted as per Section 3.3.3.2.

Samples for 2 hour incubations were first transferred into separate wells of a 6-well plate placed on dry ice. Each piece was minced with a sterile blade. To each well, 180 µl ATL buffer was added and used to capture the tissue pieces before all the contents of the wells were transferred into 1.5 ml Eppendorf tubes.

Subsequently, 20 µl of Proteinase K was added and the tubes were mixed by vortex before the 2-hour incubation at 56°C in a dry bath (Fisher Scientific, USA). During the incubation period, samples were mixed by vortex every 15-30 minutes. After 2 hours, the samples were incubated further at 95°C for 5 minutes.

One sample remained purulent thus had an addition incubation period at 95°C for 10 minutes. Once cooled, additional 10 µl of Proteinase K was added to the sample and the sample was incubated further at 56°C for 5 minutes.

Then 200 µl AL buffer and 1/6 of a vial of Lysing Matrix B beads (MPBio) were added and mixed by pulse-vortex for 15 seconds. The samples were incubated at 95°C for 10 minutes before being ribolysed at 50 oscillations/minute for 1 minute using the tissue lyser (TissueLyser LT, Qiagen, The Netherlands).

Following this, 200 µl ethanol was added to the solution and mixed by inverting. The solution was then transferred to a column and the samples centrifuged at 8,000 rpm for 1 minute. The collection tubes were discarded and replaced with fresh collection tubes.

After adding 500 µl AW1 buffer, the samples were centrifuged at 8,000 rpm for 1 minute. The collection tubes were replaced again and 500 µl AW2 buffer was added before being centrifuged at 13,000 rpm for 3 minutes.

The columns were then transferred to sterile 1.5 ml tubes and 200 µl of AE buffer were added before the final centrifugation at 8,000 rpm for 1 minute. The columns were discarded and the collected DNA was stored at -80°C.

The DNA extracted was analysed both neat and at 1:10 dilution in nf-H2O with real-time PCR using a Taqman (7300 System SDS Software) and QuantiTect Multiplex PCR Kit.
(Qiagen, The Netherlands) using the same master mix preparation and cycling conditions as detailed in Section 3.2.3.3.

CT values of the real-time PCR were analysed (Figure 3.9). S. pneumoniae and P. aeruginosa were both detected in the spiked samples, with higher CT values for the 1:10 diluted samples. No differences were seen between tissue digested over 17 hours and 2 hours. The extraction controls, unspiked tissue controls and PCR negative controls were appropriately negative.

![Figure 3.9: Digestion of Mucosal Biopsy Samples](image)

Resected mouse colon was spiked with S. pneumoniae and P. aeruginosa at 10^6 CFU/ml then digested for 2 hours after being minced or 17 hours overnight. DNA was then extracted using the QIAamp Mini Kit (Qiagen, The Netherlands) and analysed neat and at 1:10 dilutions with real-time PCT using Taqman (7300 System SDS Software) and QuantiTect Multiplex PCR Kit (Qiagen, The Netherlands). Spiked samples were compared with negative control samples (extraction controls, unspiked tissue and PCR controls). CT values are shown.
3.2.3.5 Bacterial DNA Quantification of Mucosal Biopsy Samples

Absolute quantification of bacterial load was performed to determine the quantity of DNA sample to be used. Recognising that there would also be a significant proportion of human DNA and carry-over of proteins, the resulting inhibition may necessitate dilution of the samples.

Bacterial DNA quantification was thus performed using Power SYBR Green to amplify the V5 to V6 regions of the 16S rDNA. DNA volumes of 2 µl and 5 µl from 7 patient samples (3 duodenal, 1 terminal ileal (TI) and 3 colonic biopsies) were compared. The reaction master mix had a total volume of 25 µl and consisted of the following;

<table>
<thead>
<tr>
<th>Master Mix Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>2</td>
</tr>
<tr>
<td>PCR grade water (Bioline)</td>
<td>9.5</td>
</tr>
<tr>
<td>Power SYBR Green master mix (Life Technologies)</td>
<td>12.5</td>
</tr>
<tr>
<td>785F (5’GGATTAGATACCCBRGTAGTC, 20pM)</td>
<td>0.5</td>
</tr>
<tr>
<td>1175R (5’ACGTCRTCCCCDCCTCCTC, 20pM)</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Master Mix Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>5</td>
</tr>
<tr>
<td>PCR grade water (Bioline)</td>
<td>6.5</td>
</tr>
<tr>
<td>Power SYBR Green master mix (Life Technologies)</td>
<td>12.5</td>
</tr>
<tr>
<td>785F (5’GGATTAGATACCCBRGTAGTC, 20pM)</td>
<td>0.5</td>
</tr>
<tr>
<td>1175R (5’ACGTCRTCCCCDCCTCCTC, 20pM)</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>
The negative samples and DNA extracted from a stool samples as a positive control were also prepared. The cycling conditions were as follows:

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 sec</td>
<td>40</td>
</tr>
<tr>
<td>Annealing and extension</td>
<td>60</td>
<td>1 min</td>
<td>40</td>
</tr>
<tr>
<td>Dissociation</td>
<td>95</td>
<td>15 sec</td>
<td>1</td>
</tr>
<tr>
<td>Dissociation</td>
<td>60</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Dissociation</td>
<td>95</td>
<td>15 sec</td>
<td>1</td>
</tr>
</tbody>
</table>

CT values from the real-time PCR were analysed (Figure 3.10). Samples with 5 µl of DNA were undetected, as were the TI and positive stool samples, likely due to inhibition. Samples with 2 µl of DNA were either negative or had high CT values. The 2 PCR controls were appropriately negative. Thus high DNA load was likely to lead to protein inhibition.

![Figure 3. 10: Bacterial Quantification of Extracted 2 µl and 5 µl Mucosal DNA](image)

*Extracted DNA from GI mucosal biopsies (3 duodenal, 1 terminal ileal and 3 colonic biopsies) of 7 patients were analysed. For each sample, 2 µl and 5 µl were used for bacterial quantification using Power SYBR Green to amplify the V5 to V6 regions of the 16S rDNA. Patient samples were compared with negative controls (extraction control and PCR control) and extracted DNA from a stool sample as a positive control. CT values are shown.*
As 2 µl and 5 µl of DNA were resulting in inhibition, the experiment was repeated using 1 µl of DNA neat, at 1:10 and 1:100 dilutions from 3 patient samples. Bacterial DNA quantification was performed using Power SYBR Green to amplify the V5 and 6 regions of the 16S rDNA. These samples were analysed alongside PCR negative controls and DNA extracted from a stool sample at 1:10 and 1:100 dilution as a positive control.

The cycling conditions were as in Section 3.2.3.5 above and the reaction master mix had a total volume of 25 µl consisting of the following:

<table>
<thead>
<tr>
<th>Master Mix Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>1</td>
</tr>
<tr>
<td>PCR grade water (Bioline)</td>
<td>10.5</td>
</tr>
<tr>
<td>Power SYBR Green master mix (Life Technologies)</td>
<td>12.5</td>
</tr>
<tr>
<td>785F (5' GGATTAGATACCCBRGTAGTC, 20pM)</td>
<td>0.5</td>
</tr>
<tr>
<td>1175R (5' ACGTCRTCCCDCCCTCCTC, 20pM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
</tr>
</tbody>
</table>

CT values from the real-time PCR of the samples were analysed (Figure 3.11). The CT values for neat DNA samples were similar to 1:10 diluted samples but in 1 patient, there was likely inhibition. DNA samples at 1:100 were negative, as were the PCR negative controls. The stool DNA sample was negative at 1:10 dilution, likely due to protein inhibition, and very low CT value for the sample diluted at 1:100. Thus for mucosal biopsies, 1 µl of neat DNA or at 1:10 dilution could be used.

As a result of the experiments above, DNA from GI mucosal biopsies were extracted following 17-hour overnight digestion. For the library preparation, the volume of extracted DNA used will need careful consideration due to the high likelihood of protein inhibition.
Figure 3.11: Bacterial Quantification of Extracted Mucosal DNA in Serial Dilutions

Extracted DNA of GI mucosal biopsies (1 duodenal, 1 terminal ileal and 1 colonic) of 3 patient samples were analysed using Power SYBR Green to amplify the V5 and 6 regions of the 16S rDNA. DNA extracted from a stool sample was prepared at 1:10 and 1:100 dilutions as positive controls. PCR negative controls were also prepared. CT values are shown.

3.2.4 Microbiota Analysis of Duodenal Lavages

3.2.4.1 Comparing Freeze-drying and Centrifugation to Concentrate Duodenal Lavages

To obtain a duodenal lavage, 10 ml of sterile water is injected into the duodenum through the endoscope and the lavage suctioned into a collection tube. The amount of lavage suctioned varies but is approximately 5 ml. To extract DNA from fluid using the QIAamp Mini Kit (Qiagen, The Netherlands), however, a maximum of 200 µl is used.

Initially, the lavages were freeze-dried individually (Edwards Modulyo, Thermo Scientific, UK) but there were challenges with gaining access to the machine and the machine frequently breaking down. Following discussion with Prof Georgina Hold, subsequent samples were concentrated.

This was done by dividing the sample into 2 ml tubes and centrifuging at 10 000 rpm for 20 minutes. The top 1.5 ml was removed and more lavage fluid was added before
centrifugation again. This process was repeated until all cellular contents of the fluid were concentrated to 200 µl volume. The sample was then stored at -80°C.

To understand the effects of the 2 different methods on the DNA, aliquots of sterile water were spiked with 20 µl *S. pneumoniae* and *P. aeruginosa* at 10⁴ or 10⁸ CFU/ml, and different combinations of both organisms. Duplicate spiked samples were prepared. A set of 4 ml samples and a set of 10 ml samples in 15 ml tubes (Sigma-Aldrich, Germany) were stored at -80°C then freeze-dried (Edwards Modulyo, Thermo Scientific, UK). Another set of 4 ml samples was centrifuged at 10 000 rpm for 20 minutes and subsequently concentrated as described above until 200 µl remained. Aliquots of 200 µl of sterile water were similarly spiked and included as comparison.

DNA extraction of both sets of samples was performed as per Section 3.3.3.3. Detection of the organisms was then performed by real-time PCR using the same reaction master mix and cycling conditions as per Section 3.2.3.3.

Extraction, centrifuge, freeze-dry and negative control samples were included. A 4 ml sample of sterile water was freeze-dried separately prior to freeze-drying the spiked samples (pre-freeze-dry). After the samples were freeze-dried, another 4 ml of sterile water was freeze-dried separately (post-freeze-dry) to ascertain the likelihood of contamination during the freeze-drying process. In addition, serial dilutions of *S. pneumoniae* and *P. aeruginosa* at 10⁴, 10⁶, 10⁷ and 10⁸ CFU/ml in 200 µl were also included for comparison.

CT values from the real-time PCR of the samples were compared. Results for the 200 µl, 4 ml and 10 ml aliquots were similar thus only data for the 4 ml aliquots are presented (Figure 3.12). *S. pneumoniae* and *P. aeruginosa* at 10⁴ CFU/ml were undetectable (CT values >35) in samples centrifuged and freeze-dried (from 4 ml and 10 ml). At 10⁶ CFU/ml, both organisms were detected, with the centrifuged samples having slightly higher CT values. No differences were seen between the freeze-dried 4 ml and 10 ml samples. CT values of the spiked 200 µl were similar to the freeze-dried samples.

For the serial dilutions of *S. pneumoniae* and *P. aeruginosa* at 10⁴, 10⁶, 10⁷ and 10⁸ CFU/ml, CT values reflected the dilutions with 10⁸ CFU/ml samples having the lowest CT values. All negative control samples, i.e. PCR negative controls, extraction controls, sample volume controls (200 µl, 4 ml and 10 ml), centrifuge and freeze-dry were appropriately negative.
These results suggest that there is minimal contamination, if any, during the freeze-drying process. The starting volume prior to freeze-drying did not make any difference to the bacterial content. Also, freeze-drying may preserve bacterial organisms better than centrifuged samples but the effects are small. These will need to be taken into consideration when analysing the duodenal lavage samples.

A.
Serial dilutions of S. pneumoniae and P. aeruginosa at $10^4$, $10^6$, $10^7$ and $10^8$ CFU/ml in 200 µl water were analysed (A). Aliquots of sterile water were spiked with S. pneumoniae and P. aeruginosa at $10^4$ and/or $10^8$ CFU/ml (B). The 4 ml samples were centrifuged or freeze-dried (C). Negative controls for PCR, extraction, sample volume of 4 ml, centrifuge and the (pre-) freeze-dry were included. Samples were analysed using real-time PCR using QuantiTect Multiplex PCR Kit (Qiagen, The Netherlands) and Taqman (7300 System SDS Software).

Figure 3. 12: Comparison of Centrifuged and Freeze-dried Spiked Water
3.2.4.2 Amplification of Bacterial DNA of Duodenal Lavages by PCR

The biggest challenge with duodenal lavage samples was the very low bacterial load. The aim was thus to amplify bacterial DNA without the control samples being positive too. In order to optimise the protocol for amplification of bacterial DNA in these samples, a few samples including control samples were used.

A 2-step PCR protocol was initially considered. The reaction mixture and conditions used were as follows;

<table>
<thead>
<tr>
<th>PCR Mixture Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR buffer</td>
<td>5</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1</td>
</tr>
<tr>
<td>Primer 1 V3F</td>
<td>1.25</td>
</tr>
<tr>
<td>Primer 2 V4R</td>
<td>1.25</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.25</td>
</tr>
<tr>
<td>PCR-grade water</td>
<td>36.25</td>
</tr>
<tr>
<td>Sample</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30 sec</td>
<td>25/15</td>
</tr>
<tr>
<td>Annealing</td>
<td>55</td>
<td>30 sec</td>
<td>25/15</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 sec</td>
<td>25/15</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10 min</td>
<td>1</td>
</tr>
</tbody>
</table>

This was followed by the first clean-up as discussed in Section 3.3.5.2 using 40 µl of AMPure beads. The samples were stored overnight at 4°C. Using the samples from the first step, the reaction mix was prepared as above and the cycling conditions were repeated but for 15 cycles instead of 25. Table 3.3 lists the Qubit readings of the samples for the various conditions. A 16S real-time PCR was also performed on these samples using the following reaction mixture and cycling conditions;
**PCR Mixture Components**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>2</td>
</tr>
<tr>
<td>PCR grade water (Bioline)</td>
<td>9.5</td>
</tr>
<tr>
<td>Power SYBR Green master mix (Life Technologies)</td>
<td>12.5</td>
</tr>
<tr>
<td>785F (5’ GGATTTAGATACCCBRGTAGTC, 20pM)</td>
<td>0.5</td>
</tr>
<tr>
<td>1175R (5’ ACGTCRTCCCCCDCCTCCTC, 20pM)</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>25</td>
</tr>
</tbody>
</table>

**Process**

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 sec</td>
<td>40</td>
</tr>
<tr>
<td>Annealing and extension</td>
<td>60</td>
<td>1 min</td>
<td>40</td>
</tr>
<tr>
<td>Dissociation</td>
<td>95</td>
<td>15 sec</td>
<td>1</td>
</tr>
<tr>
<td>Dissociation</td>
<td>60</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Dissociation</td>
<td>95</td>
<td>15 sec</td>
<td>1</td>
</tr>
</tbody>
</table>

**Lavage samples**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Qubit (ng/µl)</th>
<th>CT value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scope 1</td>
<td>&lt;0.5</td>
<td>38.1</td>
</tr>
<tr>
<td>Extraction Control 1</td>
<td>&lt;0.5</td>
<td>39.5</td>
</tr>
<tr>
<td>Extraction Control 3</td>
<td>&lt;0.5</td>
<td>Undetected</td>
</tr>
<tr>
<td>A5</td>
<td>0.2</td>
<td>31.12</td>
</tr>
<tr>
<td>A25</td>
<td>0.942</td>
<td>20.36</td>
</tr>
<tr>
<td>A18</td>
<td>&lt;0.5</td>
<td>33.39</td>
</tr>
<tr>
<td>A12</td>
<td>&lt;0.5</td>
<td>38.69</td>
</tr>
<tr>
<td>B3</td>
<td>&lt;0.5</td>
<td>38.79</td>
</tr>
</tbody>
</table>

*Table 3.3: Correlation between the Qubit readings and CT values for Duodenal Lavages*

As the control samples were appropriately negative, but 3 of the duodenal lavage samples were also negative, the experiment was repeated with 22 cycles in the second step. This resulted in positive Qubit readings for all samples including the control samples.
Subsequently, using the same reaction mixture and clean-up protocol, the conditions were altered to include 28 cycles in the first step then 12 cycles in the second step. The Qubit readings were negative for all samples including 3 lavage samples.

The conditions were thus altered to include a longer extension period of 1 minute, and 28 cycles initially then 17 cycles.

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Number of cycles (1st/2nd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30 sec</td>
<td>28/17</td>
</tr>
<tr>
<td>Annealing</td>
<td>55</td>
<td>30 sec</td>
<td>28/17</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1 min</td>
<td>28/17</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10 min</td>
<td>1</td>
</tr>
</tbody>
</table>

The resulting Qubit readings were positive for all samples including the control samples.

A one-step protocol was then considered using the same reaction mixture but with 40 µl of AMPure beads for the clean-up and 33 cycles.

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>3 min</td>
<td>33</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30 sec</td>
<td>33</td>
</tr>
<tr>
<td>Annealing</td>
<td>55</td>
<td>30 sec</td>
<td>33</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30 sec</td>
<td>33</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10 min</td>
<td>33</td>
</tr>
</tbody>
</table>

The Qubit readings were limited to 3 samples as some samples evaporated during storage at 4 °C. These were positive for all samples including one control sample. Keeping to the 1-step protocol and the same reaction mixture, the experiment was repeated but the conditions were altered to include 30 cycles of denaturation for 15 seconds, annealing at 54°C and extension for 90 seconds.
<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>3 min</td>
<td>30</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 sec</td>
<td>30</td>
</tr>
<tr>
<td>Annealing</td>
<td>54</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>90 sec</td>
<td>30</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10 min</td>
<td>30</td>
</tr>
</tbody>
</table>

This resulted in all samples being positive still but more appropriate Qubit readings. It became apparent that increasing the number of cycles would not produce the desired results thus the conditions were altered slightly to include denaturation for 30 seconds and annealing at 50°C.

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>3 min</td>
<td>30</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>Annealing</td>
<td>50</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>90 sec</td>
<td>30</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10 min</td>
<td>30</td>
</tr>
</tbody>
</table>

Qubit readings following amplification of bacterial DNA with 16S real-time PCR are summarised in Table 3.4. As the Qubit readings of the final protocol were acceptable, with the lavage samples being positive and the control samples being negative, this was adopted as the final protocol. All the duodenal lavages and mucosal biopsies were thus amplified using this protocol.
### Table 3.4: Qubit Readings of Samples following Bacterial Amplification with Varying PCR Conditions

<table>
<thead>
<tr>
<th>Samples</th>
<th>Qubit reading of 5 µl of sample for the following conditions (ng/µl)</th>
<th>Final protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-step, 25 cycles then 15 cycles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-step, 25 cycles then 22 cycles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-step, 28 cycles then 12 cycles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-step, 28 cycles then 17 cycles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-step, 33 cycles</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-step, 30 cycles</td>
<td></td>
</tr>
<tr>
<td>Scope 1</td>
<td>&lt;0.5 3.5 &lt;0.5 0.504 NA</td>
<td>0.0256 0.0302</td>
</tr>
<tr>
<td>Extraction control 1</td>
<td>&lt;0.5 3.2 &lt;0.5 2.26 NA</td>
<td>0.0264 0.0324</td>
</tr>
<tr>
<td>Extraction control 3</td>
<td>&lt;0.5 2.36 &lt;0.5 0.358 0.178 0.0356 0.0328</td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>0.2 13.4 0.216 4.48 1.58 0.177 0.212</td>
<td></td>
</tr>
<tr>
<td>A25</td>
<td>0.942 20 4.58 14.7 18.5 11.6 12.5</td>
<td></td>
</tr>
<tr>
<td>A18</td>
<td>&lt;0.5 3.36 &lt;0.5 1.32 NA</td>
<td>0.032 0.0944</td>
</tr>
<tr>
<td>A12</td>
<td>&lt;0.5 &lt;0.5 &lt;0.5 6.16 NA</td>
<td>0.0388 0.0676</td>
</tr>
<tr>
<td>B3</td>
<td>&lt;0.5 3.52 &lt;0.5 0.378 NA</td>
<td>0.036 0.0256</td>
</tr>
</tbody>
</table>
3.3 Materials and Methods

Following the experiments described in Section 3.2, the protocols detailed in this section were selected to achieve the best possible results.

3.3.1 Laboratory Safety and Quality Control

All laboratory techniques were carried out in accordance with good laboratory practice, ICH health and safety protocols, approved risk assessments and Control of Substances Hazardous to Health (COSHH) regulations. Materials were sterilised as required in an autoclave at a temperature of 121°C at 210 kPa above atmospheric pressure (101 kPa) for 15 minutes. Equipment and materials used for aliquoting clinical samples for microbiota analyses and for setting up a polymerase chain reaction (PCR) were exposed to UV-light 254 nm for 30 minutes.

3.3.2 Sample Collection

3.3.2.1 Collection of Gastrointestinal Mucosal Biopsies and Duodenal Lavages

Biopsies were obtained from the duodenum, terminal ileum, right colon and left colon during endoscopic mucosal assessment. A maximum of 6 biopsies were taken from a patient at any one time. The endoscope and forceps used varied depending on the weight of the patient. A sterile, single-us forceps was used for each endoscopic procedure. Using a sterile needle each time, biopsies were transferred from the forceps into a sterile universal bottle.

Duodenal lavages were collected during upper endoscopy to identify the luminal microbiota of the upper gut. Duodenal lavage was performed with 10 ml sterile water, which was suctioned into a mucous extractor (Vygon, UK) then transferred into a sterile universal bottle using a sterile needle and syringe. Samples were then transferred immediately to the laboratory and temporarily stored at 4°C.
3.3.2.2 Collection of Stool Samples

Stool samples were requested from patients prior to their hospital admission. For newly-diagnosed IBD patients, a stool sample before commencing bowel preparation for endoscopy was requested. Patients in hospital were offered a bedpan or foil bowl to aid collection. An aliquot of stool was then transferred into a sterile stool bottle using the spatula attached to the lid. The sample was then stored at 4°C until transferred to the laboratory, then stored at 4°C until weighed.

Patients being followed up and coming from home were given a stool collection kit consisting of a padded envelope, a stool bottle, gloves, icepack, FecesCatcher (Tag Hemi, The Netherlands) or foil bowl and instructions. Stool samples were produced prior to the hospital visit and kept chilled at home at 4°C. The chilled stool sample was brought to the next hospital visit, and the kit was replaced each time.

On arrival at the hospital, the sample was given to a member of the medical team. The sample was then stored in the ward fridge until being transferred to the laboratory, which takes no more than 10 minutes. Once in the laboratory, samples were stored at 4°C until weighed.

3.3.3 Storage of Sample

The laboratory hood and equipment were cleaned and irradiated with UV-light 254 nm for 30 minutes before samples were weighed. If multiple samples were obtained, samples with least bacterial load were handled first i.e. duodenal lavages, mucosal biopsies and stool samples in that order. When samples from different patients were received on the same day, the laboratory hood and equipment were cleaned and irradiated between samples. All samples were stored using a laboratory code to protect patient identity.

3.3.3.1 Storage of Stool Samples

In the clean and irradiated hood, 250-350 mg of homogenised stool was transferred into a 2 ml Eppendorf tube. The sample was briefly centrifuged to ensure that all of the sample was at the bottom of the tube before storage at -80°C.
3.3.3.2 Storage of Biopsies

Using sterile filter tips, each biopsy was placed at the bottom of separate 1.5 ml tubes and weighed. Samples were then stored at -80°C until DNA extraction.

3.3.3.3 Storage of Duodenal Lavages

Initially, the lavages were immediately stored at -80°C then freeze-dried individually (Edwards Modulyo, Thermo Scientific, UK). As there were challenges in gaining access to the freeze-drier and the machine frequently broke down, following discussion with Dr Georgina Hold, subsequent samples were concentrated by centrifugation.

To concentrate the samples, lavages were divided into 2 ml tubes and centrifuged at 10 000 rpm for 20 minutes. The top 1.5 ml was removed and more lavage fluid was added before repeat centrifugation. This process was repeated until all cellular contents of the fluid was concentrated to 200 µl volume. Samples were then stored at -80°C until DNA extraction.

3.3.4 DNA Extraction of Samples

3.3.4.1 DNA Extraction from Stool Samples

DNA extraction from stool samples was performed in batches, depending on the number of samples received using the FastDNA SPIN Kit for Soil (MPBio, UK) (Table 3.5). The recommended protocol was amended as follows;

- For frozen samples, ATL and MT buffers were added immediately and the samples were mixed with vortex intermittently and allowed to defrost in the buffers to avoid bacterial loss during defrosting
- A Tissue Lyser (TissueLyser LT, Qiagen, The Netherlands) was used at 50 osc/min for 1 minute to mechanically break down the cell wall
- Centrifugation was performed at 13 300 rpm
- After adding 100 µl DES, the final centrifugation was performed for 2 minutes to increase the volume of DNA collected

Sodium Phosphate and MT buffers were added to the frozen stool samples immediately after removal from -80 °C. The Lysing Matrix E beads (MPBio, UK) were then added.
Samples were vortexed intermittently until defrosted and thoroughly mixed. Negative control samples were included for each extraction run.

Samples were mechanically broken down with a Tissue Lyser at 50 oscillations/minute over 1 minute then centrifuged at 13 300 rpm for 10 minutes to separate the supernatants from the pellets. The supernatants were then transferred into sterile 2 ml tubes and 250 µl PPS added before mixing gently by hand, then centrifuged at 13 300 rpm for 5 minutes.

The supernatants were then transferred into sterile 15 ml tubes and 1 ml of Binding Matrix was added. These were mixed gently by inverting by hand for 2 minutes then placed on a rack for 3 minutes. Subsequently, 500 µl of the supernatants were discarded and the remaining solution re-suspended.

The Binding Matrix solutions were transferred into SPIN Filters then centrifuged at 13 300 rpm for 1 minute. The catch tubes were emptied and remaining Binding Matrix solutions were added into the SPIN Filters and centrifuged until all of the solutions had been through the SPIN Filter.

The remaining Binding Matrix in the SPIN Filters were re-suspended in 500 µl of SEWSM solution then centrifuged at 13 300 rpm for 1 minute. The catch tubes were emptied and the samples re-centrifuged with the same settings but for 2 minutes.

The Spin Filters were then transferred to a sterile catch tube. The samples were then air-dried for 5 minutes at room temperature then re-suspended in 100 µl DES before being centrifuged at 13 300 rpm for 2 minutes. The filters were then discarded and the pellets were stored in the catch tubes at -80°C.
<table>
<thead>
<tr>
<th>Container</th>
<th>Solution</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 ml eppendorf</td>
<td>978 µl NaPO₄ buffer + 122 µl MT buffer (to stool)</td>
<td>Add Lysing Matrix beads Vortex Tissue Lyser @ 50 osc/min x 1 min Spin @ 13 300 rpm x 10 min</td>
</tr>
<tr>
<td>2 ml eppendorf</td>
<td>(all) supernatant + 250 µl PPS</td>
<td>Mix by hand x 10 Spin @ 13 300 rpm x 5 min</td>
</tr>
<tr>
<td>15 ml falcon</td>
<td>(all supernatant) (re-suspend Binding Matrix) + 1 ml Binding Matrix</td>
<td>Invert by hand x 2 min Place on rack x 3 min Remove &amp; discard 500 µl supernatant Re-suspend Binding Matrix</td>
</tr>
<tr>
<td>SPIN Filter</td>
<td>600 µl Binding Matrix solution Add remaining mixture + 500 µl SEWS-M solution</td>
<td>Spin @ 13 300 rpm x 1min Empty catch tube Spin @ 13 300 rpm x 1min Empty catch tube Gently re-suspend Binding Matrix with SEWS-M solution Spin @ 13 300 rpm x 1min Empty catch tube &amp; replace Spin @ 13 300 rpm x 2min</td>
</tr>
<tr>
<td>SPIN Filter - Transfer to clean catch tube</td>
<td>100 µl DES</td>
<td>Air-dry filter x 5 min Re-suspend Binding Matrix with DES Spin @ 13 300 rpm x 2min Discard filter Store in catch tube @ -80°C</td>
</tr>
</tbody>
</table>

Table 3. 5: DNA Extraction Protocol for Stool Samples using FastDNA SPIN Kit for Soil (MPBio, UK)
3.3.4.2 DNA Extraction from Mucosal Biopsies

QIAamp Mini Kit (Qiagen, The Netherlands) was used for DNA extraction from GI mucosal biopsies as per the manufacturer’s protocol with the following alterations (Table 3.6):

- ATL buffer was added to the frozen mucosal samples and allowed to equilibrate to room temperature. The Lysing Matrix B (MPBio, UK) was then added.
- An additional 10 µl (thus total of 30 µl) of Proteinase K was added for the initial lysis period as per Thomson et al. PLOSone 2011 (622).
- Samples were first incubated with Proteinase K, ATL and AE buffers in a dry bath (Fisher Scientific, USA) at 56°C for 17 hours.

Firstly, 180 µl of ATL buffer and 30µl of Proteinase K were added to frozen biopsy samples, vortexed and then incubated at 56°C in a dry bath for 17 hours until liquefied. Negative control samples of 200 µl AE buffer (+ 30 µl Proteinase K + ATL 180 µl) were included for each extraction run.

Following digestion, samples were incubated at 95°C for 5 minutes before adding 200 µl AL buffer, mixing by pulse-vortex and incubating further at 95°C for 10 minutes. For mechanical breakdown of the samples, 1/6 of a vial of Lysing Matrix B beads (MPBio) was added to the each sample and ribolysed in a tissue lyser (TissueLyser LT, Qiagen, The Netherlands) at 50 oscillations/ minute for 1 minute.

Subsequently, 200 µl of ethanol were added to the solution and mixed by inverting. The samples were then transferred to a column and the samples centrifuged at 8 000 rpm for 1 minute. The collection tubes were discarded and replaced with fresh collection tubes. Then, 500 µl of AW1 buffer were added the samples then centrifuge at 8,000 rpm for 1 minute. The collection tubes were replaced again and 500 µl of AW2 buffer were added before being centrifuged at 13 000 rpm for 3 minutes.

The columns were then transferred to sterile 1.5 ml tubes and 200 µl of AE buffer were added before the final centrifugation at 8 000 rom for 1 minute. The columns were discarded and the pellets re-suspended and divided into 2 aliquots of 100 µl before storage at -80°C.
<table>
<thead>
<tr>
<th>Container</th>
<th>Solution</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 ml ependorph (Control)</td>
<td>200 µl Buffer AE as negative control + 180 µl Buffer ATL + 30 µl proteinase K</td>
<td>Vortex to mix Incubate at 56°C with tissue samples</td>
</tr>
<tr>
<td>In 1.5 ml ependorph containing frozen tissue</td>
<td>180 µl Buffer ATL (to tissue) 30 µl Proteinase K</td>
<td>Vortex to mix Incubate at 56°C x 17 hours</td>
</tr>
<tr>
<td>To samples and control</td>
<td>(remove from heat block, increase heat block temp to 95°C, then replace samples)</td>
<td>Incubate at 95°C for 5 minutes Spin @ 8 000 rpm x 1 min to remove drops from inside the lid</td>
</tr>
<tr>
<td></td>
<td>Add 200 µl of Buffer AL.</td>
<td>Pulse-vortex for 15 seconds Incubate at 95°C for a further 10 minutes Spin @ 8 000 rpm x 1 min</td>
</tr>
<tr>
<td></td>
<td>Add 1/6 of blue Ribolyser resin (Lysing Matrix B)</td>
<td>Ribolyse at 50 osc/min x 1 min Centrifuge briefly at 8,000 rpm</td>
</tr>
<tr>
<td></td>
<td>Add 200 µl of ethanol</td>
<td>Mix by inverting Spin @ 8 000 rpm x 1 min</td>
</tr>
<tr>
<td>Column</td>
<td>(all) solution</td>
<td>Spin @ 8 000 rpm x 1 min Repeat at higher speed if not all solution has passed through</td>
</tr>
<tr>
<td>(discard collection</td>
<td>500 µl Buffer AW1</td>
<td>Spin @ 8 000 rpm x 1 min</td>
</tr>
<tr>
<td>tube, transfer column to</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New collection tube</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(discard collection</td>
<td>500 µl Buffer AW2</td>
<td>Spin @13 300 rpm x 3 min</td>
</tr>
<tr>
<td>tube, transfer column to</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New collection tube</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(transfer column to)</td>
<td>None</td>
<td>Spin @13 300 rpm x 1 min</td>
</tr>
<tr>
<td>New collection tube</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(transfer column to)</td>
<td>200 µl Buffer AE</td>
<td>Incubate at room temperature for 5 minutes. Spin @ 8 000 rpm x 1 min Discard column Store in 2-3 aliquots in 1.5 ml ependorphs at -80°C</td>
</tr>
</tbody>
</table>

Table 3. 6: DNA Extraction Protocol for GI Mucosal Biopsies using QIAamp Mini Kit (Qiagen, The Netherlands)
3.3.4.3 DNA Extraction from Duodenal Lavages

QIAamp Mini Kit (Qiagen, The Netherlands) was used for bacterial DNA extraction from duodenal lavage samples as per the manufacturer’s protocol with the following alterations (Table 3.7):

- AL buffer was added to the frozen samples and allowed to equilibrate to room temperature before adding the Lysing Matrix B (MPBio, UK).
- After adding 500 µl of AW2 solution, the samples were centrifuged at 13 300 rpm for 3 minutes.

Duodenal lavage samples were initially freeze-dried but subsequent samples were concentrated by centrifugation (Section 3.3.2.3). To maintain the same standard, prior to DNA extraction, samples were prepared as follows;

- To frozen lavage samples of 200 µl, 200 µl AL buffer was added.
- To frozen samples of less than 200 µl, 200 µl AL buffer was added then PCR grade water was added to make up a total of 400 µl.
- To frozen samples that had mostly been freeze-dried, 200 µl AL buffer and 200 µl PCR grade water were added and the freeze-dried material resuspended.

To these samples, 20 µl of Proteinase K and 1/6 vial of Lysing Matrix B were added and mixed by pulse-vortex for 15 seconds before incubating at 56°C for 10 minutes. Negative control samples of 200 µl AE buffer (+ 30 µl Proteinase K + AL 200 µl) were included for each extraction run. Samples were ribolysed at 50 oscillations/ minute for 1 minute and briefly centrifuged.

Following this, 200 µl of ethanol were added to the solution then mixed by inverting. The solutions were then transferred to a column and the samples centrifuged at 8 000 rpm for 1 minute. The collection tubes were discarded and replaced with fresh collection tubes. Subsequently, 500 µl of AW1 buffer were added the samples then centrifuge at 8,000 rpm for 1 minute. The collection tubes were replaced again and 500 µl of AW2 buffer were added before being centrifuged at 13 000 rpm for 3 minutes.

The columns were then transferred to sterile 1.5 ml tubes and 200 µl of AE buffer were added before the final centrifugation at 8 000 rom for 1 minute. The columns were discarded and the pellets re-suspended and divided into 2 aliquots of 100 µl before storage at -80°C.
<table>
<thead>
<tr>
<th>Container</th>
<th>Solution</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 ml ependorph (Control)</td>
<td>200 µl Buffer AE as negative control + 200 µl Buffer AL + 20 µl proteinase K + 1/6 vial of blue Ribolyser resin (Lysing Matrix B)</td>
<td>Vortex x 15sec Incubate at 56°C with lavage samples</td>
</tr>
<tr>
<td>In 1.5 ml ependorph containing frozen tissue</td>
<td>Re-suspend freeze-dried lavage in 200 µl AL buffer + 200 µl AE + 20 µl Proteinase K + 1/6 vial of blue Ribolyser resin (Lysing Matrix B) Batches of 5-6 out of freezer or use icebox. Max 11 samples/extraction</td>
<td>Pulse vortex x15 sec Incubate at 56°C x 10min</td>
</tr>
<tr>
<td>To samples and control</td>
<td>(remove from heat block) Add 200 µl of ethanol</td>
<td>Ribolyse at 50 osc/min x 1 min Spin @ 8 000 rpm x 1 min to remove drops from inside the lid Mix by inverting x10 Spin at 8,000 rpm x 1 min</td>
</tr>
<tr>
<td>Column</td>
<td>(all) supernatant</td>
<td>Spin @ 8 000 rpm x 1 min Repeat at higher speed if not all solution has passed through</td>
</tr>
<tr>
<td>(discard collection tube, transfer column to) New collection tube</td>
<td>500 µl Buffer AW1</td>
<td>Spin @ 8 000 rpm x 1 min</td>
</tr>
<tr>
<td>(discard collection tube, transfer column to) New collection tube</td>
<td>500 µl Buffer AW2</td>
<td>Spin @13 000 rpm x 3 min</td>
</tr>
<tr>
<td>(transfer column to) 1.5 ml ependorph</td>
<td>200 µl Buffer AE</td>
<td>Spin @ 8 000 rpm x 1 min Discard column Store in 2-3 aliquots in 1.5 ml ependorphs at -80°C</td>
</tr>
</tbody>
</table>

*Table 3. 7: DNA Extraction Protocol for Duodenal Lavage using QIAamp Mini Kit (Qiagen, The Netherlands)*
3.3.5 DNA Quantification

3.3.5.1 Bacterial DNA Quantification by 16S Quantitative real-time PCR

Absolute quantification of bacterial load was performed first to identify samples that may contain much lower or higher DNA load which may result in template inhibition. This was performed in dilutions of 1:1000, 1:100, 1:10 and neat using Power SYBR Green to amplify the V5 and 6 regions of the 16S rDNA. The total volume of 25 µl mastermix was prepared as follows;

<table>
<thead>
<tr>
<th>Master Mix Components</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>1</td>
</tr>
<tr>
<td>PCR grade water (Bioline, UK)</td>
<td>10.5</td>
</tr>
<tr>
<td>Power SYBR Green master mix (Life Technologies, USA)</td>
<td>12.5</td>
</tr>
<tr>
<td>785F (5' GGATTAGATACCCBRGTAGTC, 20pM)</td>
<td>0.5</td>
</tr>
<tr>
<td>1175R (5' ACGTCRTCCCCCDCCTCCTC, 20pM)</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

The cycling conditions were as follows;

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 sec</td>
<td>40</td>
</tr>
<tr>
<td>Annealing and extension</td>
<td>60</td>
<td>1 min</td>
<td>40</td>
</tr>
<tr>
<td>Dissociation</td>
<td>95</td>
<td>15 sec</td>
<td>1</td>
</tr>
<tr>
<td>Dissociation</td>
<td>60</td>
<td>1 min</td>
<td>1</td>
</tr>
<tr>
<td>Dissociation</td>
<td>95</td>
<td>15 sec</td>
<td>1</td>
</tr>
</tbody>
</table>

The results were analysed using 7500 Fast System. Samples with cycle thresholds between 15-20 cycles were considered optimum as higher cycle thresholds were likely to cause template inhibition.

3.3.5.2 DNA Quantification using Qubit dsDNA HS Assay

DNA was quantified at several stages to assess the quality of the extracted or amplified DNA samples. This was performed using the Qubit dsDNA HS Assay (Invitrogen Life
Technologies, USA) as per the manufacturer’s instructions. It was used to detect DNA in 5 μl of DNA extraction control samples.

Firstly, the working solution was made by diluting the Qubit dsDNA HS reagent 1:200 with Qubit dsDNA HS buffer solution in a sterile tube. Using the working solution, 1-5 μl of samples were added to 195-199 μl working solution to make up to a total of 200 μl. Standards 1 and 2 were prepared for each batch of samples by adding 10 μl of each standard to separate tubes containing 190 μl working solution.

Samples were mixed by vortex for 2-3 seconds then incubated at room temperature for 2 minutes before being loaded onto the Qubit Fluorometer (Qubit 2.0, Invitrogen Life Technologies, USA). An absorbance reading was given and used to calculate the concentration of DNA in the original suspension.

Sample concentration (ng/ μl) = QF value x \( \frac{(200)}{\text{Volume added to the assay tube}} \)

QF value = the value given by the Qubit 2.0 Fluorometer

### 3.3.6 Library Preparation

#### 3.3.6.1 DNA Amplification with PCR

Extracted DNA was amplified by PCR using V3F and V4R primers with adapters and indexes attached. Each well had a different primer pair to enable reads to be assigned to samples. The 50 μl reaction mix were prepared as follows (see Appendix 3.B for primer sequences):

<table>
<thead>
<tr>
<th>Components</th>
<th>Mix</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Sample</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Taq</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>PCR grade water</td>
<td>36.25</td>
<td></td>
</tr>
<tr>
<td>V3F primer</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>V4R primer</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

The cycling conditions were as follows;
3.3.6.2 Size Selection using AMPure Beads

AMPure beads were allowed to equilibrate at room temperature and resuspended by vortex for 30 seconds. To each sample amplified as above, 35 μl of AMPure beads were added and thoroughly mixed by pipetting at least 10 times. A new pipette tip was used for each sample.

Samples were incubated at room temperature for 5 minutes then placed on the magnetic stand for 2 minutes until supernatants were clear. With the plate on the magnetic stand, the supernatants were carefully removed ensuring that the beads were not re-suspended. With the plate still on the magnetic stand, the beads were washed with 200 μl 80% ethanol twice, with an incubation period of 30 seconds after each wash.

Any residual ethanol was removed using a 20 μl pipette tip then the plate was left to air-dry for 15 minutes. The plate was then removed and the beads were thoroughly re-suspended in 50 μl AE buffer. New pipette tips were used for each sample at each step.

The plate was incubated at room temperature for 2 minutes then incubated again for a further 2 minutes on the magnetic stand until the supernatants were clear. The supernatants were then transferred to a new plate and stored at -80°C.

3.3.6.3 Library Preparation

Subsequently, 1 μl of amplified DNA was quantified using Qubit dsDNA HS Assay as described above and the concentration of each DNA sample was standardised to the lowest concentration measured for each plate. Following this, 5 μl of each adjusted DNA sample were pooled to form the final library.
3.3.6.4 DNA Quantification using Qubit dsDNA HS Assay

The DNA library was quantified using Qubit dsDNA HS Assay with 1 μl of DNA sample.

The library concentration was calculated using the following equation;

\[
\text{Concentration (nM)} = \frac{10^6 \times \text{DNA concentration (ng/μl)}}{649 \times \text{size of amplified PCR fragment}}
\]

3.3.6.5 DNA Quantification using Agilent Bioanalyser and Calculation of Library Concentration

The library was then quantified at 1/20 and 1/50 dilutions using the Agilent Bioanalyser (Agilent, USA) as per manufacturer’s instructions. The final library concentration was calculated using the following equation;

\[
\text{Concentration (nM)} = \frac{10^6 \times \text{DNA concentration (ng/μl) x dilution factor}}{649 \times \text{size of amplified PCR fragment}}
\]

The calculated value should match with the calculated concentration of the neat pooled library.

The pooled library was finally loaded on the MiSeq cartridge (Illumina USA) as per manufacturer's instructions for sequencing.

3.3.7 Sequencing Analysis and Bioinformatics

Sequencing data was processed using open-sourced software Mothur (V1.35.1). A published pipeline (623) was used to assemble forward and reverse reads, and remove any sequences containing ambiguous bases (reads not 100% matched were discarded). Sequences were then trimmed to 460 bp before alignment to reference genomes utilising the Silva Database (V128). Sequence reads were then filtered, removing any chimeras utilising VSEARCH (V2.0.5), non-bacterial lineage and reads with less than 97% similarity.
Subsequently, the sequences were clustered before Operational Taxonomic Unit (OTU) picking and taxonomy assignment of the sample reads. Rarefaction of all remaining samples assisted in determining the threshold for subsampling. Subsampling enables comparison of all samples. The number of reads for control samples (extraction, freeze-dry, centrifuge and endoscope) were taken into consideration. For this study, the threshold, i.e. where the rarefaction curve started to plateau, was identified as 7,000 reads thus all samples were normalised to 7,000 reads. Samples with sequencing reads of <7,000 were discarded and excluded from downstream analysis.

Further analysis and visualisation of the sequencing data was performed using RStudio (V1.0.153). This included analysis of the relative abundance of bacterial species, alpha-diversity ($\alpha$-diversity), a measure of bacterial richness (observed OTUs). In addition, the Shannon index for diversity (624) were analysed using GraphPad Prism (Version 5). Comparison was made between the groups using Mann-Whitney or Kruskal-Wallis and Dunn’s Multiple Comparison tests with $p<0.05$ being significant.
3.4 Results

One of the aims of this study was to define the intestinal microbial composition in IBD, assessing its composition at diagnosis, with disease activity and in response to treatment. To achieve this, luminal and mucosal microbial profiles of control and disease groups were investigated at phyla level.

Samples from 5 HI, 11 HC and 20 IBD patients were collected. In addition, 16 NIC, 2 allergic and 2 CAPS patients were also included (Table 2.2) Samples collected consisted of 115 stool, 145 intestinal mucosal biopsies (36 duodenum, 37 TI, 34 right colon, 36 left colon) and 28 duodenal lavages.

3.4.1. Optimisation of Methodologies

Initial experiments were conducted to ensure optimisation and delivery of reproducible data. Potential issues were taken into consideration.

3.4.1.1 Impact of Storage on Stool Microbial Profiles

This analysis was only possible for stool samples as biopsy and lavage samples were limited. Initial validation of stool sample storage at 4°C and -80°C (compared to fresh, unfrozen samples) was performed using spiked stool and real-time PCR for *S. pneumoniae* and *P. aeruginosa* (Section 3.2.2.3.4). Using 7 patient samples from this study, two aliquots of the same stool sample, of similar weights, were stored at -80°C and extracted at different times to test the effect of storage duration. Using 16S rDNA sequencing, the microbial composition was assessed in samples from Patients A2 (stored for 6 and 12 months), A8 (fresh, unfrozen and 2 months), A9 (3 and 9 months), A11 (3 days and 3 months), B3 (both 2 days) and D2 (both fresh, unfrozen) (Figure 3.13). Importantly, phyla level profiling were similar between the duplicate samples tested, suggesting that storage up to 12 months at -80°C is a viable option. The storage and stool DNA extraction were thus deemed acceptable for the purpose of this study.
Figure 3. 13: Impact of Storage on Stool Microbial Composition

The bacterial profiles of duplicate stool samples from patients (n=7) were compared at phyla level. Stool DNA was extracted from the stool samples using MPBio FastDNA SPIN for Soil (MPBio, UK), amplified and normalised before sequencing with Illumina MiSeq. Sequencing data was processed with Mothur (V1.35.1), subsampled at 7,000 and analysed using RStudio (V1.0153). Relative abundance of each phyla is presented.
3.4.1.2 Concentration of Duodenal Lavages

Lavages obtained during endoscopic procedures varied in volume, usually approximately 5-7 ml. These samples had to be concentrated to a maximum of 200 µl for DNA extraction. As discussed in Section 3.3.2.3, some samples were freeze-dried (n=5), some were centrifuged (n=6) whilst some samples were concentrated by centrifugation and freeze-drying (n=6). After 5 samples with <7,000 sequence reads were discarded, comparison of the bacterial profiles in the remaining duodenal lavages (n=17) was performed at phyla levels (Figure 3.14). The microbial composition suggested individual variations with no pattern to differentiate the methods of concentrating the lavages.

Despite the variations, the microbial profiles were dominated by Bacteroidetes or Proteobacteria, with Firmicutes also making up a significant percentage of the profiles in most patients, irrespective of their diagnosis. Given the challenges of collecting these samples, there are no reports of the microbial composition of duodenal lavages in children for comparison. The duodenal lavages of NIC compared to IBD patients are analysed further in Section 3.4.5.3.

One of the DNA extraction controls for lavage samples was positive. It was possibly contaminated during DNA extraction. This was the control for DNA extraction for samples from Patients A4, A5, A6 and A7. The bacterial profile for this control sample was dominated by Proteobacteria (51%) with Bacteroidetes (15%), Firmicutes (12%) and Actinobacteria (10%) also present. Candidatus Saccharibacteria (1%), Acidobacteria (1%) and Verrucomicrobia (1%) were also detected. The lavage profiles were highly variable between individuals, thus it was not possible to be certain that the patient samples extracted with this extraction control were contaminated. As a results, these samples were excluded from further analysis.

A. Freeze-dried

B. Centrifugation
C. Combination of Both Methods

![Figure 3.14: Comparison of Methods for Reduction of Duodenal Lavages](image)

Duodenal lavages (n=19) were concentrated for DNA extraction by freeze-drying (n=4) (A), centrifugation (n=6) (B) or a combination of both methods (n=6) (C). DNA was extracted using the QIAamp Mini Kit (Qiagen), amplified and normalised before sequencing with Illumina MiSeq. Sequencing data was processed with Mothur (V1.35.1), subsampled at 7,000 and analysed using RStudio (V1.0153). The relative abundances of samples are presented depending on the method of concentrating the samples.

3.4.1.2 Rarefaction and Subsampling of the Sequence Reads

After sequence reads were filtered, assigned OTUs and rarefied, subsampling was conducted to enable comparison of all samples. The number of reads for control samples (extraction, freeze-dry, centrifuge and endoscope) were taken into consideration. For this study, the threshold, i.e. where the rarefaction curve starts to plateau, was identified as 7,000 reads. Following these steps, 110 samples, including control samples, were discarded from further analysis.

A total of 9 stool samples (total n=115), 5 duodenal lavages (total n=28) and 48 biopsies (total n=145) were discarded due to sample failure or low sequence reads (<7,000). Possible explanations include laboratory error (1 sample), small stool aliquot and interference by chimeras. In addition, there were samples with low relative abundance from infants, patients on antibiotics, ileostomy and duodenal biopsies.
3.4.1.3 No Differences in the Number of Sequence Reads with Inflammation

As a result of normalisation during the DNA library preparation, the number of sequence reads were generally between 7,000 and 50,000 in the stool and mucosal samples. The number of sequence reads between HC (n=10), HI (n=5), allergic infants (n=2), NIC (n=12), IBD patients with inflammation (n=17) and without inflammation (n=1) were compared (Figure 3.15A). Higher number of reads were observed in stool samples of a HI with a family history of Coeliac Disease and an IBD patient with inflammation, who was also diagnosed with Coeliac Disease.

Mucosal biopsy samples were received from NIC and IBD patients. After excluding the samples with sequence reads of <7,000, which included 34 duodenal biopsy samples, the number of reads for samples with normal histology were compared to those with histological inflammation (TI: normal n=22, inflamed n=5; RC: normal n=11, inflamed n=17; LC: normal n=10, inflamed n=16) (Figures 3.15B, C, D). There were no differences observed between non-inflamed and inflamed biopsy samples, although there were greater variations in the inflamed colonic samples. Higher sequence reads in the colonic samples were observed in a NIC/IBS (Patient A10), severe CD (RC only), severe UC and VEOIBD (LC only) patients. The NIC patient was diagnosed with IBS, and found to have minor histological changes in the RC of unknown significance.
Figure 3. 15: Number of Sequence Reads with Inflammation

The number of sequence reads for stool (A), and mucosal biopsies from terminal ileum (B), right colon (C) and left colon (D), in the presence or absence of inflammation, were compared. Stool samples from HC (n=10), HI (n=5), allergic infants (n=2), NIC (n=12), IBD with inflammation (n=17) and IBD without inflammation (n=1). Mucosal biopsy samples received from NIC and IBD patients were from the terminal ileum (histologically normal n=22, with inflammation n=5), right colon (histologically normal n=11, with inflammation n=17) and left colon (histologically normal n=10, with inflammation n=16). Data is presented as the number of sequence reads.

3.4.1.5 Conclusion from Optimisation of Methodologies

Variations in methodologies were unavoidable but kept to a minimum. Differences such as duration of storage for the stool samples appeared not to affect the results. In addition, the duodenal lavages were concentrated using 2 methods i.e. freeze-drying and centrifugation, but there were no obvious differences detected due to this. The samples were normalised based on Qubit readings thus the number of sequence reads for the groups were similar with no significant differences seen with inflammation. This will enable comparison of relative abundances between the samples. The methods were thus deemed suitable for the study.
3.4.2 Stool Bacterial Profiles of the Control Groups

3.4.2.1 Stool Bacterial Profiles in Healthy Infants were Variable

Stool microbiota of children under 3 years of age are known to vary significantly before stabilising to resemble adult profiles (166). To assess if this was replicable in this study, stool samples from infants \[n=7, \text{median age 8 (7-9) months}\] were assessed. After 2 samples with <7,000 sequence reads were discarded, 5 HI samples were analysed. Demographics and factors influencing intestinal microbiota are detailed for each sample. None of the infants were exposed to antibiotics, whether it was during the antenatal, perinatal or postnatal.

Indeed the stool bacterial profiles of the 5 HI varied significantly, with Bacteroidetes \[\text{median 69 (0-91)%}\] or Firmicutes \[\text{median 76 (8-93)%}\] dominating but both being the dominant phyla (Figure 3.16A). Proteobacteria was present in all infants \[\text{median 4 (0-12)%}\], with the highest proportion seen in F3, who was a 9-month old girl, born by Caesarean section and predominantly formula-fed. In addition, Actinobacteria was detected in all infants \[\text{median 3 (0-12)%}\], with the most abundant being in the older infants F3, F6 and F8, who were 9 months at the time of sampling.

3.4.2.2 Stool Bacterial Profiles of Healthy Controls were less Variable

The stool bacterial profiles of HI and HC were compared (Figure 3.16B). The demographics for the HC accompany each sample, with additional factors detailed in Appendix 3.B. The bacterial profiles of HC stool were less variable than those of HI. Bacteroidetes \[\text{median 40 (22-79)%}\] and Firmicutes \[\text{median 51 (20-74)%}\] dominated the profiles. Proteobacteria was detected in most HC \[\text{median 2 (1-13)%}\] with the most abundant being in H7, who has a first-degree relative with CD. Similarly, Actinobacteria was detected in some HC \[\text{median 1 (0-2)%}\], with the most abundant being in H3, H4 and H9, all of whom live in rural locations. Verrucomicrobia was only present in H3, H8 and H10 \[\text{median 8 (3-15)%}\], with no clear explanation for this. Despite the variations in childhood exposures, the stool bacterial profiles at phyla level were similar within the group, even for H1, who had the most number of antibiotics courses, and H9, who had prophylactic Trimethoprim for a few months.
**A. Healthy Infants**

<table>
<thead>
<tr>
<th>Phylum</th>
<th>F1</th>
<th>F3</th>
<th>F5</th>
<th>F6</th>
<th>F8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>69%</td>
<td>0%</td>
<td>82%</td>
<td>42%</td>
<td>9%</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>24%</td>
<td>76%</td>
<td>16%</td>
<td>44%</td>
<td>81%</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>4%</td>
<td>12%</td>
<td>1%</td>
<td>2%</td>
<td>4%</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>3%</td>
<td>12%</td>
<td>1%</td>
<td>12%</td>
<td>7%</td>
</tr>
<tr>
<td>Alpha Diversity</td>
<td>2.47</td>
<td>2.39</td>
<td>1.84</td>
<td>2.86</td>
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B. Healthy Controls

The bacterial profiles of stool from HI (n=5) (A) and HC (n=10) (B) were analysed for variations in bacterial phyla. DNA was extracted using the MPBio FastDNA SPIN kit for Soil (MPBio, UK), amplified and normalised before sequencing with Illumina MiSeq. Sequencing data was processed with Mothur (V1.35.1), subsampled at 7,000 and analysed using RStudio (V1.0153). Data is presented of bacterial phyla with an accompanying table detailing the alpha diversity, Bacteroidetes:Proteobacteria (B:P) ratios and factors influencing intestinal microbiota.

(TMP – Trimethoprim, IA – inadequate, A - adequate)
**Comparison of Stool Alpha Diversity in Healthy Infants and Children**

Alpha diversity is a measure of species richness within a particular sample. More recently, different calculations have been utilised, incorporating different factors to better describe the population. These differing indices are discussed.

Observed species is a count of the different OTUs in a given sample. Chao1 is the number of observed taxa (richness) and incorporates the unknown number of species not observed, thus may be better for samples with rare OTUs. Similarly, abundance-based coverage estimators (ACE) also incorporates rare taxa. The Shannon index includes the richness and takes the evenness of the species distribution into account. The Simpson index also takes the number of OTUs and relative abundance of each OTU, giving weight to common or dominant species. The inverse Simpson is similar to Simpsons and sometimes preferred as it increases as diversity increase. The Fisher index is often used for incomplete samples as it is independent of sample size and a logarithmic distribution of species (625). Thus in summary, Chao1 is often used as a measure of richness, the Shannon index as a measure of evenness, the Simpson index as a dominance index and inverse Simpson as a measure of diversity.

Given the wide variety of species in infants, Chao1 index may be a better index for measuring alpha diversity in this group whilst the Shannon index may be better for HC (Figure 3.17A). In dysbiotic individuals, the Chao1, Simpson or inverse Simpson may be preferable. As many publications on intestinal microbiota report Shannon indices as a measure of alpha diversity, and for consistency within the study, Shannon indices were used for all samples in this study. Comparison of the Shannon indices between HC and HI revealed higher alpha diversity in HC [n=10, median 3.34 (2.52-4.19)] compared to HI [n=5, median 2.36 (1.84-2.86), p=0.0027](Figure 3.17B).

Beta diversity indicates the differences in species composition between individuals or groups. With small sample sizes, beta diversity of GI microbiota can simply reflect individual clustering and not in groups. Using a non-metric multidimensional scaling (NMDS), HC was seen to cluster together but a wide variation in alpha diversity was seen in HI, thus greater beta diversity between the infants (Figure 3.17C).
Figure 3. 17: Alpha Diversity of Stool of Healthy Infants and Healthy Controls

The alpha diversity of stool from HI (n=5) and HC (n=10) were analysed using the different methods available (A). Shannon indices of HI and HC were compared (B) using Mann-Whitney test. The NMDS demonstrates the beta diversity of bacterial composition (C).
3.4.2.3 Stool Bacterial Profiles of Non-inflammatory Control Patients were comparable to Healthy Controls

Following endoscopic assessment, the NIC patients were diagnosed with IBS (Patients A1, A3, A4, A6, A13 and 20) and food allergies (Patient A5). Stool samples were analysed for luminal bacterial content (Figure 3.18A) and compared with HC. Patient A20 received antibiotics (Cefotaxime and Metronidazole) prior to the endoscopic assessment. This is the likely explanation for the stool profile observed, thus was subsequently excluded from analysis.

As with the HC, non-inflammatory control (NIC) patients [n=6, median age 9 (5-14) years] had stool bacterial profiles dominated by Bacteroidetes [median 41 (4-59)%] and/or Firmicutes [median 50 (30-77)%]. Proteobacteria was also present but did not dominate a profile [median 2 (0-6)%]. It was most abundant in Patient A20, who was treated with antibiotics (19%). Actinobacteria was detected in Patients A1, A3, A4 and A13 [median 1 (0-4)%] while Verrucomicrobia was detected in Patient A6 (1%). Comparing the profiles of HC and NIC patients, there were no significant differences observed in the proportions for each phyla between the groups (data not shown).

The alpha diversity for HC [n=10, median 3.34 (2.52-4.19)] was compared to those of NIC [n=6, median 3.50 (2.41-3.95)], excluding Patient A20, who was exposed to antibiotics. The alpha diversity for both groups were comparable (Shannon indices, p=0.7040) (Figure 3.18B), as was the beta diversity as demonstrated by the close clustering of both groups on NMDS (Figure 3.18C).
A. **Bacterial phyla**

B. **Figure 3. 18: Bacterial Profile and Alpha Diversity of Stool of Healthy Controls and Non-inflammatory Controls**

The bacterial profiles of stool (A) from NIC patients (n=7), including those diagnosed with IBS (Patients A1, A3, A4, A6, A13 and A20) and food allergies (Patients A5) were compared with those of HC (n=10). Shannon indices of HC and NIC (n=6) were also compared (B) using Mann-Whitney test. The NMDS demonstrates the beta diversity of bacterial composition in HC and NIC patients (n=6) (C).
3.4.2.4 Summary of Bacterial Profiles in the Control Groups

Consistent with the literature, the results from this study demonstrated variable bacterial profiles in HI. There were less variations in the HC, with Bacteroidetes and Firmicutes dominating the profiles. Dysbiosis in IBS has been reported but the stool profiles of the NIC patients were similar to those of HC, except in the patient who had received antibiotics. This patient was thus excluded from further analysis. Subsequently, stool profiles from HC were used as comparison to IBD patients.

3.4.3 Dysbiosis in Inflammatory Bowel Disease

This study investigated the intestinal luminal and mucosal microbiota profiles. Stool, representing colonic luminal content, and duodenal lavages, representing proximal small bowel luminal content, were compared to mucosal biopsies.

3.4.3.1 Comparison of Stool Profiles between Healthy Children and IBD

The stool microbial profiles of HC (n=10) were compared to CD (n=10) and UC (n=3) patients in active disease (treatment-naïve and relapsed) and in remission.

The treatment-naïve and relapsed patients were combined and presented as active CD (n=8), and compared to CD in remission (n=6) (Figure 3.19A) and HC. In treatment-naïve CD, Bacteroidetes dominated [n=5, median 64 (59-75)%]. Firmicutes [median 25 (11-33)]% and Proteobacteria [median 7 (5-15)%] were also present. Actinobacteria was detected in 1 patient (A14)(Appendix 3B). Similarly, most CD patients in relapse were found to have Bacteroidetes dominating the profiles [n=4, median 57 (0-91)%], although Firmicutes [median 18 (7-63)%] dominated in 1 patient (A11, discussed in Section 3.4.7.1) Although there were no significant differences in Bacteroidetes, active CD (treatment-naïve and relapsed) had significantly less Firmicutes (p=0.027) and greater Proteobacteria (p=0.044) compared to HC. and Proteobacteria [median 3 (2-80)%] dominated in the patient with Fistulising CD (A25). In remission, both Bacteroidetes [n=6, median 55 (22-80)%] and Firmicutes [median 43 (10-66)%] dominated the profiles. Proteobacteria was present but did not dominate [median 2 (0-11)%].

Due to the small numbers, UC patients (n=3) were analysed depending on whether they were in active disease (treatment-naïve and relapse) and in remission. In active UC,
Firmicutes appeared to dominate the profiles [median 69 (30-73)%] rather than Bacteroidetes [median 25 (0-42)%](Figure 3.19B). Greater proportions of Proteobacteria were also observed [median 27 (5-28)%, p=0.037]. In contrast, in remission (n=2), Bacteroidetes dominated in 1 patient (n=2, 0-58%) while Firmicutes dominated in another (34-98%). Significantly less Proteobacteria was observed in these patients (2% in both).

In addition to CD and UC patients, there were 2 IBDU patients (A9 and D7) in active disease. Similar to UC, the IBDU patients had more abundant Firmicutes (50-92%). Bacteroidetes (1-30%) and Proteobacteria (2-19%) were also present (Appendix 3B).

**Ratios of Bacteroidetes:Proteobacteria and Firmicutes:Proteobacteria in CD and UC**

Proteobacteria is generally increased in IBD, with corresponding reduction in the proportions of Bacteroidetes and/or Firmicutes, reflecting the dynamic alterations occurring within the intestinal lumen. Ratios of the sequence reads of Bacteroidetes:Proteobacteria and Firmicutes:Proteobacteria were therefore analysed in the IBD groups in comparison to HC. Compared to HC [B:P ratio: median 24 (2.5-79); F:P ratio: median 23.8 (4.2-74)], CD patients in active disease and in remission had lower ratios of Bacteroidetes:Proteobacteria [active disease: median 10.8 (0-45.5); remission: median 14.3 (0-40)] and Firmicutes:Proteobacteria [active disease: median 3.8 (0.3-21); remission: median 7.6 (0-53)], with significantly lower Firmicutes:Proteobacteria in active disease (p=0.008).

When comparing the Bacteroidetes:Proteobacteria and Firmicutes:Proteobacteria ratios between HC and UC patients, analysis was restricted due to the small numbers however a trend was observed. The Bacteroidetes:Proteobacteria ratios in UC patients in remission [n=2, 0-29] were lower than HC and lower still in active UC [n=3, median 1.5 (0-5), p=0.063]. Similarly, the Firmicutes:Proteobacteria ratios were lower in UC [active disease: median 2.7 (1.1-13.8); remission: 17-49], with significantly lower ratios in active disease compared to HC (p=0.045).
Reduced Stool Bacterial Diversity in IBD Patients

The alpha diversity utilising Shannon indices of HC \([n=10, \text{median 3.34 (2.52-4.19)}]\) were compared to IBD patients, who were treatment-naïve \([n=12, \text{median 2.37 (0.88-3.73)}]\) and in relapsed active IBD \([n=8, \text{median 2.64 (1.36-3.44)}]\) (Figure 3.19C). In both these IBD groups, alpha diversity was lower compared to HC. Similarly, when compared to NIC \([n=8 \text{ median 3.50 (2.41-3.95)}]\), patients in active IBD had lower alpha diversity (Figure 3.19D). Given that the alpha diversities of HC and NIC were similar, this was an expected finding. Low alpha diversity was also observed when CD and UC groups were analysed separately \([CD n=10, \text{median 2.31 (0.88-2.84)}; \ UC n=3, \text{median 2.33 (2.02-2.44)}]\), with no differences seen between the groups (data not shown).

The beta diversity was then analysed, with the NMDS plot demonstrating the HC clustering together as previously seen but large variations were observed with IBD patients (Figure 3.19E). This illustrates the large deviances in a dysbiotic state.

Thus, IBD patients had stool bacterial profiles that were dysbiotic, with differences observed between active disease and remission at phyla level. In general, this can be defined as significant dominance of Firmicutes or Bacteroidetes, or greater proportion of Proteobacteria compared to HC. More specifically in active CD, there was reduction in Firmicutes associated with the increased Bacteroidetes and Proteobacteria. On the other hand, dysbiosis in active UC involves reduction in Bacteroidetes associated with the increased Proteobacteria. These profiles are associated with reduce alpha diversity compared to HC and NIC, with large variations observed.

**A. Percentages of Bacterial Phyla in CD**

![Bacteroidetes](chart1)

**Bacteroidetes**

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<th>CD Remission</th>
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<td>80</td>
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**Firmicutes**

![Firmicutes](chart2)

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</table>

**Proteobacteria**

![Proteobacteria](chart3)

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<th>CD Remission</th>
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<tbody>
<tr>
<td>Percentage (%)</td>
<td>100</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

\(p=0.027\) \(p=0.044\)
B. Percentages of Bacterial Phyla in UC

![Graphs showing percentages of Bacteroidetes, Firmicutes, and Proteobacteria in UC patient groups.](image)

C. Ratios of Bacteroidetes:Proteobacteria and Firmicutes:Proteobacteria in CD and UC

![Graphs showing ratios of Bacteroidetes:Proteobacteria and Firmicutes:Proteobacteria in CD and UC patient groups.](image)

D. Graphs showing comparisons between HC and other groups.

```
Bacteroidetes
Firmicutes
Proteobacteria

C. Ratios of Bacteroidetes:Proteobacteria and Firmicutes:Proteobacteria in CD and UC

D. Graphs showing comparisons between HC and other groups.
```
3.4.3.2 Mucosal Profiles in CD and UC

3.4.3.2.1 Bacterial Profiles of Small Bowel Mucosal Biopsies

There were 36 duodenal biopsies but 34 samples (sequence reads of <1615) were discarded, leaving 2 samples from Patients A7 (7051 reads) and C3 (11946 reads) for analysis. Bacterial profiles of the duodenal samples were compared to those of the distal small bowel i.e. terminal ileum (Figure 3.20). There were no macroscopic or microscopic inflammation reported at these sites.

In Patient A7, Bacteroidetes (48%) dominated the duodenal profile whilst both Bacteroidetes (51%) and Firmicutes (43%) dominated the TI profile. Firmicutes (23%), Actinobacteria (5%) and Fusobacteria (3%) were also present in the duodenum.
Proteobacteria was more abundant in the duodenum (19%) than the TI (5%), with Verrucomicrobia only detected in the TI (1%).

On the other hand, the bacterial profiles of both small bowel biopsies were similar in Patient C3 (colectomy for VEOIBD). Bacteroidetes (duodenum 28%, TI 25%), Fusobacteria (duodenum 11%, TI 12%) and Firmicutes (duodenum 7%, TI 8%) were present but Proteobacteria (duodenum 53%, TI 54%) dominated in both these biopsy samples. Actinobacteria (1%) was just detectable in the duodenum of Patient C3.

These findings would suggest that the difference in luminal environment, such as oxygen concentration, between the duodenum and TI in Patient A7 may influence the microbial composition. In Patient C3, formation of the ileostomy would increase oxygen concentration in the ileum, which would normally be an anaerobic environment.

![Figure 3.20: Comparison of Small Bowel Mucosal Biopsies](image)

The bacterial profiles of biopsies obtained from the duodenum (D) and terminal ileum (TI) of Patients A7 and C3 were compared. DNA was extracted from biopsies using QIAamp Mini Kit (Qiagen, The Netherlands), amplified and normalised before sequencing with Illumina MiSeq. Sequencing data was processed with Mothur (V1.35.1), subsampled at 7,000 and analysed using RStudio (V1.0153). Bacterial profiles of duodenal and terminal ileal samples are presented in pairs for each patient.

### 3.4.3.2.2 Bacterial Profiles in the Terminal Ileal and Colonic Mucosal Biopsies

Subsequently, bacterial profiles of the distal small bowel mucosal biopsies were compared to those of colonic biopsies from 11 NIC, 6 CD, 3 UC, 1 Collagenous Colitis,
1 IBDU and 1 VEOIBD patients. The NIC patients included those with IBS (Patients A1, A3, A4, A6, A10 and A13), food allergies (Patients A5, A12 and A16) or both (Patients A22 and A23). In addition, there were patients with Leukocytoclastic Vasculitis (Patient A7) and JIA (Patients A19 and A24).

Samples from 2 NIC patients and 1 CD patient with sequence reads of <7,000 were discarded (discussed in Section 3.4.1.3). The 2 patients with colectomy (Patients B3 and C3) were excluded from this analysis. Comparison was first made between the bacterial phyla of the TI, RC and LC biopsies of NIC, CD and UC patients (Figure 3.21A).

**Bacterial Phyla in Mucosal Biopsies**

In NIC patients, Firmicutes [TI n=15 median 31 (0-51)%, RC n=15 median 34 (9-56)%, LC n=15 median 35 (9-60)%] and/or Bacteroidetes [TI median 51 (14-84)%, RC median 55 (34-87)%, LC median 50 (29-87)%) dominated the mucosal bacterial profile, while Proteobacteria was minimally present [TI n=15 median 4 (1-85)%, RC median 6 (1-43), LC median 4 (1-47)%) except in patients A1, A10, A12 and A23. In addition, Verrucomicrobia was also present in some patients (Patients A4, A6 and A19) [n=5 TI median 6 (1-11)%, RC median 6 (0-12)%, LC median 7 (1-8)%].

In CD, all mucosal samples had Proteobacteria, with most having greater proportions compared to NIC [TI median 16 (2-95)%; RC median 23 (2-85)%; LC median 32 (7-92)%, p=0.048]. Proportions of Bacteroidetes [TI n=6 median 46 (0-73)%, RC n=6 median 48 (0-67)%, LC n=5 median 43 (0-49)%) and Firmicutes [TI median 29 (4-49)%, RC median 21 (1-48)%, LC median 23 (0-92)%] in the TI were similar to NIC.

Similarly, in UC, Proteobacteria was present in all patients and generally in greater proportions than in NIC, dominating in some patients [TI n=3 median 23 (13-74)5, RC n=3 median 22 (17-80)%, LC n=3 median 32 (28-66)%]. The relative abundance of Bacteroidetes [TI median 16 (0-46)%; RC median 13 (0-39)%; LC median 23 (1-30)%) appeared to be less than NIC and CD, while Firmicutes was similar in all samples [TI median 41 (9-76) %. RC median 44 (7-76)%, LC median 41 (11-65)%].

Applying the Bacteroidetes:Proteobacteria ratio to the mucosal samples, the ratios for IBD were lower than in NIC, with UC having lower ratios than CD, thereby amplifying the differences between CD and UC in the bacterial composition at phyla level.
Mucosal Microbial Profiles

Subsequently, the mucosal microbial profiles of individual NIC and IBD patients were analysed, with mucosal inflammation indicated by asterisks (Figure 3.21B). Ileal and colonic bacterial profiles of NIC patients (n=13) were similar for each patient, except for Patients A12 and A16, with inter-individual variations observed. In Patient A12, Proteobacteria dominated the TI profile (85%), with absence of Firmicutes, but Bacteroidetes dominated the colonic profiles (TI 14%, right 69%, left 79%). On the other hand, Patient A16 had greater Firmicutes in the LC compared to the TI and RC (TI 26%, RC 20%, LC 46%), with corresponding reduction in Bacteroidetes (TI 67%, RC 65%, LC 46%). In addition, Patients A1 and A10 had significant proportions of Proteobacteria (Patients A1: 39-47%, A10: 26-36%) with reduced Firmicutes in all samples (Patients A1: 10-18%, A10: 17-20%). The proportions of Bacteroidetes were stable and comparable to those of NIC (Patients A1: 38-46%, A10: 44-54%).

In CD (n=6), the mucosal profiles varied between patients. The TI and colonic profiles were similar for individual patients except for 2 treatment-naïve patients (Patients A14 and A15). The proportions of Proteobacteria in some patients were greater than those of NIC, dominating in Patients A15 (RC 84%, LC 76%) and A25 (TI 95%, RC 85%, LC 92%). On the other hand, lower Proteobacteria proportions were observed in Patients A11, A17 and D4. Complete absence of Bacteroidetes was seen in Patient A25 and Firmicutes in the colonic samples from Patient A15. It was interesting to note that inflammation did not appear to alter the colonic mucosal profiles (in Patients A15 and A17).

In UC, Firmicutes or Proteobacteria dominated the mucosal profiles. Bacteroidetes was present in the diagnostic colonic biopsies of Patient A21, who had a less severe disease course, but absent in the pre-treatment samples of Patients A8a and C1a. Although there were no histological inflammation in the TI samples, the TI bacterial profiles were similar to those of the colon.

In other IBD subtypes, dysbiosis was also observed. The TI and colonic profiles were similar in Patient A9 (IBDU), with Proteobacteria dominating (TI 80%, RC 77%, LC 74%). On the other hand, the mucosal profiles of Patient A2 (Collagenous Colitis) and B1 (VEOIBD) were comparable to NIC, with minimal or less Proteobacteria (Patient A2: 1% in all biopsies; B1: 14-15%) and Bacteroidetes dominating (Patient A2: TI 67%, RC 65%, LC 59%; B1: 59-65%).
Reduced Mucosal Bacterial Diversity in IBD Patients

The alpha diversity of mucosal biopsies in NIC (n=7) were compared with IBD patients (n=11) using Shannon indices (Figure 3.21C). Compared to NIC patients [n=7, TI median 2.62 (1.69-3.65); RC median 2.53 (1.76-3.05); LC median 2.76 (1.76-3.73)], treatment-naïve, active IBD patients were found to have lower alpha diversity of the TI [n=10, median 2.31 (0.58-3.56)], right colon [n=10, median 1.72 (0.87-3.59)] and left colon [n=9, median 1.85 (0.94-3.42)]. The NMDS plot demonstrates the greater variability seen in IBD patients compared to NIC.

Similarly, compared to NIC patients, active IBD patients appeared to have lower alpha diversity in both the colonic biopsies [TI n=13 median 2.28 (0.58-3.56); RC n=13 median 1.92 (0.87-3.59); LC n=12 median 2.11 (0.94-3.42)] but no differences were observed in the TI biopsies (p=0.2346).

When comparing the IBD types, CD [TI n=6 median 2.36 (0.58-3.56)] had greater alpha diversity in the TI compared to UC [TI n=3 median 2.1 (1.58-2.28)], while the colonic alpha diversity for both IBD types were similar [CD: RC n=6 median 2.01 (0.87-3.59); LC n=5 median 2.36 (0.94-3.42)] [UC: RC n=3 median 2.09 (1.61-2.35); LC n=3 median 2.23 (1.85-2.5)].
A. Mucosal Bacterial Phyla

Bacterial Phyla in Terminal Ileum

Bacterial Phyla in Right Colon

Bacterial Phyla in Left Colon

Patient Groups and Phyla

Bacteroidetes                     Firmicutes                  Proteobacteria             Verrucomicrobia

Percentage (%)

p=0.048
**Bacteroidetes:Proteobacteria Ratios**

- **Terminal Ileum**
- **Right Colon**
- **Left Colon**

**B. Mucosal Bacterial Profiles of Individual Patients**

*Non-inflammatory Control – Irritable Bowel Syndrome*

- **A1**
- **A3**
- **A4**
- **A6**
- **A10**
- **A13**

Phylum:
- **Actinobacteria**
- **Bacteria_unclassified**
- **Bacteroidetes**
- **Firmicutes**
- **Fusobacteria**
- **Proteobacteria**
- **Synergistes**
- **Verrucomicrobia**
Non-inflammatory Control – Food Allergies

Non-inflammatory Control – Irritable Bowel Syndrome and Food Allergies

Non-inflammatory Control – Rheumatology Disorders
C. Alpha Diversity of Mucosal Biopsies

Treatment-naïve IBD vs. Non-inflammatory Control
Active IBD vs. Non-inflammatory Control

Figure 3. 21: Microbial Profiles of Terminal Ileal and Colonic Mucosal Biopsies in IBD

The mucosal bacterial profiles of the terminal ileum (TI), the right (R) and left (L) colon of NIC (n=14), CD (n=6), UC (n=3), IBDU (n=1), Collagenous Colitis (n=1) and VEOIBD (n=1) patients were compared using Kruskal-Wallis and Dunn’s Multiple Comparison tests (A). Bacterial profiles of ileal and colonic samples at phyla level for individual patients are presented (B) (* indicates histological inflammation). The alpha diversity of mucosal biopsies from NIC (n=7) and IBD patients who were treatment-naïve (n=10) and in active disease (n=13) were analysed. The alpha diversity in CD (n=6) and UC (n=3) patients were also assessed. Analysis was performed using RStudio (V1.0153) and comparisons made using Shannon indices and Mann-Whitney test (p<0.05 being significant).

3.4.3.3 Summary of Dysbiosis in IBD

In active IBD, dysbiosis was apparent with significantly increased stool Proteobacteria compared to HC. In active CD, there was significantly less Firmicutes than HC. There appeared to be less Bacteroidetes in UC but this was not statistically significant. Dysbiosis was not always evident in active disease when the patient was on treatment.
A more dysbiotic profile pre-treatment was associated with more severe disease course. As some of the UC patients improved clinically, Actinobacteria was observed. The stool alpha diversity in active IBD was lower than HC and NIC.

Mucosal dysbiosis in IBD involves dominance of a phylum, with much reduced presence of other phyla. The reduced Bacteroidetes, and thus Bacteroidetes:Proteobacteria ratio, in UC differentiates it from CD. Although dysbiosis was observed in some NIC patients, some CD and all UC patients had more dysbiotic profiles, reflecting the more severe inflammatory state of IBD patients. The TI and colonic profiles were comparable for most patients, with histological inflammation having little effect on the profiles. All biopsy sample types in IBD had lower alpha diversity than NIC, with the increased diversity in the TI of CD patients differentiating it from UC.

3.4.4 Dysbiosis in Severe IBD is more pronounced

To understand the association between intestinal microbiota and disease severity in IBD, patients with severe IBD were also recruited. A brief clinical history to illustrate the disease severity and their profiles are presented (Figure 3.22A).

**Patient C1**

Patient C1 was diagnosed with UC and commenced first line treatment but the disease progressed despite escalating treatment. Colonic resection was being considered 2 months later but in a last bid to avoid this, the patient was commenced on Sirolimus after repeat endoscopic assessment. The mucosal microbial profiles were dominated by Proteobacteria whilst the stool profile was dominated by Firmicutes. Bacteroidetes was absent from all samples.

**Patient C2**

Patient C2 had severe CD, and subsequently went on to have intestinal resection. During the study, the patient had refractory inflammation (CRP 36 mg/dL, ESR 60 mm/hr and calprotectin 2316 µg/g). This sample was obtained 2 months after EEN was stopped and prior to starting Ustekinumab. The stool bacterial profile was significantly dominated by Proteobacteria (80%) with absence of Bacteroidetes. All mucosal biopsy samples had sequence reads <900 thus are unavailable for analysis.
**Patient C5**

Patient C5 was investigated for ongoing symptoms, which were reported to affect daily activities, despite being on Infliximab and Azathioprine. The bacterial profiles for both stool samples were dominated by Bacteroidetes, with Firmicutes also present. The profile at month 14 also included Actinobacteria (3%) and a significant proportion of Proteobacteria (19%). The first sample was obtained prior to endoscopic mucosal assessment, which was reported as normal (mucosal biopsies were not collected for the study). During the study period, the patient received psychology input for functional overlay of IBD symptoms.

**Ileostomy Samples from Patients B3 and C3**

There were 2 patients recruited to the study who had subtotal colectomy. Patient B3 had a colectomy several years prior in a bid to control the intractable panenteric inflammation associated with IL-10RB mutation. Patient C3, who was diagnosed with VEOIBD, had a colectomy during the study period.

The stool profiles for Patient B3 were significantly dominated by Proteobacteria. Following HSCT, Firmicutes began to emerge and increased at month 6 when the patient was clinically improved and tolerated enteral nutrition. The patient remained well in month 9 when Firmicutes decreased and Proteobacteria increased.

Patient C3 was diagnosed with VEOIBD after presenting at age 3 years with bloody diarrhoea and abdominal pain. The disease progressed rapidly despite escalation of conventional treatment. Due to the refractory disease, the patient underwent a colectomy 9 months post-diagnosis. The first stool sample obtained was prior to surgery and subsequent samples were from the ileostomy. Interestingly, the stool profile pre-operatively were similar to the ones obtained via the ileostomy. Proteobacteria and Firmicutes dominated the profiles. Actinobacteria, Candidatus Saccharibacteria and Fusobacteria were detectable, while Bacteroidetes was absent. With clinical improvement, there was marked reduction in Proteobacteria and emergence of Bacteroidetes.

To illustrate the severity of dysbiosis in these patients, the heatmap below highlights the profiles of these patients in comparison to HC and other less severe IBD patients (Figure 3.22B).
Analysing the bacterial profiles of these patients and comparing them to other IBD patients, significant dominance of Proteobacteria and absence of Bacteroidetes emerged as a common finding in Patients C1 and C2. Although the symptoms were reported as severe in Patient C5, the stool bacterial profile was similar to HC and NIC. Being obligate anaerobes, Bacteroidetes was also absent or minimally present in Patients B3 and C3, although its emergence was detected when the patients were clinically improved.

**A. Microbial Profiles**

**Patient C1**

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<td>1.27</td>
</tr>
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<td>1.1</td>
</tr>
<tr>
<td><strong>LC</strong></td>
<td>1.27</td>
<td>1.27</td>
</tr>
</tbody>
</table>

| **PUCAI/wPCDAI** | 70 | 5 | 5 |
| **CRP** | 8 | 36 | 6 |
| **ESR** | 5 | 60 | 6 |
| **Calprotectin** | 162 | 2316 | 515 |

**Patient C2**

**Patient C5**

**Patient B3**

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**Patient C3 - biopsies**

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<td>30</td>
<td>41</td>
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</tbody>
</table>

**Phylum**
- Actinobacteria
- Bacteria_unclassified
- Bacteroidetes
- Firmicutes
- Fusobacteria
- Proteobacteria
- Synergistetes
- Verrucomicrobia

**B. Heatmap of HC and IBD Patients highlighting Patients with severe IBD**
Summary of Dysbiosis in Severe IBD

Patients with severe IBD were observed to have more dysbiotic bacterial profiles dominated by Proteobacteria, which decreased with clinical improvement. In addition, Bacteroidetes was absent from the stool and mucosal samples but emerged as the patients began to improve clinically. A patient who presented with severe symptoms had a less dysbiotic stool bacterial profile, and this was associated with normal mucosal histology.

3.4.5 Luminal and Mucosal Microbial Profiles differ with Inflammation

3.4.5.1 Microbial Profiles of Stool and Colonic Mucosal Biopsies

There are numerous reports of stool microbiota in IBD but the question arises if the luminal profile (i.e. stool) represents that of the mucosa. Given that this is an intestinal mucosal disease, with barrier dysfunction playing a significant role, this is an important issue to clarify. In contrast, NIC patients did not have histological evidence of inflammation. Therefore, the microbial profiles of stool and mucosal biopsies of NIC and IBD patients, which were collected at the same time, were investigated (Figure 3.23).

There were data sets available for 6 NIC, 4 CD and 3 UC (5 data sets) patients in addition to the patient with Collagenous Colitis (Patient A2), IBDU (Patient A9) and VEOIBD (Patients B1 and C3). These abundance plots are presented together for ease of comparison.

In the NIC cohort (n=6), 2 patients (Patients A3 and A5) had similar profiles, while differences were observed in the remaining 4 patients. In Patients A1 and A13, there were significantly greater Proteobacteria proportions on the colonic biopsies (A1: RC 43%, LC 47%; A13: RC 6%, LC 7%) with it just detected in the stool (A1: 2%; A13: 2%).
This was associated with reduction in Firmicutes in Patient A1 and Bacteroidetes in Patient A13. Although Verrucomicrobia was not detected in the stool of Patients A4 and A6, it was present in the colonic biopsies (A4: RC 7%, LC 8%; A6: RC 6%, LC 7%). In contrast, Actinobacteria present in the stool of Patient A6 (4%) was only detected in the biopsies (1%). Thus, stool microbial composition does not always reflect the mucosal composition in NIC patients, with greater mucosal Proteobacteria and Verrucomicrobia detected in some patients.

Similarly, in CD (n=4) and UC (n=3), the stool microbial profile differs from the mucosal profiles. Unlike the NIC patients, all IBD patients harboured Proteobacteria in their stool. In addition, the proportions of Proteobacteria were much greater in the treatment-naïve mucosal samples, and dominated in some patients (Patients A15, A25, A21 and C1). In the patients with Actinobacteria in the stool (Patients A14, A8 and C1), little if any of it was detected in the mucosal samples.

The reassuring presence of Bacteroidetes in the stool may in fact be associated with significant mucosal Proteobacteria. In CD, Bacteroidetes dominance in the stool does not indicate its dominance on the mucosa. Likewise, in treatment-naïve UC, presence of Bacteroidetes in the stool may actually be associated with its absence or minimal presence in the mucosal samples. In the second set of mucosal biopsies in 2 UC patients (Patients A8 and C1), the stool and mucosal profiles were more similar, with comparable proportions of Proteobacteria. In addition, Bacteroidetes was present in the mucosal samples despite these patients being in active disease, suggesting improved bacterial handling with treatment, even if insufficient to prevent disease relapse.

In the less common IBD subtypes, the stool and mucosal profiles in Collagenous Colitis (Patient A2) were similar to NIC patients, with Bacteroidetes (stool 52%, RC 65%, LC 59%) and Firmicutes (stool 46%, RC 33%, LC 40%) dominating the stool and colonic profiles. In addition, Verrucomicrobia present in the stool (2%) was absent in the biopsies. In IBDU (Patient A9), the dysbiosis was similar to that seen in CD and UC, with Proteobacteria dominating the colonic biopsies (RC 77%, LC 74%) despite the lower proportion in stool (19%). Bacteroidetes was present in all samples (stool 30%, RC 18%, LC 20%) with Firmicutes dominating the stool profile (stool 50%, RC 5%, LC 6%).

There were 2 patients with VEOIBD. Pre-HSCT, Patient B1 had similar stool and colonic mucosal profiles dominated by Bacteroidetes (stool 52%, RC 59%, LC 65%). Proteobacteria proportions in the biopsies were less (RC 15%, LC 14%) than in the stool (30%). In addition, Synergistetes was present in similar proportions in all samples (stool
5%, RC 3%, LC 3%). Patient C3 underwent subtotal colectomy thus the profiles of ileostomy effluent was compared to the TI biopsy. Proteobacteria was dominant in both samples (stool 48%, TI 54%) but Bacteroidetes, which was absent in the stool, was present in the biopsy (25%), thus there was a significant presence of Firmicutes in the stool (52%) but not in the biopsy (8%). In addition, Fusobacteria was also present in the biopsy (12%) but undetected in the stool.

Non-inflammatory Control – Irritable Bowel Syndrome

![Graphs showing bacterial distribution](image-url)
**Figure 3.23: Comparison of Stool and Colonic Mucosal Biopsies in NIC and IBD Patients**

The bacterial profiles of stool and mucosal biopsies of the right (R) and left (L) colon of 6 NIC, 3 CD and 3 UC (4 data sets) patients, in addition to patients with Collagenous Colitis, IBDU and VEOIBD (n=2), were analysed. Bacterial profiles at phyla level are presented in sets for each patient. (* indicates histological inflammation).

### 3.4.5.2 Trends of association between Mucosal Bacterial Phyla and Stool Calprotectin

Some stool samples collected were also analysed for calprotectin concentrations, a marker of intestinal inflammation used for clinical assessment and monitoring. Correlation between calprotectin and Bacteroidetes, Firmicutes and Proteobacteria proportions in the TI (n=10)(Figure 3.24A) and left colonic (n=10)(Figure 3.24C) biopsies were analysed in a cohort consisting of NIC (n=4), UC (n=2), CD (n=3) and Collagenous colitis (n=1). For the right colonic biopsies (n=11), the same IBD patients were analysed alongside 5 NIC patients (Figure 3.24B). None of the correlations were statistically significant although negative trends of association with Bacteroidetes and positive trends of associations with Proteobacteria were observed in the colonic biopsies. There were no obvious trends observed with Firmicutes.

**A. TI Mucosal Biopsies**

![Graphs showing correlation between calprotectin and bacterial phyla in TI biopsies](image)

**B. Right Colonic Mucosal Biopsies**

![Graphs showing correlation between calprotectin and bacterial phyla in RC biopsies](image)
C. Left Colonic Mucosal Biopsies

Figure 3.24: Correlation between Mucosal Bacterial Phyla and Calprotectin

Percentages of bacterial phyla in mucosal TI and LC samples were analysed for potential correlation with calprotectin in of NIC (n=4), UC (n=2), CD (n=3) and Collagenous colitis (n=1). Analysis was performed in the same IBD patients and NIC (n=5) for the RC samples. Linear regression was conducted using GraphPad Prism V5 with 95% confidence interval.

3.4.5.3 Microbial Profiles of Duodenal Lavages and Mucosal Biopsies

Duodenal lavages were analysed to investigate the proximal small bowel luminal content. Comparison was made between the NIC (IBS n=5, food allergies n=1, Rheumatology patients n=2) and IBD patients (CD n=6, UC n=3, IBDU n=1, IL-10RB mutation n=1) (Figure 3.25A).

The lavages from NIC patients (n=8) were also variable with Proteobacteria dominating in Patients A16, A20 and A24 (37-77%), Bacteroidetes dominating in Patient A23 (34%) and Firmicutes dominating in Patient 22 (41%). In Patient A12, who was diagnosed with food allergies, the profile was dominated by Bacteroidetes (33%) and Proteobacteria (32%).

The lavage profiles for IBD patients also varied between patients with no obvious differences observed between CD (n=6) and UC (n=3). In these patients, Proteobacteria (7-61%), Bacteroidetes (10-80%) or Firmicutes (1-45%) dominated. In addition, Actinobacteria (1-14%), Verrucomicrobia (13%), Fusobacteria (1-12%), Acidobacteria (17%), SR1 (1%), Candidatus Saccharibacteria (1-2%) and Bacteria Unclassified (1-5%) also detected. The patient with IBDU (Patient A9) had a similar lavage profile. Interestingly, Patient C1 (severe UC) was found to also have SR1 (10%) and Spirochaetes (1%). In addition, the lavage profile of Patient B3 (IL-10RB mutation) was
dominated by Bacteroidetes (38%) and Proteobacteria (34%), with Actinobacteria (6%), Verrucomicrobia (6%), Firmicutes (3%), Acidobacteria (2%), Fusobacteria (1%), SR1 (1%), Candidatus Saccharibacteria (1%) and Bacteria Unclassified (6%) detected.

There were, however, 2 CD patients with histological evidence of duodenal mucosal inflammation, Patients A14 (duodenitis with aphthous ulcers present) and A25 (mild increase in chronic inflammatory cells in the lamina propria). The lavage profile in Patient A14 was dominated by Proteobacteria, with less Bacteroidetes (10%) and Firmicutes (1%) compared to other CD patients. Unusually, there were also higher proportions of Acidobacteria (17%), Actinobacteria (14%) and Verrucomicrobia (13%), with double the alpha diversity compared to NIC and other IBD patients. In Patient A25, Firmicutes dominated the lavage profile (45%), with Proteobacteria (25%) and Bacteroidetes (22%) also present. Actinobacteria (4%), Fusobacteria (2%) and Candidatus Saccharibacteria (1%) were also detected.

Next, duodenal luminal content was compared to the corresponding duodenal mucosal biopsy of Patient A7 (NIC) (Figure 3.25B). This analysis was severely restricted due to the number of duodenal mucosal biopsies with sequence reads of less than 7,000 being discarded. It was thus only possible to analyse the 1 biopsy with its corresponding duodenal lavage. In both samples, Bacteroidetes dominated (lavage: 54%; biopsy 48%), with similar proportions of Firmicutes (lavage: 19%; biopsy 23%) and Proteobacteria (lavage: 17%; biopsy 19%) present. In addition, Fusobacteria (lavage: 6%; biopsy: 3%), Actinobacteria (lavage: 3%; biopsy: 5%) and Candidatus Saccharibacteria (1% in both). In addition, the alpha diversity was the same in both samples (3.48).

A. Non-inflammatory Control

| Alpha Diversity |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 2.23 | 3.13 | 3.74 | 3.29 | 2.72 | 3.62 | 3.07 | 3.65 |
Figure 3.25: Bacterial Profiles of Duodenal Lavages

The bacterial profiles of duodenal lavages in NIC (n=8), CD (n=6), UC (n=3), IBDU (n=1) and IL-10RB mutation (n=1) were analysed. Lavage profiles are presented with the corresponding alpha diversity (* indicate corresponding histological duodenitis). In addition, bacterial profiles of the duodenal lavage and mucosal biopsy in Patient A7 were analysed (B). The relative bacterial abundances are presented at phyla level.
3.4.5.4 Summary of Luminal and Mucosal Bacterial Profiles

In NIC, the bacterial profiles of stool and intestinal mucosa were mostly similar, although greater mucosal abundance of Proteobacteria was observed in 2 patients. In contrast, in IBD, the stool and mucosal bacterial profiles can differ significantly. High abundance of Bacteroidetes can in fact be associated with increased abundance of mucosal Proteobacteria. In addition, there was generally greater abundance of mucosal Proteobacteria while the mucosal Bacteroidetes was less than in the stool. In severe IBD, Bacteroidetes was absent from the mucosal samples despite being present in the stool. Similarly, in Collagenous Colitis, Verrucomicrobia which was present in the stool was not detected in the mucosal samples. Differences were also seen between TI and ileostomy samples. Bacteroidetes and Fusobacteria in the TI samples were absent from the ileostomy sample.

Although not statistically significant, there were trends apparent between stool calprotectin concentrations and the bacterial phyla of colonic samples. A negative trend was observed with Bacteroidetes and a positive trend with Proteobacteria.

The lavage profiles were highly variable despite similar alpha diversities, however, Bacteroidetes and/or Proteobacteria were the prominent phyla in these samples. It is possible that most of these profiles are dysbiotic but without samples from HC, this aspect could not be further investigated.

There were no particular differences observed between CD and UC patients. Compared to NIC there were no differences observed in alpha diversity between treatment-naïve IBD (n=7) and Active IBD, nor between CD and UC. Nevertheless, there was increased alpha diversity with inflammation (Patient A14), and the number of phyla detected in the patient with IL-10RB mutation. It was possible to compare the bacterial profiles of a duodenal lavage and biopsy (uninflamed) in only 1 patient. The bacterial composition, relative abundances of the bacterial phyla and alpha diversities were similar for these samples.
3.4.6 Alterations in Bacterial Profiles with Treatment

In active disease, induction of remission with mucosal healing is the aim of treatment. Given the changes occurring at mucosal level, alterations in bacterial profiles with treatment was investigated. Stool and/or mucosal profiles before and after treatment are presented with accompanying clinical summary.

3.4.6.1 Exclusive Enteral Nutrition

Patients A15 and A17 were commenced on EEN following their diagnosis with CD. Stool bacterial profiles before and after treatment with EEN were analysed (Figure 3.26).

In the pre-treatment samples, Bacteroidetes dominated the profiles of both patients (A15: 73%; A17: 75%). Firmicutes (A15: 11%; A17: 20%) and Proteobacteria (A15: 15%; A17: 5%) were also present. Patient A17 responded to the treatment, and this was associated with reduction in Proteobacteria (2%). After 6 weeks of EEN, Patient A17 continued having the feed as a supplement, and remained well during the study period.

Patient A15 was diagnosed with Norovirus gastroenteritis a week after diagnosis. The patient was admitted for intravenous fluids for 2 days, and then gradually resumed EEN. Formed stools were noted 7 days after the start of the gastroenteritis. At the same time, the patient was also found to have a urinary tract infection for which a 5-day course of Trimethoprim was given. The patient continued EEN until 6 weeks post-diagnosis, after which time a normal diet was reintroduced.

Although Patient A15 achieved remission after the EEN course, the Bacteroidetes proportion was greatly reduced (23%) and the Proteobacteria persisted (20%), perhaps due to the course of Trimethoprim or the gastroenteritis. The Bacteroidetes proportion gradually increased and remained stable despite the subsequent relapse. The patient was commenced on Infliximab at month 9, during which time, Bacteroidetes dominated with Proteobacteria detected. At month 11, at the end of the Infliximab induction course, Bacteroidetes was seen to decrease although continued to dominate, and Proteobacteria just detected.

Comparing the profiles of these patients, there was greater Proteobacteria and a marked reduction in Bacteroidetes after the EEN course in Patient A15. In addition, there were
greater proportions of mucosal Proteobacteria in this patient compared to Patient A17 (Figure 3.26).

Exclusive Enteral Nutrition

**Patient A15**

**Patient A17**

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<td>54</td>
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Azathioprine

EEN

Infliximab | 130 | 135

wPCDAI

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**Figure 3.26: Stool Bacterial Profiles of Patients on Exclusive Enteral Nutrition (EEN)**

Stool bacterial profiles of Patients A15 and A17 were analysed in conjunction with wPCDAI, CRP (mg/dL), ESR (mm/hr) and calprotectin (µg/g) before and after EEN. Bacteroidetes: Proteobacteria ratios were calculated using the sequencing reads for Bacteroidetes and Proteobacteria.
3.4.6.2 Prednisolone

Patients A8 and A21 were diagnosed with UC while Patient A9 was diagnosed with IBDU. All patients received Prednisolone at diagnosis for induction of remission. Stool bacterial profiles before and after treatment are presented (Figure 3.27). In addition, there was also a set of mucosal biopsies from Patient A8 during relapse 14 months later.

Patient A8
There was some degree of clinical improvement with the course of Prednisolone, which was stopped after a gradual dose reduction over 2 months. Active disease persisted in this patient despite Pentasa and an increased dose of Azathioprine. This was partly due to poor compliance. Due to persisting inflammation, the patient received a second course of Prednisolone at month 6. Although there was some response to this, there was ongoing inflammation thus this patient underwent endoscopic mucosal assessment at month 14 and subsequently had an escalation of treatment with Infliximab.

The pre-treatment stool profile included raised Proteobacteria (28%), which decreased following treatment (5%), despite the ongoing active disease. This was associated with increased Firmicutes (30 to 78%), reduced Bacteroidetes (42 to 8%) and emergence of Actinobacteria (10%). With subsequent clinical deterioration at 14 months, there was increased Bacteroidetes (44%) and Proteobacteria (15%) with decreased Firmicutes (38%) and Actinobacteria (3%).

Comparing the stool and mucosal profiles at diagnosis, the higher relative abundance of Proteobacteria was similar in both profiles (TI 23%, RC 22%, LC 32%), with the LC having the highest abundance of the mucosal samples. Bacteroidetes, which was present in the stool, was absent in the mucosal samples, thus Firmicutes dominated the mucosal profiles (TI 76%, RC 76%, LC 65%). At 14 months, still in active disease but on treatment, Bacteroidetes was present in the stool and mucosal (TI 68%, RC 74%, LC 42%) samples, with less abundant Proteobacteria in the colonic samples (TI 2%, RC 10%, LC 12%). These findings suggest that the treatments received had altered the mucosa and thus bacterial profiles, accounting for differences seen in the samples pre-diagnosis and at 14 months despite mucosal inflammation being present during both endoscopic assessments.

Patient A21
Patient A21 presented with symptoms that began with an episode of gastroenteritis whilst visiting family in Pakistan. The pre-treatment stool profile was similar to those of HC;
Firmicutes dominated (69%) with Bacteroidetes (25%) and Proteobacteria (5%) present. This patient responded well to induction of remission with Prednisolone, and this was associated with increased Bacteroidetes (58%) and decreased Firmicutes (34%) and Proteobacteria (2%). Actinobacteria increased at month 3 (6%), when the patient was in remission. This patient remained well on Azathioprine and probiotics, which was started soon after diagnosis.

**Patient A9**

Patient A9 had a background history of atopy and severe multiple food allergies associated with raised IgE levels. This patient was diagnosed with IBDU following endoscopic findings of continuous colitis from the rectum to the hepatic flexure and aphthous ulcers in the duodenum. At diagnosis, the stool profile was dominated by Firmicutes (50%). With response to treatment (reported clinical improvement but no clinical data was available), Actinobacteria increased (12%) while Proteobacteria decreased and was undetectable. This patient subsequently relapsed thus received Infliximab a few months later.

**Patient A8**

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Figure 3. 27: Luminal and Mucosal Bacterial Profiles of Patients on Prednisolone

Luminal (stool and duodenal lavages) and mucosal bacterial profiles of UC Patients A8 and A21, and IBDU Patient A9 were analysed in conjunction with wPCDAI, CRP (mg/dL), ESR (mm/hr) and calprotectin (µg/g) before and after Prednisolone. Bacteroidetes: Proteobacteria ratios were calculated using the sequencing reads for Bacteroidetes and Proteobacteria. The * indicates mucosal inflammation.

3.4.6.3 Infliximab

Due to ongoing active disease, Patients D2, D4, D6 and D7 had escalation of treatment and received Infliximab, while Patient A25 received Infliximab for treatment of perianal fistula (Figure 3.28).
Patient A25
Patient A25 was diagnosed with CD thus was initially treated with EEN and Azathioprine. At diagnosis, Bacteroidetes dominated the profile (64%). Two months after diagnosis, this patient was found to have perianal fistula, thus Infliximab and Ciprofloxacin were commenced. The patient responded to treatment and a second stool sample collected at 7 months was associated with clinical improvement. In this sample, Firmicutes increased (25 to 53%) and Proteobacteria (11 to 1%) was replaced by Verrucomicrobia (12%).

Patient D2
Patient D2 was initially diagnosed with Coeliac Disease at less than a year of age, and CD was diagnosed at the age of 9 years. The patient was commenced on Azathioprine and Budesonide but despite an increased Azathioprine dose, the patient was unwell 3 months later. After responding to a course of Prednisolone, this patient relapsed at a year post-diagnosis thus Azathioprine was increased and Infliximab was commenced.

Prior to receiving Infliximab, Bacteroidetes dominated the stool bacterial profile (59%). During the Infliximab induction period, Bacteroidetes (29%) and Proteobacteria (7 to 4%) decreased. The patient had short-lived responses with each dose of Infliximab but remained in active disease. Bacteroidetes (42-52%) and Firmicutes (43-51%) dominated the stool profile during this period with minimal Proteobacteria (4-6%) (3 and 4 months). Worsening disease was associated with increased Proteobacteria in month 5 (18%), thus Pentasa was commenced, following which Proteobacteria decreased (4%). The profile remained largely stable but due to ongoing symptoms, the Infliximab dose was doubled. The effects were short-lived thus Infliximab was switched to Adalimumab. The subsequent clinical improvement at month 14 was associated with a profile dominated by Bacteroidetes (62%) with raised Proteobacteria (24%). Although raised, the Proteobacteria may actually be decreasing with the clinical improvement but without stool profiles between months 9 and 14, it was not possible to confirm this. It was interesting to note that the profile of this patient had greater presence of Firmicutes, more similar to Coeliac Disease.

Patient D4
Patient D4, diagnosed with CD, had a relapse on maintenance Azathioprine 18 months after diagnosis. Induction of remission was attempted with Prednisolone but due to ongoing symptoms, Infliximab was commenced and Prednisolone was weaned and stopped 3 months later.
A stool sample was unavailable prior to starting Infliximab, however, the stool sample collected after the first induction dose, at 2 weeks, was dominated by Bacteroidetes (77%) with Firmicutes (16%) and Proteobacteria (7%) also present. The initial improvement with Infliximab at month 4 was associated with reduced Proteobacteria (3%) and Bacteroidetes (51%), however, a viral infection then triggered another relapse (no clinical parameters available). A month later, increased Bacteroidetes was observed (81%), and with subsequent improvement at month 7, there was significant reduction in Bacteroidetes (10%) with emergence of Actinobacteria (3%). There was increased Proteobacteria compared to month 5 (3 to 10%) but this could actually be decreasing from the previous month.

Due to ongoing active disease, Patient D4 underwent mucosal assessment after 12 months of Infliximab. Despite histological inflammation, Bacteroidetes dominated the mucosal profiles, with relatively less Proteobacteria.

**Patient D6**
Patient D6 commenced on Infliximab due to ongoing active CD and persisting radiological evidence of strictures in the TI, despite treatment with Azathioprine and Prednisolone. The stool profile after the first Infliximab dose at week 2 was dominated by Bacteroidetes (91%). Reported clinical improvement at week 6 was associated with reduced Bacteroidetes (79%) and persisting Proteobacteria (10%). This patient subsequently reported recurrence of symptoms after eating popcorn, a known trigger. Treatment was escalated but due to persisting active disease, surgical resection was performed 12 months after commencing Infliximab.

**Patient D7**
Patient D7 had a long history of GI issues which began following an episode of gastroenteritis after visiting Turkey. An inflammatory mass in the right colon resolved after treatment with antibiotics (Cefuroxime and Metronidazole then Augmentin only). Following a year of ongoing GI symptoms, the patient was diagnosed with IBDU after endoscopic findings of pancolitis with multiple ulcers and chronic gastritis. The patient responded well to a course of EEN and continued the feed as a supplement in addition to Azathioprine. Subsequently, there were intermittent relapses triggered by various food. Due to pancreatitis, Infliximab was commenced and Azathioprine was stopped. There was initial clinical improvement but after the last sample was collected for the study, treatment was escalated to include Salofalk enema, then Infliximab was switched to Adalimumab.
The first stool sample received after induction of Infliximab was dominated by Firmicutes (92%) with Actinobacteria present (3%) and Bacteroidetes (1%) and Fusobacteria (2%) just detectable. Despite clinical improvement, this patient had persisting active disease. At month 4, dominance of Firmicutes (89%) and presence of Proteobacteria (2%) and Actinobacteria (2%) persisted, however Bacteroidetes increased (7%).

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**Phylum**
- Actinobacteria
- Bacteria_Undclassified
- Bacteroidetes
- Firmicutes
- Fusobacteria
- Proteobacteria
- Synergistetes
- Verrucomicrobia
**Patient D2**

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**Medications**

- Azathioprine
- Prednisolone
- Infliximab
- Pentasa
- Adalimumab

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**Patient D4 – Stool Mucosal Biopsies**

![Graph showing bacterial abundance](image)

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**Figure 3. 28: Luminal and Mucosal Bacterial Profiles of Patients on Infliximab**

Luminal (stool and duodenal lavages) and mucosal bacterial profiles of Patients A25, D2, D4, D6 and were analysed in conjunction with wPCDAI, CRP (mg/dL), ESR (mm/hr) and calprotectin (µg/g) before and during treatment with Infliximab. Bacteroidetes: Proteobacteria ratios were calculated using the sequencing reads for Bacteroidetes and Proteobacteria. The * indicates mucosal inflammation.

**3.4.6.4 Sirolimus**

As discussed in Section 3.4.4, Patient C1 was diagnosed with UC, which progressed despite escalating treatment. Sirolimus was commenced after repeat endoscopic mucosal assessment.
Prior to Sirolimus, Bacteroidetes was absent from the stool and mucosal profiles (Figure 3.29). After a month, the clinical improvement was associated with significant reduction in Proteobacteria in the stool (27 to 2%), although Bacteroidetes remained absent. The patient achieved remission but then relapsed.

Whilst on Prednisolone for this at 6 months, increased Bacteroidetes (74%) was associated with detectable Proteobacteria (1%) and Actinobacteria (1%). With response to Prednisolone, Bacteroidetes decreased (39%) while the presence of Actinobacteria persisted (1%). Although the patient achieved remission, the calprotectin concentration was still high, suggesting ongoing mucosal inflammation and heralding the subsequent relapse at month 12. Similar to the pre-treatment stool profile, Bacteroidetes was absent and Proteobacteria increased (17%). Interestingly, Actinobacteria increased significantly (17%). Unlike the pre-treatment mucosal profiles, Bacteroidetes was present (TI 46%, RC 39%, LC 30%). Although there was high relative abundance of Proteobacteria (TI 13%, RC 17%, LC 28%), it did not dominate the post-treatment mucosal profiles. It was most abundant in the LC sample, associated with more severe inflammation distally, as characteristic of UC. This patient went on to have Adalimumab with good response.

Stool

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Mucosal Biopsies

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**Figure 3. 29: Luminal and Mucosal Bacterial Profiles of Patient C1**

Luminal (stool and duodenal lavages) and mucosal bacterial profiles of Patient C1 were analysed in conjunction with wPCDAI, CRP (mg/dL), ESR (mm/hr) and calprotectin (µg/g) before and during treatment with Sirolimus. The * indicates mucosal inflammation.

3.4.6.5 Haematopoietic Stem Cell Transplantation

Patients with severe, refractory infantile IBD underwent HSCT. Their microbial profiles before and after HSCT were analysed (Figure 3.30).

**Patient B1**

Patient B1 was diagnosed with VEOIBD. Although a genetic mutation was not detected, given the severity of the gastrointestinal inflammation, HSCT was recommended. Prior to HSCT, the stool and mucosal profiles were similarly dominated by Bacteroidetes (stool 52%, RC 59%, LC 65%). In addition, Firmicutes (stool 13%, RC 22%, LC 17%), Proteobacteria (stool 30%,RC 15%, LC 14%) and Synergistetes (stool 5%, RC 3%, LC
3% were present in all samples. Following HSCT, at 7 months, mucosal assessment was performed to investigate the possibility of graft versus host disease. Despite the mild, patchy chronic inflammation throughout the colon, Bacteroidetes dominated the mucosal profiles (TI 70%, RC 71%, LC 70%) with Firmicutes also present. Proteobacteria was much reduced and Synergistetes was no longer detectable. The patient subsequently improved and was readmitted for routine mucosal assessment, revealing stable mucosal profiles associated with mild distal colitis.

**Patient B3**

Patient B3 presented in infancy with severe, intractable enterocolitis. In a bid to control the severe inflammation, the patient underwent subtotal colectomy. Years later, the patient was diagnosed with IL-10RB mutation and proceeded to have HSCT.

This analysis was limited by the number of mucosal samples being discarded due to sequence reads of <7,000. All mucosal samples pre-HSCT and at 4 months, in addition to the duodenal and redundant colonic biopsies at 9 months were unavailable for analysis.

Proteobacteria dominated the stool profile pre-HSCT and throughout the study period (97-100%). Firmicutes was detectable (1-2%) but Bacteroidetes was absent. Although significantly dysbiotic, this profile was stable during the study period, despite intestinal GvHD at 3 months, which resolved the following month with Budesonide. The patient was then able to commence and establish enteral nutrition gradually. At 9 months, the patient underwent endoscopic mucosal assessment whilst clinically well. In the TI biopsy, only Proteobacteria was detected.
B1 – Stool and Mucosal Biopsies

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**Figure 3. 30:** Luminal and Mucosal Bacterial Profiles of Patients with HSCT

Luminal (stool and duodenal lavages) and mucosal bacterial profiles of Patients B1 and B3 were analysed in conjunction with CRP (mg/dL) and ESR (mm/hr) before and after HSCT. Alpha diversity of the samples are also presented. The * indicates mucosal inflammation.
3.4.6.6 Cryopyrin-Associated Periodic Syndrome (CAPS)

As comparison to IBD patients, stool microbial profiles with treatment in CAPS patients were analysed (Figure 3.31). Patient E1 was recruited just after receiving the first dose of Canakinumab at 3 years of age. The patient was symptomatic with loose stools and abdominal pain, both gradually settling with Canakinumab. The stool microbial profiles and alpha diversities were similar to HC and stable throughout the study period. Bacteroidetes dominated the profiles (66-83%), and Proteobacteria was detected in all samples (1-6%), with the most observed at 1 months. Verrucomicrobia was detected at 4 months (1%) and Actinobacteria (1%) was detected at 6 months, with associated improvement after an increased dose of Canakinumab.

Patient E2 was relatively well at the start of treatment, reporting mild, intermittent abdominal pain. As with Patient E1, the stool bacterial profile was dominated by Firmicutes (61%) and Bacteroidetes (36%), and was comparable to the profiles of HC. Proteobacteria (1%) and Actinobacteria (1%) were detected. With Canakinumab, improved clinical symptoms were associated with increased Bacteroidetes (65%) and Proteobacteria (4%), and emergence of Verrucomicrobia (8%). The alpha diversities were similar at both time points.

In comparison to IBD patients, CAPS patients had similar stool microbial profiles to those of HC. These profiles remained largely stable despite fluctuations in disease activity.

**Patient E1**

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Patient E2

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**Figure 3. 31: Stool Bacterial Profiles of Patients with CAPS**

Stool bacterial profiles of Patients E1 and E2 were analysed in conjunction with the alpha diversities and Disease scores, CRP (mg/dL), Amyloid A (µg/ml) before and during treatment with Canakinumab.

3.4.6.7 Summary of Alterations in Bacterial Profiles with Treatment

Treatment appeared to alter the bacterial profiles of stool and mucosal biopsies in IBD patients. In patients who subsequently relapsed or remained in active disease, being on treatment appeared to lessen dysbiosis. The profiles and alterations also differed between CD and UC patients.

In CD, Bacteroidetes appeared to dominate the pre-treatment samples, and decreased as the patients clinically improved and/or achieved remission. Proteobacteria also decreased with treatment, clinical improvement and in remission. On treatment, Bacteroidetes and Firmicutes were generally stable despite changes in clinical status. Interestingly, Proteobacteria in active Fistulising CD was replaced by Verrucomicrobia when remission was achieved with treatment.
In UC, Firmicutes dominated the bacterial profiles prior to treatment. The low or absent Bacteroidetes pre-treatment and during relapse emerged and/or increased as the patient clinically improved. In addition, Actinobacteria emerged with clinical improvement or remission. As with CD, decreased Proteobacteria was associated with clinical improvement and remission with treatment. In 2 UC patients, Bacteroidetes was strikingly absent from the pre-treatment mucosal samples but present during relapse i.e. in active disease during both time points but on treatment when relapsed.

In a patient who underwent HSCT, Proteobacteria and Synergistetes present in the stool and mucosal samples pre-HSCT decreased post-HSCT. In another patient, Proteobacteria dominated the stool and mucosal profiles although Firmicutes was detected with clinical improvement.

In comparison to IBD patients, CAPS patients had more stable stool bacterial profiles mostly dominated by Bacteroidetes before and during treatment despite fluctuating disease activity. Low relative abundances of Proteobacteria were detected in the samples, the most abundant being in a patient who had just started treatment. As in UC, Actinobacteria was detected in a patient who improved with treatment. Similarly, Verrucomicrobia was observed in another patient when well post-treatment.

A CD patient received a course of Trimethoprim, and concurrently was found to have Norovirus gastroenteritis. Increased Proteobacteria and marked reduction of Bacteroidetes was observed in the following post-treatment stool sample from this patient.

3.4.7 Bacterial Profiles in Other GI Inflammatory Diseases
3.4.7.1 Microbial Profiles of Other IBD Patients

Within the study cohort, there were patients who were diagnosed with other IBD subtype or additional diseases that may influence the microbial profiles. These patients are presented (Figure 3.32).

Patient A11 was diagnosed with CD but was further investigated when there was little response to conventional IBD treatment, which included EEN, Azathioprine, Prednisolone, Infliximab and Adalimumab. This patient had persisting inflammation during the study period, and was later diagnosed with Granulomatosis Polyangiitis. Although the stool microbial profiles were dysbiotic at times, with significant dominance.
of Bacteroidetes or Firmicutes, Proteobacteria abundance remained relatively low or was absent. Similarly, the mucosal profiles were dominated by Bacteroidetes with minimal Proteobacteria (Figure 3.20). Following the diagnosis of Granulomatosis Polyangiitis, the patient was commenced on Methotrexate with good effect.

**Patient A11**

![Stool Bacterial Profiles of Patients with Other Types of IBD](image)

<table>
<thead>
<tr>
<th></th>
<th>Pre-Tx</th>
<th>Mth 2</th>
<th>Mth 3</th>
<th>Mth4</th>
<th>Mth6</th>
<th>Mth8</th>
<th>Mth9</th>
<th>Mth 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha Diversity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TI</td>
<td>2.36</td>
<td>1.88</td>
<td>2.84</td>
<td>0.47</td>
<td>2.27</td>
<td>0.62</td>
<td>1.93</td>
<td>2.27</td>
</tr>
<tr>
<td>RC</td>
<td>2.28</td>
<td>2.2</td>
<td>11.3</td>
<td>i</td>
<td>i</td>
<td>i</td>
<td>6.3</td>
<td>61</td>
</tr>
<tr>
<td>B:P Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azathioprine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EEN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Humira</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infliximab</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisolone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wPCDAI</td>
<td>47.5</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>40</td>
<td>40</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>7</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>ESR</td>
<td>73</td>
<td>95</td>
<td>66</td>
<td>67</td>
<td>70</td>
<td>60</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Calprotectin</td>
<td>291</td>
<td>9</td>
<td>1766</td>
<td>3083</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.32: Stool Bacterial Profiles of Patients with Other Types of IBD**

The stool bacterial profiles of Patients A2 (Collagenous Colitis) and A11 were analysed and compared to other IBD patients. Clinical data, percentages of bacterial phyla, alpha diversity and Bacteroidetes:Proteobacteria (B:P ratios) ratios are presented. Bacteroidetes: Proteobacteria ratios (B:P ratios) were calculated using the sequencing reads for Bacteroidetes and Proteobacteria.
Patient D2 (CD and Coeliac Disease) was discussed in more detail in Section 3.4.6.3. It was interesting to note that most of the stool profiles had increased Firmicutes, which is more characteristic of Coeliac Disease.

Patients A9 and D7 were diagnosed with IBDU, and discussed in the sections above. Their dysbiotic profiles during active disease were more similar to UC than CD with Firmicutes dominating the profiles. The Bacteroidetes:Proteobacteria ratios were also similar to active UC.

Patient A2 was diagnosed with Collagenous Colitis. The pre-treatment stool received had a profile similar to that of HC, with Bacteroidetes and Firmicutes dominating and Verrucomicrobia present (Figure 3.22). The reported colitis was associated with calprotectin of 1223 µg/g.

### 3.4.7.2 Cow’s Milk Protein Allergy

The bacterial profiles of allergic infants (n=2, aged 7 and 12 months)(Figure 3.33A) were compared to HI [n=5, median age 9 (7-9) months](Figure 3.16A). There were no particular trends correlating bacterial profiles with contributing factors (mode of delivery, feeding, weaning age and sibling) (Section 3.4.2.1) in HI, thus comparison with allergic infants was not possible, especially given the small numbers.

As with the HI, the bacterial profiles of the allergic infants were variable. Bacteroidetes or Firmicutes dominated, with Proteobacteria detected. Actinobacteria was also detected in Infant F10. The alpha diversity of stool samples between the groups was also compared. Statistical analysis was not possible given the small cohort numbers, however, compared to HI [n=5 median 2.36 (1.84-2.86)], allergic infants appeared to have lower alpha diversity (1.71, 2.17)(Figure 3.33B) despite one allergic infant being a few months older and thus expected to be on a more varied diet. Given the wide variations in alpha diversity, meaningful analysis of the beta diversity was not possible.
Figure 3. 33: Bacterial Profiles and Alpha Diversity of Stool of Allergic Infants
The bacterial profiles and alpha diversity of stool from allergic infants (n=2) (A) were analysed. The alpha diversity for HI and allergic infants was compared using Shannon indices (B).

3.4.7.3 Summary of Bacterial Profiles in Other GI Inflammatory Diseases

The pathogenesis for these GI conditions differ from IBD, and associated with this are differences in the bacterial profiles, in which Proteobacteria does not have a dominant presence. An exception is IBDU, which appears to be more similar to UC than CD.
### 3.4.8 Summary of Results

The findings of the microbiota analysis are summarised below;

**Stool Microbial Profiles of Control Patients**

<table>
<thead>
<tr>
<th></th>
<th>Healthy Infants</th>
<th>Healthy Children</th>
<th>Non-inflammatory Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>5</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Age</td>
<td>7-9 months</td>
<td>4-17 years</td>
<td>4-15 years</td>
</tr>
<tr>
<td>Dominant Phyla</td>
<td>Bacteroidetes or Firmicutes</td>
<td>Bacteroidetes and Firmicutes</td>
<td>Bacteroidetes and Firmicutes</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>High abundance</td>
<td>High abundance</td>
<td>High abundance</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>High abundance</td>
<td>High abundance</td>
<td>High abundance</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Present in all infants</td>
<td>Present in all HC, generally 1-3%</td>
<td>Present in most patients</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>Present in all infants</td>
<td>Detected in most HC, most abundant in those living in rural locations</td>
<td>Detected in 2 patients</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>N/A</td>
<td>Detected in 3 HC</td>
<td>Detected in 1 NIC</td>
</tr>
<tr>
<td>Alpha Diversity</td>
<td>Lower than HC</td>
<td>N/A</td>
<td>Similar to HC</td>
</tr>
<tr>
<td>Addition Comments</td>
<td>Significantly variable profiles</td>
<td>Child with highest Proteobacteria (13%) had a first-degree relative with CD</td>
<td>Highest Proteobacteria (19%) in a patient who had antibiotics</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low beta diversity</td>
</tr>
</tbody>
</table>

**Stool Dysbiosis (n=20, age 4-16 years)**

- Dysbiosis in IBD can be described as significant reduction of Firmicutes or Bacteroidetes, or greater relative abundance of Proteobacteria compared to HC.
- Dysbiosis was not always associated with disease activity, especially when on treatment.
- A profile similar to HC at diagnosis (pre-treatment) was associated with good response to treatment whilst a more dysbiotic profile was associated with more severe disease course.
Duodenal Lavages NIC (n=8) vs. IBD (6 CD, 3 UC, 1 IBDU, 1 IL-10RB mutation)
The profiles varied widely between patients, with Proteobacteria or Bacteroidetes dominating most profiles. There were no differences observed between IBD and NIC, and CD and UC patients.

**Stool Microbial Profiles in IBD**

<table>
<thead>
<tr>
<th></th>
<th>Active CD Remission</th>
<th>CD Remission</th>
<th>Active UC Remission</th>
<th>UC Remission</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dominant Phyla</strong></td>
<td>Bacteroidetes</td>
<td>Bacteroidetes and Firmicutes</td>
<td>Firmicutes</td>
<td>Firmicutes and Bacteroidetes</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>High abundance</td>
<td>High abundance</td>
<td>Lower than in remission</td>
<td>Higher than in active UC</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Lower than in remission</td>
<td>Higher abundance</td>
<td>High abundance</td>
<td>High abundance</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Higher than HC and CD Remission</td>
<td>Similar to HC</td>
<td>Higher than in HC, CD and UC remission</td>
<td>Similar to HC</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Associated with clinical improvement or remission</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>Present in patient with Fistulising CD</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Alpha Diversity</td>
<td>Lower than HC</td>
<td>N/A</td>
<td>Lower than HC</td>
<td>N/A</td>
</tr>
<tr>
<td>B:P Ratios</td>
<td>Similar to HC</td>
<td>Similar to HC</td>
<td>Lower than HC</td>
<td>Similar to HC</td>
</tr>
<tr>
<td>F:P Ratios</td>
<td>Lower than HC</td>
<td>Similar to HC</td>
<td>Lower than HC</td>
<td>Similar to HC</td>
</tr>
<tr>
<td><strong>Addition Comments</strong></td>
<td>Proteobacteria most abundant in patient with Fistulising CD</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

B:P – Bacteroidetes:Proteobacteria; F:P – Firmicutes:Proteobacteria
Stool vs. Colonic Mucosal Biopsies

In most NIC patients, stool and mucosal profiles were similar.
In IBD, microbial profiles of stool and colonic mucosal biopsies differed.
Greater mucosal Proteobacteria than seen in NIC.
Higher mucosal Proteobacteria, lower mucosal Bacteroidetes than in the stool.

TI and Colonic Mucosal Biopsies

(11 NIC, 6 CD, 3 UC, 1 Collagenous Colitis, 1 IBDU, 1 VEOIBD)

<table>
<thead>
<tr>
<th></th>
<th>NIC</th>
<th>CD</th>
<th>UC</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>11</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Dominant Phyla</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>High abundance</td>
<td>Absent in Fistulising CD</td>
<td>Lower, present in patient who achieved remission</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>High abundance</td>
<td>Absent in a treatment-naïve patient</td>
<td>Higher abundance</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Present in some NIC patients</td>
<td>Present in all IBD patients, dominating in some</td>
<td>Higher abundance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Higher in CD than NIC</td>
<td>Lower abundance associated with remission and being on treatment</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>Present in 3 NIC</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Alpha Diversity</td>
<td>N/A</td>
<td>Lower in mucosa of treatment-naïve and active/relapsed IBD than NIC</td>
<td>Similar to UC in colon</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Similar to CD in colon</td>
<td></td>
</tr>
<tr>
<td>B:P Ratios</td>
<td>N/A</td>
<td>Lower than NIC</td>
<td>Lower than NIC and CD</td>
</tr>
<tr>
<td>Addition Comments</td>
<td>Similar TI and colonic profiles (n=7) except in a patient with food allergies</td>
<td>Similar TI and colonic mucosal profiles for most patients</td>
<td>Bacteroidetes absent in treatment-naïve patients</td>
</tr>
</tbody>
</table>
Disease Severity
Patients with severe IBD harboured more dysbiotic stool and mucosal profiles with greater presence/dominance of Proteobacteria than other IBD patients. In addition, Bacteroidetes was absent in stool and mucosal samples. With clinical improvement, the proportions of Proteobacteria decreased and Bacteroidetes increased.

In the ileostomy samples of 2 patients (Patients B3 and C3), Proteobacteria dominated the profiles. With clinical improvement, Proteobacteria decreased and Firmicutes increased. Bacteroidetes emerged in a patient when well.

Alterations of Bacterial Profiles with Treatments
Alterations in bacterial profiles were observed with treatment, even with persisting active disease.

When already on treatment, Bacteroidetes and Firmicutes were generally stable despite changes in clinical status

Increased Proteobacteria and marked reduction of Bacteroidetes was seen after Norovirus gastroenteritis and a course of Trimethoprim (Patient A15)

HSCT
Proteobacteria and Synergistetes in the stool and mucosa of Patient B1 pre-HSCT decreased or was absent post-HSCT. In another patient (Patient B3), Proteobacteria dominating the ileostomy profiles decreased while Firmicutes was detected with clinical improvement.
<table>
<thead>
<tr>
<th></th>
<th>CD</th>
<th>UC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteroidetes</strong></td>
<td>Domated pre-treatment</td>
<td>Emerged and/or increased with clinical improvement</td>
</tr>
<tr>
<td></td>
<td>Decreased with clinical improvement and in remission</td>
<td>Absence in stool pre-treatment and at relapse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Absent in mucosa pre-treatment but present in relapse (Patients A8, C1)</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td>N/A</td>
<td>Dominated pre-treatment</td>
</tr>
<tr>
<td><strong>Proteobacteria</strong></td>
<td>Higher abundance in mucosal at diagnosis associated with more severe disease (EEN, Patient A15)</td>
<td>Decreased with treatment, clinical improvement and in remission</td>
</tr>
<tr>
<td></td>
<td>Decreased with treatment, clinical improvement and in remission</td>
<td>High abundance during severe, active disease</td>
</tr>
<tr>
<td></td>
<td>Increased during disease exacerbation (Patient D2)</td>
<td></td>
</tr>
<tr>
<td><strong>Actinobacteria</strong></td>
<td>N/A</td>
<td>Emerged with clinical improvement or remission</td>
</tr>
<tr>
<td><strong>Verrucomicrobia</strong></td>
<td>Proteobacteria in active disease was replaced by Verrucomicrobia in remission (Fistulising CD)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Other IBD Subtypes**

With the exception of IBDU, the bacterial profiles of other GI inflammatory conditions were less dysbiotic with less abundance of Proteobacteria. The dysbiotic stool profiles in IBDU were dominated by Firmicutes and were comparable to active UC. The mucosal profiles in these patients were similar to CD and UC with Proteobacteria dominating the profiles.
3.5 Discussion

The study of intestinal microbiota has provided new insights in the interactions between genetics, environmental factors and the host in health and disease. It has also highlighted risk factors in multifactorial diseases. This study has further explored intestinal microbiota in PIBD, and the influence of the host and treatments in altering the microbial populations in these children. In addition, this study set out to characterise dysbiosis in IBD.

3.5.1 Analysis of the Methodology

The methodology for investigating the microbiota vary widely. Variations in sample collection, storage, DNA extraction, library preparation, regions sequenced, bioinformatics pipelines and analysis are able to alter the results thus comparison between studies can be challenging (626, 627). Careful consideration and optimisation was thus performed for this study to ensure that potential biases and confounding factors were minimised (Section 3.2). Protocols for sample collection, storage, DNA extraction and library preparation were developed with the identified limitations in mind. Suitable control cohorts were recruited alongside disease groups and all the samples were treated and analysed the same way, thus making it easier to compare between the groups.

Additional experiments were conducted to assess validity of the results. Duplicate aliquots of the same stool samples were extracted after varying storage durations. The results of these were comparable. In addition, there were samples that did not sequence. A major cause may be interference by chimeras, especially for samples of low bacterial abundance. This included stool samples from patients who were on antibiotics, infants and those with ileostomies

The duodenal lavages collected varied in volume and had to be concentrated for DNA extraction. Depending on the laboratory machines available, the lavages were concentrated by freeze-drying or centrifugation. Despite the 2 methods of concentrating the duodenal lavages, there were no clear patterns distinguishing the methods. In addition, there were no patterns distinguishing inflammation or CD from UC. As there are no reports of duodenal lavages in PIBD, perhaps due to the challenges of collecting these samples, analysis of these samples were limited.
A significant number of biopsies were discarded. Again, interference by chimeras may have been an issue, particularly with samples of low bacterial abundance such as duodenal biopsies and biopsies of patients who received antibiotics or parenteral nutrition (PN). The intestinal microbes of the patient who received PN was effectively deprived of substrates, thus was likely of low relative abundance. Reduction of the dysbiotic stool, and consequent pro-inflammatory stimuli, may have been a factor for the brief improvement in symptoms.

In summary, steps were taken to ensure that the data subsequently analysed were as robust as can be. In addition to novel findings, several results of this study were consistent with published reports, as discussed in the following sections.

3.5.2 Microbiota Profiles in Control Groups

A healthy cohort is essential for identifying deviations from health. Ideally, stool and intestinal mucosal biopsies were obtained from healthy controls but given the ethical considerations, non-invasive stool samples were received from HC while opportunistic stool, lavages and intestinal mucosal biopsies from NIC patients were collected.

3.5.2.1 Stool Microbial Profiles of Healthy Controls (HC)

HC [n=10, median age 12 (4-17) years] recruited to the study had not received antibiotics for at least 2 years preceding the sample collection. In general, the microbial profiles were similar despite the variable factors that may have influenced intestinal microbiota, such as mode of delivery, antibiotics received and diet. As previously reported (166, 628, 629), Bacteroidetes [median 40 (22-79)%] and Firmicutes [median 51 (20-74)%] dominated the healthy profiles.

Proteobacteria [median 2 (1-13)%] was present in all HC, accounting for 1-3% of the microbial profile. It was most abundant (13%) in a HC with a first-degree relative with CD. Family members of IBD patients are known to harbour dysbiotic bacterial profiles. Hedin et al. demonstrated that genotype was the most significant factor in the microbial variations observed in healthy individuals (630). The ATG16LI and NOD2 genotypes have been associated with shifts in Faecalibacterium and Escherichia taxa (432). Being
in the same environment and sharing the same diet are other factors that can influence the intestinal microbiota (631).

Actinobacteria was detected in most HC (>0.5% in 6 HC) and most abundant (2%) in 3 HC living in rural locations. Actinobacteria includes Gram-positive bacteria found in soil and water. Many species of the Actinomycetes produce almost 2/3 of the natural antimicrobial drugs used including Streptomycin, Vancomycin, Neomycin and Chloramphenicol (632, 633), therefore suggesting its beneficial presence in health.

Verrucomicrobia was also detected in 3 HC. Verrucomicrobia is a little-known phylum of bacteria found in fresh water, soil and animal stool. Fujio-Vejar et al. reported higher abundance of this phyla in healthy Chilean adults compared to adults in U.S.A (634). Akkermansia muciniphila is a Gram-negative, strict anaerobe and is a major genus of Verrucomicrobia found in human intestinal mucus. It is detected (1-4%) in healthy individuals (635) and utilises mucin as the only source of carbon and nitrogen (636). With the mucin degradation, A. muciniphila produces acetate and propionate for the host and stimulates mucin expression and secretion (41, 636, 637). Thus this symbiotic relationship benefits the human host and may explain its presence in the HC.

3.5.2.2 Stool Microbial Profiles of Healthy Infants (HI)

Intestinal microbiota is known to alter with age (166). To understand the changes better, healthy, antibiotic-naïve infants, who were at various stages of weaning, were also recruited [n=5, median 9 (7-9) months].

As previously seen, there were individual variations within the HI cohort. Depending on the age, mode of delivery and milk feed, Firmicutes, Actinobacteria or Bacteroidetes dominated the stool profiles (383, 638, 639).

The small cohort number limited this analysis but 2 patterns were recorded. Firstly, Proteobacteria was present in all the HI (1-12%) with the highest abundance observed in an infant who was born by Caesarean section and predominantly bottle-fed. Mode of delivery influences the microbial profile, with Bacteroides (family of Bacteroidetes) and Bifidobacteria (family of Actinobacteria) being less abundant or absent from those born by Caesarean section compared to those born via the vagina (379, 640). Bacterial diversity is also lower in the infants born by Caesarean section (146, 380). In addition, breast milk is known to stimulate the proliferation of Bifidobacteria and Lactobacillus,
both known to be more abundant in breastfed infants, likely accounting for the more acidic stools rich in SCFA in these infants (641, 642).

Secondly, Actinobacteria was present in all infants (1-12%), with the highest abundances (7-12%) observed in the older infants (9 months). As Actinobacteria is generally found in soil and water (643), the more mobile and inquisitive 9-month old infants may have wider environmental exposures that explains this finding. The microbial diversity is known to expand with increasing environmental exposures (139). Compared to the older HC, as expected, the alpha-diversity was lower in HI with high variability. The interactions with increasing variety of microbes is thought to “educate” and programme host immunity thereby influencing risk factors for disease in later life.

3.5.2.3 Stool Microbial Profiles in Non-inflammatory Controls (NIC)

NIC patients [n=16, median age 11 (4-15) years] were recruited prior to the diagnostic endoscopic assessments. Stool samples (n=7) were collected alongside intestinal mucosal biopsies (n=16 sets) prior to the procedure. This enabled comparison of luminal and mucosal bacterial content.

These patients did not have histological evidence of inflammation; they were diagnosed with IBS and/or food allergies. Both these conditions are associated with dysbiosis (343, 644). In addition, there were 4 patients with Rheumatological diseases who presented with GI symptoms. Although IBD was not diagnosed in these patients, dysbiosis also occurs in JIA (n=2) (345, 346).

Similar to HC, Bacteroidetes and Firmicutes dominated the stool profiles in NIC (Section 3.4.2.3). In addition, Proteobacteria was present in most NIC (n=5, 1-19%), with the highest proportion seen in a patient who received antibiotics prior to the endoscopy. In this patient (Patient A20), Bacteroidetes was significantly reduced (4%), with Firmicutes dominating the profile (77%). This patient was subsequently excluded from further analysis. The detrimental effects of antibiotics on intestinal microbiota are well-reported (56). This reiterates the impact of antibiotics and reaffirms the recommended caution with antibiotic use.

Actinobacteria was detected (2-4%) in 2 NIC patients, both of whom presented with anaemia and bleeding per rectum, and thus were on a proton pump inhibitor (PPI). In contrast to this, Reveles et al. reported decreased Actinobacteria following a course of
PPI thus suggesting another potential cause for this result (645). Another possible explanation is greater exposure to outdoor environments in these patients.

Verrucomicrobia was detected (1%) in 1 patients, who was investigated for anaemia and found not to have any GI abnormalities. The anaemia in this patient resolved with the cause unidentified. This patient was otherwise well and reported to have a healthy, well-balanced diet, potentially explaining the presence of Verrucomicrobia in only this patient.

The age ranges for HC and NIC were similar thus may be the reason for the similar alpha diversities seen. The beta diversities were also similar thus suggesting that the stool bacterial diversity in these groups were comparable.

### 3.5.3 Dysbiosis in Inflammatory Bowel Disease

In this study, luminal and mucosal dysbiosis were characterised in IBD. This offers the possibility of furthering our knowledge of the pathogenesis of CD and UC.

#### 3.5.3.1 Clinical Factors influencing Microbial Profiles

As discussed in more detail in Section 2.4, early exposures influence GI microbiota. More HC were breastfed for longer and received home-cooked weaning food rather than commercially produced food in comparison to IBD patients. The increased intestinal permeability in infants in addition to the relatively lower IgA levels (646), make them susceptible to antigenic stimulation. At the time of sample collection, HC had higher intake of dietary plant fibres. In addition, IBD patients were more likely to have dietary restrictions due to food intolerances, with both conditions associated with dysbiosis. There were more IBD patients than HC reporting a history of atopy, also known to be associated with dysbiotic microbial profiles (647). Antibiotic usage was clearly higher in IBD patients compared to HC, another well-reported risk factor in developing IBD (52, 53, 366-368).

Stress was reported to precede the development of IBD in a few of the study patients. Children experiencing neglect, physical and sexual abuse were found to have increased risk of developing IBD (648, 649). There have been very few studies investigating the
effects of stress on the microbiota in IBD. Intestinal microbiota has been demonstrated to influence the gut-brain axis (GBA).

Interestingly, depression from chronic stressful life events has been associated with increased Enterobacteriaceae, and psychological stress has been associated with decreased Lactobacilli spp. and increased E. coli and Pseudomonas spp. (650, 651). In murine models, chronic stress in early life or adulthood can alter intestinal microbial composition via increased pro-inflammatory cytokines, impaired intestinal barrier and increased activity of the hypothalamus-pituitary-adrenal (HPA) axis (343). Intestinal microbes also programmes the HPA axis in regulating responses to stress in the postnatal period (652).

3.5.3.2 Microbial Profiles of Duodenal Lavages in IBD

Luminal upper and lower intestinal microbial profiles of IBD patients were assessed using stool and duodenal lavages, respectively. Duodenal lavages of IBD patients were compared to NIC patients. With very little published on duodenal lavages in PIBD, analysis of these samples was limited.

Despite the variability, most profiles were dominated by Bacteroidetes or Proteobacteria. Firmicutes was the next abundant phylum with several other phyla detected. This was the case in IBD as well as NIC patients, with no significant differences observed between the groups, nor between CD and UC. This may be due to the absence of inflammation in these patients. There were 2 IBD patients with histological evidence of duodenitis. The alpha diversity was highest in the patient with significant inflammation, suggesting increased mucosal colonisation with impaired barrier function in inflammation. In the IBD and NIC groups, there were patients presenting with upper and lower GI tract symptoms thus perhaps both groups have dysbiotic duodenal luminal profiles. Without a cohort of truly healthy controls it was not possible to differentiate a dysbiotic profile from that of a healthy one.

Duodenal bacterial content has been estimated at $10^{1-3}$ CFU/ml (653). Perhaps due to the challenges in collecting and analysing these samples, there are very few reports of the duodenal luminal bacterial composition. Mu et al. reported dominance of Firmicutes followed by Proteobacteria, in piglets both consisting of species that are facultative
anaerobes (654). Minimal Bacteroidetes, consisting of strict anaerobic species, were present. This is in contrast to the findings of this study.

### 3.5.3.3 Microbial Profiles of Small Bowel Mucosal Biopsies

Analysing the bacterial profiles of duodenal mucosal biopsies proved challenging. All the samples were subsampled at 7,000 sequence reads, excluding 94% of the duodenal biopsies (total n=34). A corresponding duodenal lavage profile was available from a NIC patient. The lavage and mucosal profiles were similar with Bacteroidetes dominating both profiles. Firmicutes, Proteobacteria and Actinobacteria were also present in similar proportions in these samples. The only difference apparent was the presence of Fusobacteria in the duodenal biopsy.

Fusobacteria are gram-negative, obligate anaerobic bacilli associated with a wide spectrum of human diseases. It colonises mucosal membranes of the upper respiratory and GI tract. It is relatively well-known to be associated with Lemierre Syndrome, which is septic thrombophlebitis of the internal jugular vein. In addition, species of Fusobacteria are associated with infections of the oral cavity and central nervous system intra-abdominal and perirectal abscesses (655, 656) and Fusobacterium nucleatum specifically, has been associated with IBD (385, 657).

Comparing the mucosal profiles of the duodenum (proximal small bowel) to the TI (distal small bowel) in this patient, differences were observed. Although Bacteroidetes also dominated the TI profile, Proteobacteria was more abundant in the duodenum. It may be that Proteobacteria is more adaptable to the higher oxygen concentration and/or rapid reduction in acidity (from pH 2 to 5) in the duodenum (658).

Corresponding duodenal and TI profiles were also available in a patient diagnosed with VEOIBD, and with an ileostomy. The profiles for both sites were similar, with Proteobacteria dominating. Firmicutes, Fusobacteria and Bacteroidetes were also present in similar proportions in both biopsies suggesting that the duodenal and TI environments were similar in this patient. This was in contrast to the previous patient, who was diagnosed with NIC and had an intact GI tract. The higher oxygen concentration being more favourable for facultative bacteria such as Proteobacteria, and/or the underlying diagnosis of IBD influencing bacterial handling, may account for these findings.
In summary, analysis of the duodenal lavages was limited as there were no samples from healthy children to compare with. Analysis of the duodenal mucosal biopsies was restricted as the majority of the samples were discarded. However, it was noted that duodenitis was associated with increased bacterial diversity of the duodenal lavage (n=1), perhaps due to less regulated colonisation. In addition, the microbial composition may reflect the luminal environment; the duodenal and TI profiles were similar in the patient with an ileostomy but differed in the patient with an intact GI tract.

3.5.4 Dysbiosis in Crohn’s Disease

3.5.4.1 Dysbiotic Stool Profile in CD

Stool samples, being easier to collect, has been the predominant biological sample studied for microbiota profiles. Dysbiosis in IBD has been well reported, however, as yet, the dysbiosis has mainly been characterised qualitatively as relative abundances compared to control groups. Gevers et al. calculated the Microbial Dysbiosis Index (MD-index) to describe dysbiosis (Figure 3.1.1). The equation included selected families and orders with no obvious biological relationship (56).

In this study, further characterisation of the dysbiosis was attempted based on microbial alterations at phyla level. Published reports have identified various species, genera, families and orders implicated in IBD pathogenesis. The intestinal microbiota, however, exists as a community thus alterations of a particular species, genera, etc. reflect wider changes within the lumen and/or on the mucosa. Whilst these may be helpful biomarkers, they do not necessarily explain the changes occurring with inflammation and treatment. Thus, in this study, the luminal and mucosal profiles at phyla level were investigated.

In IBD, the dysbiosis observed consisted of significant dominance of Firmicutes or Bacteroidetes, or greater proportion of Proteobacteria compared to HC, consistent with previous studies (56, 270, 334, 385, 435). In active CD, Bacteroidetes dominated many of the profiles whilst in remission, the proportion of Firmicutes appeared to increase although this was not statistically significant (Figure 3.4.19, Appendix 3C). Clostriadales is the most abundant order of Firmicutes found in human stool but IBD patients have been reported to have decreased relative abundances of this order, particularly the Ruminococcaceae, Lachnospiraceae and Clostriaceae families (56, 659).
These families of Firmicutes are oxygen sensitive while *Bacteroides*, accounting for a significant proportion of Bacteroidetes, can survive in nanomolar quantities of oxygen concentration (660). With inflammation, oxidative burst occurs due to the release of reactive oxygen species by neutrophils (661). In addition, increased vascularisation with inflammation increases oxygen delivery to support the inflammatory process (662, 663). In remission, reduction in dysbiosis is associated with increase in the families of Firmicutes mentioned above.

The abundance of Proteobacteria was higher with inflammation in active CD and UC compared to HC as well as in remission, although a larger UC cohort would be needed to confirm this finding. The increased relative abundance of Proteobacteria in IBD and with disease severity in CD has been previously reported (389, 659). Whether this dysbiosis existed prior to IBD development or as a consequence of IBD has yet to be determined.

Genetic mutations affecting bacterial handling have been associated with IBD (664, 665). In addition, healthy siblings and family members of IBD patients also harbour dysbiotic bacterial profiles, perhaps due to the same genetic defects and/or environmental exposures (666). Together, this suggests that the dysbiosis may exist prior to developing IBD, which may have been triggered by an event not shared by other members of the family. This would explain the higher abundance of Proteobacteria seen in one of the HC of this study, who has a family member with CD. On the other hand, in support of inflammation leading to increased Proteobacteria, the increased oxygen concentration with inflammation encourages facultative anaerobes such as Proteobacteria whilst being a disadvantage to obligate anaerobes such as some species of Firmicutes and Bacteroidetes (661, 667). This could be a factor that exacerbates dysbiosis.

Dysbiotic profiles were observed mostly in treatment-naïve patients, however, there was also dysbiosis seen in reported remission. In addition, relatively normal stool profiles were also observed in patients who relapsed on treatment. Although a seemingly inconsistent finding, this would be similar to other studies, some reporting dysbiosis with inflammation and some reporting dysbiosis irrespective of disease activity (659, 668). The persisting dysbiosis in remission may be due to underlying genotype and/or ongoing environmental exposures. Furthermore, the patients with less dysbiotic stool profiles were more likely to achieve remission with treatment whilst a more dysbiotic profile was associated with more severe disease course, as previously reported (56, 659). The effects of treatment are discussed in Section 3.5.4.4.
The proportions of Bacteroidetes, Firmicutes and Proteobacteria were highly variable between IBD patients, which reflected the wide variability in beta diversity. This could be the result of uncontrolled colonisation associated with dysregulated immune function. In addition, the alpha diversities with inflammation, be it pre-treatment or during relapse, were lower in IBD compared to HC. The alpha diversity in relapsed active IBD was also lower when compared to NIC. This finding concurs with larger studies in PIBD (659, 669).

In addition to the 3 main phyla, Verrucomicrobia was only present in remission in the patient with Fistulising CD, but like Proteobacteria, the relative abundance was higher than in HC. It may be that the presence of these less abundant phyla are detectable due to the relatively lower abundances of other phyla. There were no other IBD patients harbouring Verrucomicrobia. Generally, it is reportedly less abundant in IBD (670) and obese patients (671). Verrucomicrobia, namely *Akkermansia muciniphila*, when increased with prebiotics resulted in improved barrier function and insulin sensitivity (634, 672), thus potentially explaining its beneficial presence.

Therefore, stool dysbiosis with inflammation in CD was characterised by the dominance of Bacteroidetes, and associated with reduced bacterial diversity. Similarly, proportions of Proteobacteria were higher in active disease. In remission, Bacteroidetes and Firmicutes dominated with the relative increase in Firmicutes.

### 3.5.4.2 Dysbiotic Distal Intestinal Mucosal Profile in Non-inflammatory Bowel Disease and Crohn’s Disease

The luminal and mucosal environments differ thus differences in microbial profiles may exist. Variations between the TI and colonic mucosal microbiota were investigated as the difference between their structure and function may be associated with different microbial composition. Also, inflammation could alter the microbiota as many of the CD patients had histological evidence of TI inflammation, and by definition, the UC patients had normal TI histology. Comparison was conducted between NIC and IBD patients.

Similar TI and colonic microbial profiles were observed in NIC patients, except for a patient (Patient A12) with a history of food allergies and preceding episode of gastroenteritis, which may account for the significant dominance of Proteobacteria in the TI. The majority of NIC patients had mucosal profiles dominated by Firmicutes and/or Bacteroidetes. Verrucomicrobia was absent from the mucosa of IBD patients but present.
in 3 NIC patients. These findings may well be the normal profile in healthy children but without a truly healthy cohort for comparison, this could not be concluded.

In IBD, despite the individual variations in mucosal microbial profiles, the TI and colonic mucosal profiles were similarly dysbiotic in all UC and most CD patients, as has been previously reported (56). This was associated with reduced bacterial diversity in the colonic samples of treatment-naive and relapsed active IBD patients. There was also reduced diversity in the TI samples of treatment-naive IBD patients compared to NIC. Comparison between CD and UC found similarly low bacterial diversity of the colonic samples but higher diversity in the TI of CD patients than UC, pointing towards the dysregulated bacterial colonisation in the inflamed TI of CD but not UC patients.

In CD, the dysbiotic mucosal profiles included absence of Firmicutes in the colonic samples of a patient (Patient A15) and absence of Bacteroidetes in all biopsies of another patient (Patient A25 with Fistulising CD). In these mucosal biopsies, the facultative anaerobic Proteobacteria significantly dominated the profiles. Families of Firmicutes are known to decrease in CD, namely Lachnospiraceae and Ruminococcaceae, of which Faecalibacterium prausnitzii is commonly reported on (56, 385). In the remaining CD patients, Bacteroidetes dominated the TI and colonic profiles, except in a patient with mild disease course (Patient A17), in whom Firmicutes dominated.

Proteobacteria was present in the mucosal samples of some NIC patients but present in all IBD patients, dominating in some of the IBD patients. The higher abundance of Proteobacteria is a consistent finding that is widely published (56, 659). This accounts for the lower Bacteroidetes:Proteobacteria ratios in CD compared to NIC. Proteobacteria was, however, relatively less abundant in a patient who achieved remission with EEN (Patient A17) compared to the other CD patients, thus suggesting less severe disease with higher likelihood of responding to treatment. Proteobacteria was also relatively less abundant in a patient already on treatment and commencing Infliximab (Patient D4). This suggests that treatment influences bacterial handling in addition to ameliorating inflammation, although was insufficient to prevent disease relapsed.

Differences between TI and colonic profiles were observed in a treatment-naïve CD patient. These variations likely reflected the severity of mucosal inflammation, as more significant inflammation was reported in the colonic samples. In patients who had regions spared from inflammation (Patients A17 and D4), the profiles remained similar (at phyla level) to the inflamed sites, thereby suggesting that dysbiosis exists prior to development
of inflammation as it is evident even in unaffected sites. There have been reports of similar dysbiosis observed in IBD patients in active disease and in remission (385, 668). In these 2 study patients, their mucosal profiles were observed to be less dysbiotic with relatively less Proteobacteria at all sites, thus may be another explanation for the similarly dysbiotic mucosal profiles irrespective of inflammation.

Thus, in general, the mucosal dysbiosis in CD was similar to the stool profiles; Bacteroidetes dominated although was absent in the patient with Fistulising CD. Mucosal Proteobacteria was also increased. These mucosal profiles were associated with low bacterial diversity compared to NIC with CD patients having increased diversity in the TI compared to UC.

3.5.4.3 Bacteroidetes:Proteobacteria (B:P) and Firmicutes:Proteobacteria (F:P) Ratios in CD

The symbiotic commensal community exists in balanced proportions that aids beneficial functioning in health. The disruption of the microbial balance favouring health i.e. dysbiosis can be quantified as a ratio of the taxa of functional importance.

Given the observed shifts in bacterial phyla in the IBD cohort, Bacteroidetes:Proteobacteria (B:P) and Firmicutes:Proteobacteria (F:P) ratios were investigated and found to be different in CD and UC patients. In the stool, the B:P ratios were lower in active CD (n=9) and in remission (n=6) compared to HC. In addition, F:P ratios were also lower in CD, and was significantly lower in active disease. The lower Firmicutes and Bacteroidetes, and higher Proteobacteria in IBD discussed above would account for these results.

In the mucosal samples, the B:P ratios were similarly lower in CD (n=6) at all 3 sites compared to NIC (n=15). These, however, were not statistically significant, perhaps due to the small number of samples.

3.5.4.4 Alterations in Microbial Profiles with Treatment

IBD treatment can be effective at controlling the inflammatory processes. This study investigated how this could affect the intestinal microbiota.
Exclusive Enteral Nutrition

EEN is the first-line treatment for CD, as discussed in Section 3.1.4.1. Two patients in this study received EEN, one of whom received a course of Trimethoprim (Patient A15), as discussed above. The other patient (Patient A17) responded well to EEN and achieved remission. Comparing these patients pre-treatment, Proteobacteria was less abundant in the stool and mucosa of the patient who achieved remission. Proteobacteria was absent during EEN, and was detected after the EEN course, although Bacteroidetes continued to dominate the profiles. Thus microbial profiles may be useful in predicting treatment response (659).

EEN is superior to Prednisolone in achieving mucosal healing thus is recommended as the first-line treatment for PIBD (67, 70). The molecular mechanisms for this remains largely unknown. Intestinal inflammation can be alleviated by removing pathogenic stimulus, such as by diversion of stool with ileostomy formation (673) and fasting with parenteral nutrition (674). EEN appears to have a similar effect.

Gerasimidis et al. reported reduction of pathogenic as well as beneficial organisms with EEN (437). However, Lactobacillus, a homo-fermenter, increased with associated reduction in pH, presumably with increased production of lactic acid. This protects the host from many pathogenic organisms during recolonization with food reintroduction, similar to breastfed infants (675). A healthy balanced diet after EEN would encourage recolonization with beneficial microbial community but an unhealthy diet would facilitate the return of the dysbiotic profile. In addition, C. coccoides, another commonly found species in infants and a known butyrate-producer, also increased with EEN (676-678).

A similar effect can be achieved with fasting but this would not be appropriate for children with CD, many of whom would have lost weight and require adequate nutrition for catch-up growth and development. Nevertheless, patients with fistulising CD were previously treated with a period of parenteral nutrition with no enteral intake. Anti-TNF agents have now replaced this.

Immunosuppressive Agents

In contrast to EEN, Prednisolone and Infliximab alters host immune responses. This was associated with beneficial alterations in stool microbial profiles in patients post-treatment, specifically with reduction or absence of Proteobacteria. Corticosteroids and anti-TNF
agents have been reported to restore bacterial diversity and ameliorate intestinal dysbiosis in IBD patients (389, 458, 463). The more favourable microbial profiles suggest better bacterial handling in these patients.

Antibiotics

The effects of antibiotics on the intestinal microbiota have been well documented (56, 172, 174, 176). This was also observed in this study in Patient A20, as discussed in Section 3.5.2.3. In addition, a CD patient (Patient A15) had an episode of Norovirus gastroenteritis and urinary tract infection, which was treated with Trimethoprim, during the EEN course. Despite clinical improvement with EEN, this patient had decreased Bacteroidetes associated with increased Proteobacteria and Firmicutes abundances in the stool after EEN. The stool dysbiosis, perhaps in addition to the Proteobacteria dominance in the mucosal samples, may have contributed to the subsequent relapse in this patient.

In short, EEN alters the microbial composition, reducing intestinal bacterial burden and immune responses. Immunosuppressive agents control the dysregulated immune responses, enabling restoration of mucosal integrity and barrier function. In addition, antibiotics distort the microbial composition unfavourably, increasing the proportion of Proteobacteria.

3.5.5 Dysbiosis in Ulcerative Colitis
3.5.5.1 Dysbiotic Stool Profile in UC

In contrast to CD, Bacteroidetes was lower in active UC as well as in remission, with Firmicutes dominating the stool profiles in active UC (Figure 3.23 Ulcerative Colitis). Many of the butyrate-producers are from the phylum of Firmicutes (679). The relative increase of Firmicutes could be an attempt to increased butyrate production to facilitate mucosal healing in UC, which is characterised by diffuse, continuous inflammation of the colonic mucosa.

As with CD, increased Proteobacteria was observed in active UC. In addition, as mentioned above, the stool alpha diversities were lower with inflammation compared to HC. Another trend was increased Actinobacteria with clinical improvement or remission. This was evident in 3 UC (total n=4) and 1 IBDU (total n=2), as well as 1 CD (total n=20)
patients. Increased Actinobacteria (discussed in Section 3.5.2.1), incorporating the orders *Bifidobacteriales* and *Actinomycetales*, has been reported in IBD (432, 680). *Bifidobacteriales* is also beneficial and has been developed commercially as probiotics. The expansion of Actinobacteria with clinical improvement and early remission may reflect increased antimicrobial defence mechanisms at play during this period.

### 3.5.5.2 Dysbiotic Distal Intestinal Mucosal Profiles in UC

The main difference between CD and UC in the TI and colonic mucosal samples were the proportions of Bacteroidetes and Firmicutes. In contrast to CD, Bacteroidetes was lower or absent in treatment-naïve UC. Interestingly, the patient with Bacteroidetes present in the mucosal biopsy achieved remission with a course of Prednisolone, suggesting a milder disease course with a less dysbiotic profile.

As a result, Firmicutes appeared to be more abundant in UC patients, perhaps as a response to the needs of the host with mucosal inflammation. As mentioned above, many of the butyrate producers belong to the phylum of Firmicutes. As inflammation reduces, and potentially oxygen concentration declines, the luminal environment may then be more hospitable for the obligate anaerobic Bacteroidetes to emerge and thrive, further aiding mucosal healing and normal function.

In addition, as with CD, mucosal Proteobacteria was increased in the TI and colonic samples, dominating in 2 of the patients. One of these patients responded well to Prednisolone and remained in remission, thus the significant dominance by Proteobacteria was not associated with a severe disease course for this patient, perhaps due to the concurrent presence of Bacteroidetes in all mucosal samples. As with the stool samples, the TI and colonic mucosal samples also had reduced alpha diversities.

Comparison between TI and colonic mucosal biopsies in UC resulted in a surprising finding. Despite the absence of histological inflammation in the TI, the microbial profiles were similar to the colon, with reduced or absent Bacteroidetes. This confirms that dysbiosis can exist without inflammation, thus does not in itself cause inflammation. This finding also suggests that the need for Bacteroidetes varies between TI and colon. A major difference is the need for butyrate as a source of energy for colonocytes. Small bowel mucosa utilises glutamine, glutamate and aspartate (681). Thus, this potentially implicates inadequate butyrate as a significant factor in UC pathogenesis.
3.5.5.3 Bacteroidetes:Proteobacteria (B:P) and Firmicutes:Proteobacteria (F:P) Ratios in UC

In the stool, the Bacteroidetes:Proteobacteria ratios were significantly lower in active UC (n=3) compared to HC (Figure 3.21A). These are the result of the lower Bacteroidetes and higher Proteobacteria as discussed above. Due to the small numbers, it was not possible to analyse UC in remission.

Similarly in the TI and colonic mucosal samples, the combination of decreased Bacteroidetes and increased Proteobacteria resulted in the lower Bacteroidetes:Proteobacteria ratios in UC compared to NIC. The ratios were significantly lower in the LC, where inflammation is characteristically worse in UC. The ratios also appeared lower than CD but this was not statistically significant.

3.5.5.4 Alterations in Microbial Profiles with Treatment

The UC patients recruited to this study received Prednisolone for induction of remission. In addition, a patient with severe UC, who received Sirolimus, was also recruited. Associated with clinical response to treatment, Proteobacteria was observed to decrease in the stool profiles. In addition, Bacteroidetes increased in a patient who achieved remission. Treatment, therefore appears to shift the microbial composition towards symbiosis. Interestingly, in some patients, mostly UC, Actinobacteria emerged with clinical improvement or remission, as discussed above.

Mucosal samples pre- and post-treatment in 2 UC patients (one who received Prednisolone and another who received Sirolimus) also demonstrated similar findings. Bacteroidetes was absent in the mucosal pre-treatment samples but present post-treatment. This occurred despite Bacteroidetes being present in the pre-treatment stool of 1 patient, and Bacteroidetes being absent from the post-treatment stool in the other patient. In addition, Proteobacteria decreased in the post-treatment samples. These findings suggest that treatment favourably alters the mucosal microbial composition, even with disease relapse. Treatment thus appears to improve bacterial handing at the mucosal level, even if that effect is insufficient to prevent relapse of disease.

Corticosteroids are usually prescribed alongside a proton pump inhibitor (PPI). Although PPI is known to influence intestinal microbiota (460, 682), this was not evident from the current analysis.
In summary, the dysbiosis in UC is characterised by reduced Bacteroidetes and increased Proteobacteria, associated with reduced alpha diversity in the stool and mucosal samples. The alterations observed suggest reduced butyrate production as a potential factor in disease pathogenesis. Treatment with immunosuppressive agents appears to influence stool and mucosal bacterial colonisation, increasing Bacteroidetes presence and reducing Proteobacteria. In addition, Actinobacteria may play a role in disease amelioration in UC. The significantly lower stool and mucosal Bacteroidetes:Proteobacteria ratios may be a useful clinical tool for disease monitoring, although greater patient numbers would be required to confirm this finding.

3.5.6 Correlation between Stool and Colonic Microbial Profiles

Several studies report stool microbial profiles but how representative these are of the profiles at the mucosa level, at the interface with the host has not been clearly elucidated. In this study, this aspect was investigated further in patients with histological evidence of inflammation compared to those without inflammation.

Most studies report the microbial profiles of stool or intestinal mucosal biopsies but few report both profiles of a patient obtained at the same time. In these studies, the mucosal profiles were similar but differed from the profiles of stool or colonic lavages. The authors reported greater bacterial abundances and diversity in the luminal samples, while another study reported similar diversity and proportions (56, 683, 684).

Even in the absence of mucosal inflammation, stool microbial profiles can differ from the colonic profiles. This was evident in some NIC patients (Patients A1, A4, A6 and A13), in whom there were increased mucosal Proteobacteria or Verrucomicrobia compared to the stool samples.

Similarly, in most IBD patients, the proportions of mucosal Proteobacteria were higher than in the stool, and generally more abundant than in NIC patients. In addition, in IBD, Bacteroidetes was less abundant in the mucosal samples than in stool. Despite being present in the stool, Bacteroidetes was absent from colonic samples of a patient with CD and in another with UC. In addition, high proportions of stool Bacteroidetes can be associated with greater mucosal Proteobacteria, thus high Bacteroidetes abundance does not protect the mucosa from significant colonisation with Proteobacteria. This therefore suggests barrier dysfunction being a strong factor in these patients, and the
advantage Proteobacteria appears to have with inflammation. Indeed, inflammation seems to exacerbate dysbiosis as Bacteroidetes present in milder disease was then absent in relapse.

In addition, the presence of Verrucomicrobia in the mucosal biopsies did correlate with its detection in stool, being more abundant in the mucosal biopsies than in stool. Thus, stool microbial profile is not always representative of mucosal bacterial composition in the absence of inflammation. In IBD, stool and mucosal microbial profiles differ with the dysbiosis generally being more marked in the mucosal samples, thus stool microbiota in IBD patients cannot be used to represent mucosal microbiota. This highlights the mucosal barrier dysfunction in these patients.

**Correlation between Mucosal Bacterial Phyla with Calprotectin**

Calprotectin is a soluble protein contained in neutrophils. With inflammation, neutrophils are recruited to the inflamed site and released into the lumen with neutrophil cell death. It is now recognised to have bacteriostatic and antifungal properties (685, 686), and is widely used as a marker of intestinal inflammation, assisting in IBD diagnosis and monitoring. From this dataset, no correlation between calprotectin and bacterial phyla was evident.

**3.5.7 Microbial Profiles in Severe IBD**

Patients with severe IBD were recruited to the study to investigate the possibility of a more dysbiotic profile with severe disease. Consistent with published reports (56, 659, 687), this was the case with the study patients who had severe IBD. Greater Proteobacteria dominance and the absence of Bacteroidetes in the stool and mucosal profiles were observed in these patients compared to those with milder disease. With clinical improvement, Proteobacteria decreased. The association of disease severity and degree of dysbiosis points to the crucial role of the intestinal microbiota in IBD.
3.5.7.1 Microbial Profiles of Ileostomy and TI Mucosal Samples

There were 2 patients in the study with refractory IBD, one who previously had subtotal colectomy (Patient B3) and another who had a colectomy during the study period (Patient C3). Both patients had ileostomy profiles consisting predominantly of Proteobacteria. Firmicutes emerged in Patient B3 when clinically improved after HSCT for IL-10RB mutation. In Patient C3, Firmicutes was also present and increased as Proteobacteria decreased with clinical improvement. Bacteroidetes, which was absent, emerged when the patient was well. It was interesting to note that the profile of the stool per rectum prior to the subtotal colectomy was similarly dysbiotic as the subsequent ileostomy samples.

Formation of ileostomy disrupts the luminal environment. One of the changes is the increased oxygen concentration which favours facultative organisms that can adapt to this change, such as Proteobacteria, facilitating their expansion. This alteration disadvantages the obligate anaerobes such as Bacteroidetes, preventing them from thriving (688). Furthermore, oxygen concentration is lower at the mucosal surface than within the lumen. This may potentially enable growth of the anaerobic Bacteroidetes and Fusobacteria, explaining their presence in the TI sample of Patient C3 despite its absence from the ileostomy sample.

3.5.7.2 Alterations in Microbial Profiles with Treatment

The study patients with severe IBD received Sirolimus, as discussed in Section 3.5.5.4, and HSCT for VEOIBD and IL-10RB mutation. Sirolimus alters host immune responses. This alteration is permanent with HSCT, following which the immune system is essentially re-programmed or replaced with that of the donor.

In this study, beneficial alterations in stool microbial profiles were observed in patients post-treatment, with reduction or absence of Proteobacteria and absence of Synergistetes in a patient post-HSCT. In addition, Bacteroidetes increased in 2 of the patients and continued to dominate in another. The less dysbiotic microbial profiles associated with clinical improvement suggest improved bacterial handling with treatment, in addition to more regulated immune responses.
3.5.8 Microbial Profiles in Other Diseases

**IBDU**
This study also included analysis of other IBD subtypes. There were 2 IBDU patients in active disease, whose profiles were similar to active UC. Firmicutes dominated the profiles with B:P ratios that were also low, similar to active UC. Mucosal biopsies received from a patient revealed similar TI and colonic profiles which all exhibited dysbiosis as seen in IBD. These findings point towards a pathogenesis more similar to UC than CD in IBDU. As yet, little is known of this less common IBD subtype.

**Collagenous Colitis**
A patient with Collagenous Colitis was also recruited and observed to have a stool profile similar to HC. Bacteroidetes and Firmicutes dominated the stool profile, similar to the findings by Halfvarson et al. (270). Also, the mucosal profiles were similar to NIC, and comparable between the biopsy sites irrespective of inflammation. The reported symptoms in this patient resolved without any treatment, suggesting mild disease associated with the normal stool and less dysbiotic mucosal microbial profiles.

**Coeliac Disease**
One of the CD patients was also diagnosed with Coeliac Disease (Patient D2). Interestingly, the profile was more characteristic of Coeliac Disease. The stool profiles were mostly dominated by Firmicutes or Bacteroidetes, with increased Proteobacteria associated with active disease. In infants who developed Coeliac Disease, increased Firmicutes and Proteobacteria, associated with reduced Actinobacteria, have been reported (564, 565). In contrast, increased Bacteroidetes was reported in older children (568). Patients with Coeliac Disease are also reported to have increased bacterial abundance. Despite normalisation, the highest number of sequence reads were from an infant with a family history of Coeliac Disease and Patient D2, although this was not significantly so.

**Granulomatosis Polyangiitis**
This CD patient who was also diagnosed with Granulomatosis Polyangiitis was in active disease throughout the study. Despite this, the stool and mucosal profiles were dominated by Bacteroidetes and/ or Firmicutes with absent or lower Proteobacteria, unlike other IBD patients and more similar to HC and NIC respectively. Although the GI symptoms were similar to IBD, the intestinal microbial profiles suggest a different
pathogenesis occurring in this patient, and highlights the dysregulated bacterial handling in IBD patients.

**Cryopyrin-Associated Periodic Syndrome**

CAPS is a systemic inflammatory condition, and these patients with CAPS reported GI symptoms, thus was investigated for microbial dysbiosis. Their stool profiles were in fact similar to HC, although Proteobacteria was higher in a patient soon after the first dose of Canakinumab. In addition, Actinobacteria and Verrucomicrobia were detected as disease improved on treatment. Despite these mild alterations, the microbial profiles were largely stable with changes in clinical status. This would suggest that microbial dysbiosis is not a major factor in this disease although mild shifts towards dysbiosis was observed with ill health. However, these shifts could be within the expected range of HC with common, mild illnesses but these have yet to be better defined.

**Cow’s Milk Protein Allergy**

The analysis of intestinal microbiota in CMPA was restricted by the patient numbers but the results of this study is consistent with published reports (584, 689, 690). HI were younger and at various stages of weaning. Like the HI cohort, allergic infants had profiles dominated by Bacteroidetes or Firmicutes. However, unlike the HI, Proteobacteria was just detectable or absent. In addition, beneficial Actinobacteria was not detected in an allergic infant but was detected in all HI. The alpha diversity was also lower in an allergic infant compared to the HI despite being older and having more food. Together, these findings support the Hygiene Hypothesis. Early exposure to a diverse community of organisms, together with genetic factors, shape the colonising intestinal microbiota that educates and programmes the immune system.

Comparison between these patients and the IBD cohort has highlighted the dysfunctional mucosal barrier and dysregulated bacterial handling associated with IBD. The dysbiosis in IBD is different to other inflammatory conditions such as Coeliac Disease and CAPS, despite these patients also having GI symptoms.

**3.5.9 Clinical Implications**

The differences in microbial compositions observed between CD and UC, as well as between IBD and other inflammatory conditions, make this a potential diagnostic tool that could be utilised alongside current diagnostic investigations.
There were common findings in patients with severe IBD, therefore enabling their identification. This could alter their management to include closer monitoring and potentially more aggressive approach to their treatment.

Stool microbial profiles offer another non-invasive method of disease monitoring. Admittedly, calprotectin is a reliable marker and more established in clinical practice. Nevertheless, microbiota profiles offer insight into the bacterial shifts occurring within the intestine rather than more general, systemic markers such as calprotectin and ESR. Experience of microbial profiles in clinical practice may help determine its reliability as a monitoring tool.

The study results have highlighted two mechanisms in treating IBD. Conventional medications control the inflammatory responses to enable mucosal healing and intestinal barrier repair. EEN, however, alters the microbial profiles to reduce the burden of pathogenic microbes. In addition, the increased Lactobacillus and lactic acid production favour recolonization with beneficial microbes. Thus, it may be possible to reset the intestinal microbiota to a less dysbiotic profile. Therefore, potentially EEN could be used in other conditions with associated dysbiosis. One such example is JIA. Berntson et al. reported on 13 children with JIA who had significant anti-inflammatory effects from EEN over 3-8 weeks (691). The role of EEN in JIA is being further explored.

This study has also highlighted the detrimental effects of antibiotics. Whilst necessary in some cases, repeated administration and the long-lasting effects on the colonising microbiota could have significant impact in early childhood, with health consequences in later life. Measures such as routine use of probiotics after courses of antibiotics ought to be considered for younger children to minimise this impact.

Although this was only observed in one child, the finding was striking. This child, who has a family member with CD, had the highest Proteobacteria abundance. Family members and those living with IBD patients are known to harbour dysbiotic profiles (432, 631, 666, 692) but this may also suggest that this child is genetically at risk of IBD. This raises the question of whether disease risk can be altered during early childhood if a genetic mutation was identified, as demonstrated in other conditions such as CMPA (693). With genetic analysis increasingly used in the clinical setting, this information may be made available in the future.
3.5.10 Research Implications

This study has demonstrated differences between the microbial profiles of stool and intestinal mucosal biopsies. As there are many studies analysing one of these samples in isolation, it is important to bear in mind that the stool profiles do not always correlate with mucosal profiles in IBD.

As some of the NIC patients had dysbiotic microbial profiles, albeit milder than IBD, this ought to be taken into consideration when these patients are used as control groups.

3.5.11 Study Limitations

This study would have benefitted from larger cohorts of control and diseased groups. Despite this, differences were identified and the smaller cohorts enabled more detailed analysis to be done. As there are potential applications from these results, these findings would benefit from confirmation and validation with bigger numbers in each group.

Having two methods for concentrating duodenal lavages was not ideal but occurred out of necessity. Although there were no apparent differences observed because of this, ideally consistency was maintained.

Despite these limitations, this study has highlighted important differences, some of which are significant and many worth exploring further.

3.5.12 Conclusion

The intestinal microbiota is a dynamic entity, its composition continually shifting in response to environmental and host factors. The samples collected offer insight into processes occurring at that time within the intestinal lumen and at mucosal surfaces. Longitudinal samples from the same patients enabled better understanding of these processes.

With carefully optimised methods, comparison with HC and NIC enabled characterisation of the microbial composition of stool and mucosal biopsies of IBD patients. In so doing,
differences between CD and UC were identified and variations with treatment and
disease severity observed. Reduced luminal bacterial diversity results in less organisms
for particular functions such as butyrate production, which decreases host resilience to
the effects of dysbiosis. On the other hand, increased mucosal bacterial diversity points
to the defective mucosal barrier function in IBD, leading to hyper-stimulation of the
dysregulated immune system. In addition, these findings have highlighted the possible
mechanism for EEN. Together, these reaffirms the crucial role of the intestinal microbiota
in IBD development, pathogenesis, treatment and disease progression.
4. Chapter 4

Short Chain Fatty Acids

4.1 Introduction

Short chain fatty acids (SCFA) are saturated aliphatic organic acids consisting of 2-6 carbons. These include acetate (C2), propionate (C3), butyrate (C4), valerate (C5) and caproate (C6) (Figure 4.1).

More than 95% of SCFA in the human gut are acetate, propionate and butyrate (694) and their functions are more widely-known. These SCFA exhibit unique and overlapping functions which are discussed in further detail below.
4.1.1. SCFA Production

SCFA are produced by anaerobic bacteria during fermentation of food, a metabolic process in the absence of oxygen which converts NADH to the coenzyme NAD$^+$ to be utilised for glycosis and releases 2 ATP per glucose molecule (compared to 32 ATP per glucose molecule during aerobic respiration). Fermentation mostly occurs in the proximal colon where substrate availability and bacterial activity are highest. The extraction of free water increases diffusion of substrates and microbial products. SCFA, gases (CO$_2$, CH$_4$ and H$_2$) and heat are the end products of this anaerobic fermentation (695).

SCFA production is determined by several factors as discussed in the following sections.

4.1.1.1 Substrate

The amount and type of substrate i.e. indigestible fibre content of host diet greatly affects SCFA production (694). Approximately 75-100% of plant dietary fibres are complex carbohydrates (oligosaccharides and polysaccharides) resistant to hydrolysis. They escape small bowel digestion and absorption, entering the colon (696, 697) where they serve as substrates (698). Examples of edible indigestible plant fibres include pectin, resistant starch (milled grains and seeds, some legumes), wheat bran and oats (699, 700).

4.1.1.2 Gut Microbiota

The composition of gut microbiota i.e. abundance and types of bacteria influences the quantities as well as the type of SCFA being produced (701). Despite these variations in individual profiles, the microbial metabolic functions remain fairly constant among individuals as studied by the Human Microbiome Consortium. This is mainly due to redundancy in biochemical pathways as they can be activated by alternative members of the microbiome (268, 702-705).

Composition of the microbiota is influenced by pH, which increases from caecum to rectum due to the exchange of SCFA anions for bicarbonate (Figure 4.2) (236, 706, 707). In addition, this environment prevents overgrowth of pH-sensitive pathogenic bacteria e.g. Enterobacteriaceae and Clostridia (268, 704, 705).
Acetate, propionate and butyrate are produced by organisms from the two main phyla, Bacteroidetes and Firmicutes (Table 4.1). These phyla are hypothesised to exist in symbiosis whereby Bacteroidetes produce acetate, which is then utilised by Firmicutes to produce butyrate and propionate (708), (709). Some species such as Eubacterium rectale, Faecalibacterium prausnitzii, and Roseburia intestinalis preferentially colonize the mucus layer thus increasing butyrate availability for colonocytes (710, 711).

Bacteria themselves are hydrolysed in the distal colon by host urea to produce bacterial proteins and amino acids (Figure 4.2). These are broken down further by secondary fermenters, resulting in branched-chain fatty acids and potentially toxic metabolites (712).

**Figure 4.2: Gut microbial and Functional Variations in the Proximal and Distal Colon**

Firmicutes make up most of the proximal microbial population, producing mostly butyrate. Bacteroides, which dominate in the distal colon, predominantly produce acetate and propionate. In addition, bacteria, mainly Bacteroidetes, are hydrolysed by host urea producing bacterial proteins and amino acids in the proximal colon. These are later broken down further by secondary fermenters in the distal colon producing branched-chain fatty acids and other metabolites (695, 703, 712).
<table>
<thead>
<tr>
<th>Genera of Acetogenic Bacteria *</th>
<th>Propionate-producing Bacteria (713)</th>
<th>Butyrate-producing Bacteria (714, 715)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum: Firmicutes</td>
<td>Phylum: Firmicutes</td>
<td>Phylum: Firmicutes</td>
</tr>
<tr>
<td>Class: Negativicutes</td>
<td>Class: Negativicutes</td>
<td>Class: Clostridia</td>
</tr>
<tr>
<td>Order: Selenomonadales</td>
<td>Species: <em>Phascolarctobacterium</em></td>
<td>(esp Clostridial clusters IV and XIVA)</td>
</tr>
<tr>
<td>Species: <em>Acetonema longum</em></td>
<td>succinatutens</td>
<td>Order: Clostridiales</td>
</tr>
<tr>
<td><em>Sporomusa ovata</em></td>
<td>Veillonella parvula</td>
<td>Family: <em>Clostridiaceae</em></td>
</tr>
<tr>
<td>Class: Clostridia</td>
<td><em>Dialister succinatophilus</em></td>
<td>Species: <em>Faecalibacterium praunitzii</em> **</td>
</tr>
<tr>
<td>Order: Thermoanaerobacterales</td>
<td><em>Megasphaera elsdenii</em></td>
<td>**Species: <em>Eubacterium rectale</em> **</td>
</tr>
<tr>
<td>Species: <em>Moorella thermoacetica</em></td>
<td></td>
<td>**Genus: <em>Butyricicoccus</em></td>
</tr>
<tr>
<td><em>Thermoacetogenium phaeum</em></td>
<td></td>
<td>**Family: <em>Ruminococcaceae</em></td>
</tr>
<tr>
<td><em>Thermoanaerobacter kivui</em></td>
<td></td>
<td>**Family: <em>Lachnospiraceae</em></td>
</tr>
<tr>
<td>Order: Halanaerobiales</td>
<td>**Species: <em>Roseburia inulinivorans</em> (B)</td>
<td>**Genus: <em>Roseburia</em></td>
</tr>
<tr>
<td>Species: <em>Acetohalobium arabaticum</em></td>
<td></td>
<td><strong>rectalis</strong> **</td>
</tr>
<tr>
<td><em>Fuchsiiella alkaliacetigena</em></td>
<td>**(B) also produce butyrate</td>
<td>**Genus: <em>Anaerostipes</em></td>
</tr>
<tr>
<td><em>Natroniella acetigena</em></td>
<td>**(B) also produce butyrate</td>
<td>**(in colonic lumen)</td>
</tr>
<tr>
<td>Order: Clostridiales</td>
<td>**</td>
<td>**On colonic mucus layer</td>
</tr>
<tr>
<td>Family: <em>Lachnospiraceae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species: <em>Acetitomaculum ruminis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Blautia productus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Marvinbryantia</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>formatexigen</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Syntrophococcus sucromutans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family: <em>Peptostreptococcaceae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species: <em>Acetoanaerobium noterae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family: <em>Eubacteriaceae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species: <em>Acetobacterium woodii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Eubacterium limosum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family: <em>Clostridiaceae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species: <em>‘Butyrnbacterium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>methylotrophicum</em>, <em>Caloramator fervidus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium aceticum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Oxobacter pfennigii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Natronincola</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>histidinovorans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tindallia californiensis</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
*First species of each genus classified as acetogen displayed

Table 4.1: Organisms producing Acetate, Propionate and Butyrate in the Human Colon

*Most reported acetogens are Gram positive. Genera Acetobacterium and Sporomusa are exclusive acetogens while genera Clostridium, Blautia, Eubacterium, Thermoanaerobacter Treponema produce acetogens and non-acetogens (716).

The gut microbiota works in symbiosis with the host and with each other. An example of this is mutual cross-feeding (Figure 4.3). Archaea utilise CO₂ and H₂, which are produced during fermentation, to produce methane (CH₄). Acetate-producing bacteria, known as acetogens, convert the CO₂ and H₂ produced during fermentation into acetate (Table 4.1). The H₂ from acetate production is then used by other bacteria thus avoiding excessive accumulation of H₂. As a mutually beneficial role, the host provides the CO₂ needed for electron transfer chain, part of which is the exchange of HCO₃⁻ with SCFA anions in the gut lumen. In addition, almost 50% of host-derived urea is hydrolysed in the colon to release nitrogen, which is essential for bacterial growth (717).
Food escaping small bowel digestion is fermented in the proximal colon by acetogenic primary fermenters to produce acetate, $\text{CO}_2$ and $\text{H}_2$. The acetate can be further utilised by secondary fermenters more distally to produce additional SCFA. Acetate-producing bacteria, acetogens, utilises the $\text{CO}_2$ and $\text{H}_2$ generated during fermentation to produce more acetate. Butyrate-producers utilises acetate to produce butyrate whilst also generating Acetyl coA.

4.1.1.3 Gut Transit Time

The duration of time that the substrate is in the gut i.e. gut transit time can also influence the production of SCFA (718, 719). As substrate availability changes during gut transit, it may influence the bacterial population and number within the length of the gut (720).

4.1.2 Functions of Short Chain Fatty Acids in Health

SCFA play a significant role in gut health thus it is unsurprising that diversion colitis occurs when faecal stream is halted following colectomy (721). Most studies report on the main SCFAs, acetate, propionate and butyrate, thus we have a greater
understanding of their roles compared to valproate and caproate. Here, their known functions are discussed.

### 4.1.2.1 SCFA as an Energy Source

SCFA provide ~10% of our daily caloric requirements (722), and 60-70% of the energy required by colonocytes (723-725). More than 95% of SCFA in the human gut are acetate, propionate and butyrate (694), generally present in molar ratio of 57:22:21 in the caecum, distal colon and stool (236). Butyrate is used as colonocyte fuel preferentially over propionate and acetate (694), and is preferred over glucose or glutamine supplied by blood (726). Following whole gut transit time of over 50 hours, butyrate is no longer detected as 70-90% of butyrate is rapidly taken up and metabolised by colonocytes (694, 695).

Once absorbed into the systemic circulation, the ratio alters to 71:21:8 in portal veins and 81:12:7 in hepatic veins (236). SCFA is metabolised at two other major sites, the liver and muscles. Hepatocytes extract 90% of propionate, which is used for gluconeogenesis. Approximately 75% of acetate is also utilised by the hepatocytes (727, 728). In muscles, myocytes also generate energy from oxidation of residual acetate (701).

### 4.1.2.2 SCFA in Gut Ion Transport

SCFA uptake from gut lumen occurs in a concentration-dependent manner (729). SCFA crosses the apical membrane of colonocytes by passive diffusion in its protonated form or via active mechanisms. In its ionised form, it can be taken up with H+ by monocarboxylate transporter 1 (MCT1) (730), with Na+ by Sodium-dependent monocarboxylate transporter 1 (SMCT1) (731) or exchanged with HCO3- by an unknown transporter (732-734). SMCT 1 has been reported to transport butyrate faster than propionate and acetate (735). SCFA anion transport via SMCT1 is coupled to Na+ in a ratio of 1:2 (731). Its uptake can also occur via transport of Na+ and K+ and transport of water (736), which occurs more distally than proximally (737).

SCFA stimulates Na+ and water absorption by modulating the expression and activity of Na hydrogen exchanger-3 (NHE3). SCFA activates this apical Na+/H+ transporter leading to water and salt absorption as needed to adapt to changes in salt and
carbohydrate intake (699, 738). Unoxidised SCFA leaves colonocytes through the basolateral membrane via MCT4 (739), and is also exchanged with HCO$_3^-$ via an unknown mechanism (734, 740).

4.1.2.3 SCFA as Signalling Molecules

SCFA exerts several effects as a signalling molecule. Many of its effects are mediated through the inhibition of histone deacetylases (HDACs). These are enzymes that remove acetyl groups from lysine amino acid on a histone. Histones are proteins in cell nuclei that arrange DNA into nucleosomes. By removing the acetyl groups, HDACs enable DNA to wrap themselves tightly around the histones (Figure 4.4). The effects of SCFA appear to be concentration-dependent with higher concentrations required (741).

In addition, SCFA are also ligands for G protein-coupled receptors (GPRs). These are:

1. GPR41, also known as free fatty acid receptor 3 (Ffar3)
2. GPR43, also known as Ffar2
3. GPR109A, also known as HCAR2 (742, 743)

These receptors differ in affinity for SCFAs, physiological roles, cell and tissue distribution (744, 745). Their varied interactions assist with host regulation of inflammation and metabolism, thus playing a role in maintaining homeostasis. GPR41 is widely expressed in the spleen, pancreas, lymph nodes, bone marrow and polymorphonuclear cells with the highest expression in adipose tissue. It has an affinity to butyrate and propionate (744, 745).

GPR43 expression is highest in immune cells e.g. monocytes, B-lymphocytes and polymorphonuclear cells (744-746), and present in bone marrow, spleen, pancreas, colon, white and brown adipose tissue (747). It has a higher affinity to propionate (744, 745). Its expression is critical for SCFA-induced neutrophil chemotaxis (748) and Treg cell function (195).

The activation of adipose-specific GPR43 by acetate and propionate (749, 750) suppresses insulin signalling which inhibits fat storage in adipose tissue and promotes lipid and glucose metabolism in other tissues (751). Both GPR41 and GPR43 co-localise with Peptide-YY (PYY), a satiety hormone, and appear to have a role in its expression and glucose tolerance (752-754).
GPR109A is expressed by numerous cell types, such as pancreatic beta cells, in the skin, adipose tissue and colonocytes, and responds to butyric acid. It is reported to prevent colitis and colon carcinogenesis by inducing differentiation of Treg cells and IL-10-producing T cells (755). In the gut, GPR43 and GPR109A activate the inflammasome cascade and increase IL-18 production (756), which has pro-inflammatory effects and involved in epithelial repair (757).

Figure 4.4: Acetylation and Deacetylation of Histones
Histone deacetylases (HDACs) remove acetyl groups from histone, enabling tighter wrapping of the DNA around it. On the other hand, histone acetyltransferases (HAT) acetylate the lysine amino acids on histone proteins. This is achieved by transferring an acetyl group from acetyl-CoA to form ε-N-acetyllysine. Histone acetylation generally increases transcriptional activity and gene expression. SCFA are able to inhibit the effects of HDAC thereby making DNA more accessible to transcription factors.

4.1.2.4 SCFA and the Regulation of Glucose Metabolism

SCFA appear to have a beneficial effect on glucose metabolism by normalizing plasma glucose concentrations and increasing glucose handling either via hepatic AMPK regulation pathway or indirectly via the gut derived hormones PYY and GLP-1 as discussed below.
Acetate and propionate are thought to reduce fasting plasma glucose via the AMPK pathway. In KK-A(y) mice, an animal model of human type 2 diabetes, which were fed acetate, reduced fasting plasma glucose, insulin, HbA1c concentrations and reduced expression of genes involved in gluconeogenesis and lipogenesis were observed (758). In normal rats fed propionate, decreased fasting glucose was also seen (759).

SCFA may also affect plasma glucose concentrations by increasing the gut hormones PYY and GLP-1 via activation of GPR41 and GPR43, which co-localize with enteroendocrine L cells containing PYY (752-754). The satiety hormone PYY (760), reinforces insulin action on glucose disposal in muscle and adipose tissue (761, 762). GLP-1, on the other hand, indirectly regulates blood glucose concentrations by increasing insulin secretion and glucagon secretion by the pancreas (763).

Propionate is better absorbed in the colon than acetate (764, 765), and transported to the liver where 90% of propionate is extracted during a single pass (727, 728). It is a substrate for hepatic gluconeogenesis and is also an inhibitor of gluconeogenesis (766). It enhances glycolysis in the liver and influences glucose metabolism indirectly by lowering plasma fatty acid concentration (767). The hypolipidaemia in turn inhibits cholesterol synthesis (768).

### 4.1.2.5 SCFA and the Regulation of Fatty Acid Metabolism

SCFA regulate the balance between fatty acid (FA) synthesis, FA oxidation and lipolysis. They activate FA oxidation and inhibit lipolysis resulting in reduction of free FA concentrations in plasma (750). It also increases AMPK activity in liver and muscle tissue (769-771). Activation of AMPK triggers peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α expression, which is known to control the activity of several transcription factors important in the regulation of cholesterol, lipid, and glucose metabolism (772, 773). As a consequence, FA oxidation is enhanced and de novo fatty acid synthesis in the liver is decreased.

In muscle and liver, SCFA stimulates FA oxidation by activating AMPK (pAMPK) directly by increasing the AMP/ATP ratio and indirectly via the Ffar2-leptin pathway. Leptin is a hormone that regulates energy expenditure and food intake. In white adipose tissue, via GPR43, SCFAs decrease insulin sensitivity and fat storage (774, 775).
Acetate is readily absorbed and transported to the liver, where 75% of it is extracted (727, 728) (694). Acetyl-CoA synthetase present in the cytosol of adipose and mammary glands facilitates lipogenesis from acetate. Through GPR43, SCFA also decreases lipolysis and reduces plasma free FA (750).

4.1.2.6 SCFA and the Regulation of Cholesterol

SCFA have been associated with reduced serum LDL-cholesterol, likely via AMPK activation, although in humans, the cholesterol reduction appears to be restricted to those with Type 2 diabetes mellitus, not in healthy subjects (776-779).

In rats receiving 1% (w/w) cholesterol, the increased serum cholesterol concentrations were significantly lower when they received supplemental 0.3% (w/w) acetate (780). Acetate supplementation also decreased hypercholesterolemia in humans (781). In the liver, increased mRNA level of cholesterol 7 α-hydroxylase (CYP7A1) was seen with supplemental acetate. CYP7A1 converts cholesterol to bile acid, thus reducing excess circulating cholesterol. Similarly, rats administered propionate had reduced rate of cholesterol synthesis thought to be due to decreased activity of Hydroxymethylglutaryl-CoA synthase (HMGCS) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) (782-784).

4.1.2.7 SCFA in Maintenance of Gut Immune Homeostasis

SCFA play an important role in modulating gut and systemic immune responses thereby maintaining immune homeostasis (Figure 4.5).

4.1.2.7.1 Intestinal Epithelial Cells

SCFA are essential for maintaining intestinal epithelial cell (IEC) barrier functions. Activation of GPR43 and GPR109a by SCFA activates NLRP3, which promotes the conversion of pro-IL-18 to IL-18, thus increasing this key cytokine involved in repair and maintenance of epithelial integrity (225, 755, 756). SCFA also increases IEC oxygen consumption resulting in reduction in oxygen tension and stabilisation of hypoxia-
inducible factor (HIF), namely HIF-1α and HIF-1β, which are transcription factors that regulates intestinal epithelial tight junctions (785, 786).

Butyrate has a major role in the regulation of cell proliferation and differentiation (695, 701, 724, 725). At low concentrations, butyrate at the crypts appears to induce colonocyte proliferation and at the villi tip, higher concentrations of butyrate has a pro-apoptotic effect inducing colonocyte exfoliation (787) thus regulating cell turnover and maintaining the physical barrier between luminal contents and the host.

IEC senses pathogenic microorganisms or their molecules and responds accordingly. Butyrate specifically increases the expression of β-defensins, cathelicidins, LL-37 and CAP-18, which are antimicrobial peptides produced by colonocytes (785, 786, 788-790). SCFA also stimulates goblet cell differentiation and mucus production, produced by B. thetaiotaomicron or F. prausnitzii in GF mice (791).

In response to inflammatory or infectious stimulation, IEC produce immune mediators involved in recruitment and regulation of immune cells (792). SCFA appear to have anti-inflammatory responses (755). For example, CXCL8, a neutrophil chemoattractant, and CCL20, a lymphocyte and dendritic cell chemoattractant, are expressed by Caco-2 cells in response to flagellin but are reduced by butyrate and propionate (793). However, it can also have pro-inflammatory effect (794). This contradiction is not fully understood and may be due to the different models used to study SCFA functions.

4.1.2.7.2 Neutrophils

Neutrophils are recruited first to the site of inflammation, where they mount a response to the infectious agents and produce cytokines that initiate recruitment and activation of other immune cells. SCFA alter neutrophil recruitment by regulating the production of inflammatory cytokines TNF-α and IL-17 and chemoattractants (748, 755, 793-796).
SCFA are products of bacterial fermentation of undigested or partially digested food fibres in the colon. They stimulate sodium and water absorption by modulating the expression and activity of Na hydrogen exchanger 3 (NHE3). Butyrate particularly is a...
major source of energy for colonocytes. It also has a major role in the regulation of cell proliferation and differentiation, and maintaining epithelial barrier. SCFA are ligands for G protein-coupled receptors (GPRs). GPR41, GPR43 and GPR109A are expressed on epithelial cell surface and in multiple organs. They are involved in gut motility and regulation of fatty acid, glucose and cholesterol metabolism. SCFA regulates the differentiation, recruitment and activation of immune cells including dendritic cells (DC), macrophages, neutrophils and T lymphocytes. They modulate the proliferation and differentiation of T lymphocytes into Th effector and Treg cells, resulting in increased anti-inflammatory cytokines TGFβ and IL-10. They also suppress DC maturation, decreasing production of pro-inflammatory cytokines, such as TNFα and IL-12, by macrophages and DCs. SCFA enhance mucus production by goblet cells. They increase IEC expression of antimicrobial peptides and increase secretion of secretory IgA by B cells. SCFA effects also include activation of inflammasomes and subsequent production of IL-18, and inhibition of NF-κB and apoptosis. Adapted from Rooks, 2016 (325)

4.1.2.7.3 Monocytes, Macrophages and Dendritic Cells

Monocytes respond to inflammatory signals, migrate to inflammatory sites and differentiate into macrophages and dendritic cells. SCFA play an important role in regulating pro-inflammatory innate immune responses. In the presence of SCFA, human monocytes stimulated with LPS have increased PGE2 and reduced IL-10 production. These changes were also seen in peripheral blood mononuclear cells, in addition to inhibition of LPS-induced TNF-α and IFN-γ production (797). SCFA have anti-inflammatory effects on macrophages (798, 799) and DCs via HDAC inhibition (279, 800). When murine macrophage cell line RAW264.7 was incubated with LPS and different concentrations of SCFA, decreased TNF-α, IL-1β, IL-6 and increased IL-10 production were seen with SCFA (801). DCs treated with butyrate resulted in attenuated T cell stimulation, reduced production of IL-12p40 and IFN-γ, and increased production of IL-10 (801)(42). Butyrate also increases the production of IL-23 by DCs, affecting polarization of T cells (802). In addition, when incubated with TNF-α+PGE2, LPS or TNF-α+IL-1β, butyrate inhibited DC maturation (801, 803). The ability to suppress DC development was also seen with propionate but not acetate (800).
4.1.2.7.4 SCFA and the Adaptive Immune System

SCFA mainly induces a tolerogenic T-lymphocyte profile by affecting the activation and effector function of T cells. This is thought to occur via inhibition of HDAC activity (399, 804-806).

DCs exposed to butyrate, and to a lesser extent to propionate, expressed indoleamine 2,3-dioxygenase 1 and aldehyde dehydrogenase 1A2. These enzymes cause tryptophan depletion and generation of retinoic acid, an immunosuppressive molecule, thus suppressing immune activation. SCFA also promoted the conversion of naïve T cells into FoxP3+ regulatory T cells (Tregs) in a HDAC-dependent manner, and suppress their conversion into pro-inflammatory T cells (IFN-γ+ T cells) (805). Thus, SCFA are capable of regulating the size and function of colonic FOXP3+ Treg cell pool (195, 399, 806).

Butyrate specifically plays an essential role in maintaining the balance between pro- and anti-inflammatory CD4+ T cells. It has been shown to inhibit the proliferation of both CD4+ and CD8+ T cells in vitro (59). GPR109a activation in macrophages and DC appear to mediate Treg generation. In its absence, there are reduced IL-10 producing CD4+T-cell and increased IL-17-producing T cells (755) Park et al. demonstrated that acetate, propionate and butyrate enhance the naïve T-cell polarization to Tregs but also promoted the generation of Th17 and Th1 effector cells in vitro. On the other hand, in an in vivo colitis model, less inflammation was seen in the presence of SCFA, perhaps due to its ability to induce IL-10 production (807). GPR43 activation also appear to mediate Treg generation (195).

Although studies support the idea that SCFAs induce a tolerogenic and anti-inflammatory profile of T cells, evidence also indicates that under some conditions they may induce Th1 and Th17 responses. Depending on the disease/model and other factors including time, route and concentrations of treatment used, they can both ameliorate or worsen the disease severity (802, 807-809).

4.1.2.8 SCFA Influences Gut Motility

SCFA regulates intestinal motility via the actions of Peptide YY (PYY) (810). SCFA receptors GPR41 and GPR43 are co-located in the mucosal enteroendocrine cells with PYY (811). PYY is synthesised in the enteroendocrine “L” cells of distal ileum, colon and rectum (812).
PYY is known to inhibit stomach acid and pancreatic exocrine secretion. In the post-prandial phase, it appears to mediate satiety signals and is associated with reduced food intake. PYY causes longitudinal gastric muscle relaxation and inhibition of distal gastric motility, thus leading to slowing of gastric emptying. Interestingly, decreased concentrations of PYY was reported with fasting. It is mostly expressed in the distal ileum where it inhibits intestinal transit thus is also known as an "ileal brake". PYY-containing cells are increased in the ascending colon of patients with chronic slow transit constipation. This leads to increased water absorption and decreased water and electrolyte secretion, thus contributing further to the slow intestinal transit.

4.1.3 Short Chain Fatty Acids in Intestinal Infections and Inflammatory Diseases

4.1.3.1 Intestinal Infections

As discussed above in Section 4.1.1.2, SCFA reduces the luminal pH thus influencing microbiota composition and protecting the host from pathogenic organisms. The intestinal microbiota and the SCFA they produce work in symbiosis with the host in maintaining homeostasis.

SCFA inhibits the growth of Salmonella, and, at low pH, influences Salmonella virulence factor production, thus its use as a food preservative and in poultry feed to minimise contamination. The low pH also influences virulence factor produced by Listeria monocytogenes, and has a protective role against C. jejuni. Shigella down-regulates cathelicidin and AMP production but butyrate-containing enema administered to patients with Shigella infection resulted in higher expression of cathelicidin and clinical improvement.

4.1.3.2 Inflammatory Bowel Disease

Dysbiosis in IBD appear to affect SCFA concentrations in these patients. Indeed, SCFA has been implicated in IBD pathogenesis. For example, in adults with CD and UC, decreased F. prausnitzii and Roseburia hominis have been reported, both of which are butyrate-producers.
Butyrate, as a major colonocyte fuel, appears to play a role in gut health and disease. Vernia et al. reported lower concentrations of butyrate in adult UC patients, especially in those with severe disease (829). Instead, glucose oxidation was found to be higher in active and quiescent UC, with increased glutamine oxidation and lactate production in the acute-colitis specimens isolated from human colons studied by Roediger (830). This switch in oxidation may be driven by the availability of free CoA, which is necessary for FA oxidation in tissues (830). In adults with UC, there is decreased free CoA in the mucosa (831). The altered acetyl-CoA:free CoA ratio affects pyruvate dehydrogenase activity, which in turn affects glucose oxidation (832).

Increased sulphide production may also play a role in UC pathogenesis. Patients with UC usually have greater protein intake, thus more sulphur amino acids. This appears to encourage proliferation of sulphate-reducing bacteria (833, 834) and in combination, increased production of sulphide compared to controls. Sulphides are known to inhibit oxidation of n-butyrate thus may explain the reduced capacity to utilise it as an energy source (835). Although this does not explain the lower concentrations of butyrate in UC patients, microbial shifts within the colon may be the cause of both these findings and potentially related.

The diet and thus available substrate for fermentation inevitably influences the SCFA produced. Huda et al. reported a significant reduction in butyric and propionate concentrations in IBD patients compared to healthy adults. Acetate was also reduced although not statistically significant. In this Malaysian cohort, where UC is more common than CD, the proportion of butyrate was higher than propionate, hypothesised to be due to environmental factors such as a predominant rice diet (836).

4.1.3.3 Inflammatory Bowel Disease in Children

Treem et al. compared 17 children with UC, 22 children with CD and 12 healthy age-matched controls in U.S.A in the early 1990’s (837). Children with UC and CD were found to have decreased stool acetate and increased n-butyrate concentrations compared with control children. A subgroup analysis of UC children revealed increased n-butyrate concentrations in inactive and mild UC, and decreased total SCFA and acetate in moderate and severe UC. Treem et al. postulated the possibility of impaired transport
mechanism, defective cellular metabolism or self-imposed dietary/fibre restriction as explanation for these findings.

Gerasimidis et al. more recently reported on stool SCFA changes with exclusive enteral nutrition (EEN) in children with CD. As expected, following a course of EEN, reduction in acetic acid, propionic acid, butyric acid, valeric acid and caproic acid were observed, associated with reduction in F. prausnitzii. These levels reverted to pre-treatment levels on free diet associated increased microbial diversity (437).

Thus far, there have been limited studies reporting SCFA in paediatric IBD with none reporting changes in response to treatment.

4.1.3.4 Short Chain Fatty Acids as Treatment for IBD

The diet greatly influences SCFA production thus it is unsurprising that dietary manipulations have been used to alter SCFA in patients (838). In rats, Henningsson et al. manipulated SCFA production by modifying the diet for 13 days. Increase in indigestible carbohydrates resulted in greater production of acetic acid, propionic acid and butyric acid (839). In humans, a similar effect was seen with a diet supplemented with oat bran (838).

Plant extracts have been made commercially available in the form of prebiotics. Kanauchi et al. reported increased Bifidobacteria and reduction in PUCAI scores whilst Hanai et al. reported significantly prolonged remission in UC patients on prebiotics (840-842).

SCFA enemas have been used as a treatment (Table 4.2). It was first trialled in diversion colitis, for which rectal irrigation of SCFA appeared to be beneficial (721, 827). Harig et al. found marked endoscopic improvement with SCFA enema used twice daily for 4-6 weeks for diversion colitis (721). Improvements were also seen by Cummings et al. with SCFA enema for treating distal UC (843). Scheppach et al. conducted a trial with the administration of butyrate only enemas for 2 weeks. After this period, they found decreased stool frequency and cessation of blood per rectum. There was also endoscopic and histological improvement seen (844).
Senagore *et al.* performed a randomised prospective study in patients with non-specific proctosigmoiditis. The recovery rate was similar between the groups of patients receiving corticosteroid enemas, mesalamine enemas and SCFA enemas (845). The cost savings, however, greatly favoured SCFA enemas. Breuer *et al.* conducted a randomised, placebo controlled trial in patients with UC using SCFA enema twice daily for 6 weeks. No significant differences were seen during this period although improvements were seen in patients with short (< 6 months) episodes of colitis and those who complied with treatment. In the open-label extension trial, 65% of the patients improved clinically and histologically with SCFA enema (846). Therefore, for improvements to be seen, longer duration and greater frequency of administration may be necessary.

<table>
<thead>
<tr>
<th>SCFA route and dosage</th>
<th>Effect Reported</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet with resistant starch at 1.53 kg/10 kg of diet administered to rats</td>
<td>Symptomatic improvement: epithelial cell proliferation, laminin regeneration, intestinal bacterial growth</td>
<td>(847)</td>
</tr>
<tr>
<td>Cellobiose (9%) supplementation to diet of mice</td>
<td>Reduced weight loss, diminished tissue oedema, attenuated inflammatory cytokine concentrations</td>
<td>(479)</td>
</tr>
<tr>
<td>Fibre (5%) supplementation before and after TNBS colitis in rats</td>
<td>Reduced MPO and NO synthase activities, restored colonic glutathione concentrations, diminished TNF-α concentrations</td>
<td>(848)</td>
</tr>
<tr>
<td>Sodium butyrate enema (100mM) administered to rats</td>
<td>Increased duration of pain in rats with colitis</td>
<td>(849)</td>
</tr>
<tr>
<td></td>
<td>Improved clinical symptoms and inflammatory scores</td>
<td>(850)</td>
</tr>
<tr>
<td></td>
<td>Minor effects on colonic inflammation and oxidative stress, increased IL-10/IL-12 ratio, increased CCL5 concentrations in adults</td>
<td>(851)</td>
</tr>
<tr>
<td>2 g 5-ASA + 80mM Sodium Butyrate enemas administered to adults</td>
<td>Improvement seen, one patient went into remission</td>
<td>(852)</td>
</tr>
<tr>
<td>10 mg/kg oral Sodium Butyrate administered to mice</td>
<td>Improvement of mucosal lesions and attenuated mucosal inflammation in a model of DSS-induced colitis</td>
<td>(853)</td>
</tr>
</tbody>
</table>
**Table 4. 2: SCFA as treatment in IBD**
*(Adapted from Table 2, Vinolo et al.)* (748) MPO-myeloperoxidase; TNBS-Trinitrobenzenesulfonic acid

### 4.1.3.5 Food Allergy

In allergic patients, SCFA concentrations were also found to be lower than healthy controls. Stool acetic and propionic acids decreased from 1 to 4 years of age in all children, while valeric acid increased. Children with low butyric and valeric acids at a year of age were associated with food allergy and lower stool butyric and valeric acids at 4 years of age (854).

SCFA supplementation in the form of high-fibre diet or acetate administration appeared to protect mice against development of allergic airway disease. This protection was also seen in the offspring of pregnant mice fed SCFA-supplemented feed. Thus, SCFA appears to play a role in the pathogenesis of allergy and can be manipulated to alter disease expression (855).

### 4.4.3.6 Coeliac disease

In contrast, children with Coeliac disease have been found to have higher stool SCFA concentrations, associated with the microbial dysbiosis seen in these patients, compared to healthy children. With a gluten-free diet, the concentrations of acetic acid, i-butyric acid and i-valeric acid decrease to levels seen in healthy controls (856).

In addition, it is interesting to note that the altered metabolomic profile of Coeliac disease patients are detectable prior to disease onset. Serena *et al.* hypothesised that the increased lactate production, and consequently increased FOXP3 expression, associated with *Lactobacilli* spp. enrichment may represent a preclinical compensatory mechanism in genetically predisposed children to down-regulate the inflammation. *Lactobacilli* spp. have been shown to enhance intestinal barrier (857), perhaps partly through its production of butyrate, which is detected in increased concentrations in these at-risk children. The increased lactate concentrations then drops, perhaps contributing to the definitive loss of gluten intolerance with disease onset. This metabolomic profile was not seen in children who did not develop Coeliac disease (160).
4.1.4 Aims of the Study

In summary, the symbiotic relationship between the gut microbiota and its host includes the production of SCFA from fermentation of dietary fibre in the colon. SCFA has wide-ranging functions in health and disease.

With few studies looking at SCFA in children with IBD, and with very limited information available on the effects of IBD treatment on SCFA concentrations and function, there is a need to improve our understanding of the role of SCFA in paediatric gut health and disease. To achieve this, the aims of the present study were:

4. To investigate the SCFA concentrations and the relative proportions of SCFA in control and disease groups;
5. To identify the changes in SCFA concentrations and the relative proportions of SCFA in response to treatment in children with IBD; and
6. To define potential associations between SCFA concentrations and the relative proportions of SCFA with clinical status.
4.2 Materials and Methods

With the aim of investigating stool samples for microbiota (Chapter 3) and its corresponding metabolites, stool SCFA were analysed utilising an established protocol by Dr Gerasimidis in his laboratory in University of Glasgow, UK. I collected the samples, stored them in aliquots and freeze-dried them before taking them to Glasgow. I learnt the protocol and extracted the SCFA there for analysis by gas chromatography.

Although researchers also analyse urinary and serum metabolites, stool SCFA was felt to be more appropriate for pairing with stool microbiota analyses. It is worth noting that Kolho et al. observed that stool metabolomics (excluding SCFA) profile was more superior to serum in discriminating between NIC and IBD as well as between CD and UC (858).

4.2.1 Sample Storage

For SCFA, 1g of stool in a 2 ml eppendorf tube was preserved with 1 ml of 1M NaOH in order to stabilise the SCFA. The sample was vortexed and stored at -20°C until SCFA extraction.

4.2.2 Extraction of Short Chain Fatty Acids by Diethyl Ether:Orthophosphoric Acid Methodology

Stool samples were transferred to -80°C overnight and freeze-dried (Maxi dry Iyo, Heto-Holten, Denmark) at 1 mbar and -100°C for 24-48 hours. The freeze-dried samples were stored at room temperature until analysis in Glasgow.

SCFA were extracted using diethyl ether (Sigma, U.S.A) and orthophosphoric acid (OPA) (Sigma, U.S.A). When stool samples are mixed with diethyl ether and OPA, 2 phases are formed, an upper ether phase, which contains diethyl ether and SCFA, and a lower stool phase, which contains the stool sample and OPA.

For each sample, 100 mg of freeze-dried stool was transferred into blue-capped 15 ml falcon tubes. Subsequently, 300 µl of distilled water and 100 µl of diethyl butyric acid (73.6 mmol, Sigma, U.S.A) were added. Diethyl butyric acid was used as an internal standard with each sample to account for loss of SCFA with handling and processing.
In the fume cupboard, 100 µl of OPA was added to the mixture. Thus, the ratio of stool sample:water:diethyl butyric acid:OPA was 1:3:1:1. To minimise loss of volatile gases, caps were replaced immediately after addition of further reagents.

Still in the fume cupboard, 1.5 ml of di-ethyl ether (Sigma, U.S.A) was added to the mixture. The tubes were vortexed before being placed on a shaker (IKA VIBRAX VXR Basic, IKA-Werke GmbH & Co. KG, Germany) at 1200 osc/min for 1 minute. Following shaking, 500 µl of the upper clear phase was transferred into the pre-labelled red-capped 15 ml falcon tubes.

Another 1.5 ml diethyl ether was added to the blue-capped 15 ml falcon tube and the mixture vortexed for 1 minute as above. Then 1 ml of the upper phase was transferred into the red-capped 15 ml falcon tube. This was repeated once more with another 1.5 ml diethyl ether.

Finally, 1.5 ml of the solution was transferred from the red-capped 15 ml falcon tube into 1.5 ml glass vials (Agilent technologies, USA) and crimped with silicone rubber seal crimp tops (Fisher Scientific, UK).

In addition to the internal controls, a control stool sample extracted as above was analysed after every 12th sample to determine consistency of the analysis with other runs. This control stool sample was provided by a healthy volunteer as per University of Glasgow regulations.

4.2.3 Analysis of Stool SCFA Extraction by Gas Chromatography

Bacterial metabolites can be extracted from blood, urine, stool and exhaled gas samples by chromatography (859-861). This is a technique used for separating compounds of a mixture.

Essentially, a mixture of a matter (gas or liquid), called the mobile phase, moves over the surface of another matter (liquid or solid), called the stationary phase (Figure 4.6). As the mobile phase moves over the stationary phase, it undergoes repeated adsorption and desorption at different speeds thus separating out the different components. Further details are as described in Figure 4.6. Based on this principle, a few types of
Chromatography exist with paper, column and gas chromatography being the more commonly used ones.

**Figure 4.6: Gas chromatography**

With gas chromatography, a carrier gas (Nitrogen), which is the mobile phase, is introduced. The sample being analysed is injected into the carrier gas and vaporises. The constituent gases of the sample separate out by the process of repeated adsorption, and thus travel at different speeds along the coiled column, which is the stationary phase. The entire coil is in an oven with a temperature high enough to ensure that the sample remains as a gas. At the end of the column, the gases are eluted and detected by a detector generating electrical signals that are used to record relative amounts of the constituents with corresponding peaks on a chart called chromatogram.

The 1.5 ml glass vials containing extracted and control samples were placed onto the AS2000 Autosampler (Thermo Quest CE instruments, Manchester, UK) for gas chromatography (Figure 4.7). The second batch of samples were run on the 7820A with DB-Wax ultra-inert capillary column (Agilent, U.S.A). The parameters of the gas chromatograph are summarised in Table 4.3. At the start of the analysis, 2 ether injections were run to ensure that the column was clean. This was shown on the chromatogram as single peaks. After each sample, the Autosampler needle was washed with ether and 100% methanol.
**Figure 4. 7: AS2000 Autosampler (Thermo Quest CE instruments, Manchester, UK)**

<table>
<thead>
<tr>
<th>Programme</th>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven</td>
<td>Ramp rate</td>
<td>15°C/min</td>
</tr>
<tr>
<td></td>
<td>Initial temperature</td>
<td>80 °C</td>
</tr>
<tr>
<td></td>
<td>Ramp temperature</td>
<td>210 °C</td>
</tr>
<tr>
<td></td>
<td>Oven maximum temperature</td>
<td>260 °C</td>
</tr>
<tr>
<td></td>
<td>Prep run time out</td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td>Equilibration time</td>
<td>0.25 min</td>
</tr>
<tr>
<td></td>
<td>Acquisition time</td>
<td>10.67 min</td>
</tr>
<tr>
<td>Ring inlet</td>
<td>Inlet temperature</td>
<td>230 °C</td>
</tr>
<tr>
<td></td>
<td>Mode</td>
<td>Splitless</td>
</tr>
<tr>
<td></td>
<td>Purge</td>
<td>Constant septum purge</td>
</tr>
<tr>
<td>Right carrier</td>
<td>Ramps, Flow</td>
<td>12 ml/min</td>
</tr>
<tr>
<td></td>
<td>Flow mode</td>
<td>Constant flow</td>
</tr>
<tr>
<td>Autosampler</td>
<td>Sample volume</td>
<td>1 µl</td>
</tr>
<tr>
<td></td>
<td>Sample speed</td>
<td>100 µl/sec</td>
</tr>
<tr>
<td>Column parameters</td>
<td>Length</td>
<td>15 M</td>
</tr>
<tr>
<td></td>
<td>Internal diameter</td>
<td>0.53 mm</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>100 °C</td>
</tr>
<tr>
<td></td>
<td>Inlet pressure gauge</td>
<td>70 kPa</td>
</tr>
<tr>
<td></td>
<td>Outlet absolute pressure</td>
<td>10 kPA</td>
</tr>
<tr>
<td>Carrier gas parameter</td>
<td>Carrier gas</td>
<td>Nitrogen</td>
</tr>
<tr>
<td></td>
<td>Flow standard</td>
<td>1.89 cc/min</td>
</tr>
<tr>
<td></td>
<td>Velocity</td>
<td>35.14 cm/sec</td>
</tr>
<tr>
<td></td>
<td>Hold up time</td>
<td>85.38 sec</td>
</tr>
<tr>
<td>Right detect</td>
<td>Base temperature</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>Hydrogen pressure</td>
<td>25Psi</td>
</tr>
<tr>
<td></td>
<td>Nitrogen pressure</td>
<td>30 kPa</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>350 kPa</td>
</tr>
</tbody>
</table>

**Table 4. 3: Gas Chromatography Parameters**
4.2.4 Preparation of External Standards

In parallel, known concentrations of SCFA were prepared as described above (Section 4.2.2) to be used as external controls (862). These contained 11 short, medium (MCFA) and branched (BCFA) chain fatty acids of known molarities (Table 4.4). Dilutions (10, 25, 50, 100, 200 and 300) of the mixture were prepared and extracted using the same protocol as for the unknown samples. These standards were used to obtain calibration curves. All reagents were from Sigma Aldrich, UK, except acetic acid (Fisher Scientific, UK). In order to ensure that the gas chromatography is calibrated corrected, the external standard was run after every 12th sample. In addition, 2 quality control samples were run at the beginning and at the end of the whole run.

<table>
<thead>
<tr>
<th>No of carbons</th>
<th>Acid Name</th>
<th>Molarity used (g/L)</th>
<th>Calculated Molarity (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Acetic acid</td>
<td>60.05</td>
<td>183.5</td>
</tr>
<tr>
<td>3</td>
<td>Propionic acid</td>
<td>74.08</td>
<td>134.52</td>
</tr>
<tr>
<td>4</td>
<td>Butyric acid</td>
<td>88.11</td>
<td>111.74</td>
</tr>
<tr>
<td>5</td>
<td>Valeric acid</td>
<td>102.13</td>
<td>89.92</td>
</tr>
<tr>
<td>6</td>
<td>Caproic acid</td>
<td>116.16</td>
<td>80.12</td>
</tr>
<tr>
<td>7</td>
<td>Enanthic acid</td>
<td>130.18</td>
<td>68.53</td>
</tr>
<tr>
<td>8</td>
<td>Caprylic acid</td>
<td>144.21</td>
<td>57.59</td>
</tr>
<tr>
<td>9</td>
<td>Iso-butyric acid</td>
<td>88.11</td>
<td>104.22</td>
</tr>
<tr>
<td>10</td>
<td>Iso-valeric acid</td>
<td>102.13</td>
<td>85.51</td>
</tr>
<tr>
<td>11</td>
<td>Iso-caproic acid</td>
<td>116.16</td>
<td>52.41</td>
</tr>
</tbody>
</table>

*Table 4.4: Concentrations of External Standards*

4.2.5 Calculation of SCFA Concentrations

From the chromatograms (Figure 4.8), the ratio of area of individual SCFA to the area of the internal standard is calculated.

\[
\text{Area ratio of individual SCFA} = \frac{\text{Area under the curve for individual SCFA}}{\text{Area under the curve for internal standard}}
\]

Relative response factor gives a measure of area ratios between 2 points. For each SCFA and BCFA in internal and external standards, the relative response factor was calculated using the formula below:
Relative response factor = Area ratio of individual SCFA in 100 µl of external standard

(Concentration of the SCFA in the final vial)

Concentration of the internal standard in the final vial

Finally, the concentration of SCFA was calculated using the following formula:

Concentration of SCFA
= (Area ratio of individual acid) x Concentration of internal standard

Relative response factor

Figure 4.8: Chromatogram of a Sample St50 on 21.2.17

4.2.6 Analysis

The SCFA results were analysed with corresponding clinical data. Analysis was performed using GraphPad Prism 5.0 and Excel 2013. Correlation between SCFA concentrations and clinical data was assessed using linear regression with 95% confidence intervals. Comparisons between patient groups were analysed using Kruskal-Wallis and Dunn’s Multiple Comparison tests. Statistical significance was considered for p values of less than 0.5.
4.3 Results

A total of 82 stool samples were analysed for SCFA from healthy infants (HI) (n=5) and children (HC) (n=11) in the community, and patients with CD (n=9), UC (n=3), IBDU (n=2), VEOIBD with ileostomy (n=1), IL-10RB defect (n=1), Granulomatosis polyangiitis (n=1), Cryopyrin-Associated Periodic Syndrome (CAPS) (n=2), cow’s milk protein allergy (n=2) and non-inflammatory intestinal symptoms (NIC) (n=5) (Table 2.2, Chapter2).

Depending on their management, patients were seen in hospital at variable intervals. Research samples were collected opportunistically alongside clinical samples thus the number of SCFA samples correlated with clinical samples and parameters varied in time intervals and numbers for each patient.

Comparison between groups were performed using Kruskal-Wallis and Dunn’s Multiple Comparison tests. Given the small numbers, statistical significance was often not achieved. On the other hand, direct comparison between 2 groups using Mann-Whitney test was more likely to result in statistical significance. These are also reported when relevant.

4.3.1 Variations in Duplicate Stool SCFA Extraction

To assess for consistency in SCFA extraction, duplicate samples from the same stool sample were analysed. SCFA was extracted and quantified by gas chromatography in different batches. The results reported demonstrated small variations in the total SCFA concentrations for most samples except for Patients A16 (583, 818 µmol/g), C1 (280, 291 µmol/g) and D2 (363, 437 µmol/g; 449, 349 µmol/g) (Figure 4.9). Despite this, percentages of acetate, propionate and butyrate in the duplicate samples were comparable for these patients.
Figure 4.9: Duplicate Samples of SCFA Extraction
Total SCFA concentration (A) and percentages of acetate (B), propionate (C) and butyrate (D) were compared in duplicate samples. SCFA was extracted in duplicates from the same stool sample and quantified by gas chromatography. Data is presented in pairs of duplicates.

4.3.2 SCFA Concentrations and Percentages in Control Patients and Children

The intestinal microbiota is known to vary with age (166), thus for ideal comparison, control samples were obtained from HI (aged 5-9 months) and sex- and age-matched HC (aged 4-17 years) in the community. In addition, a group of patients with no mucosal inflammation NIC (aged 8-17 years) were also recruited.

First, total SCFA and the individual SCFA were compared according to age (Figure 4.10). Differences in SCFA profiles between HI and children over 4 years of age were apparent,
as detailed subsequently in Section 4.3.1.1. The total SCFA concentrations of each control group were then compared (Figure 4.11). There were no significant differences in total SCFA concentrations between the HI [n=5, median 591 (475-895) µmol/g], HC [n=11, median 457 (227-1264) µmol/g] and NIC patients [n=5, median 583 (467-1000) µmol/g].

![Figure 4.10: SCFA Concentrations in the Control Groups with Age](image)

*Figure 4.10: SCFA Concentrations in the Control Groups with Age*

Total and individual stool SCFA concentrations in healthy infants (HI, n=5, age 5-9 months), healthy children (HC, n=11, age 4-17 years) and non-inflammatory control (NIC, n=5, age 8-17 years) patients were measured using gas chromatography. Data are presented in µmol/g according to age.
Figure 4.11: Total SCFA Concentration in the Control Groups

Total stool SCFA concentrations in HI (n=5, age 5-9 months), HC (n=11, age 4-17 years) and NIC (n=5, age 8-17 years) patients were measured using gas chromatography. Comparisons were performed using Kruskal-Wallis and Dunn’s Multiple Comparison tests with p<0.05 as significant. Data are presented in µmol/g and shown as median and standard error of median.

4.3.2.1 Individual SCFA in Healthy Infants and Children

No significant differences were seen when SCFA concentrations were compared. When comparing the percentages of individual SCFA, however, differences became apparent (Figure 4.12).

The HI had higher percentages of acetate [n=5, median 89 (74-99) %], thus correspondingly lower propionate [median 6 (1-19)%) and butyrate [median 3 (0-15)]% percentages.

Percentages of acetate in HI were higher when compared to percentages of acetate in HC [n=11, median 70 (64-74)%]. Although the percentages of propionate and butyrate in HI were lower than those of HC [propionate median 14 (11-20)%; butyrate median 12 (6-19)%), they were not significantly different.
The cumulative percentages of isovalarate and valerate [median 0.2 (0-1.9)\%] and isocaproate and caproate [median 0.1 (0.1-0.3)\%] for HI were also found to be less than those of HC [iso/valerate median 2.7 (1.6-6.7)\%, p=0.016; iso/caproate median 0.4 (0.1-0.9)\%, p=0.036] but admittedly, the values were small.

**4.3.2.2 Individual SCFA in Healthy Children and Non-inflammatory Control Patients**

Comparison was also made between HC and NIC patients (Figure 4.12). Percentages of acetate in HC [median 70 (64-74)\%] were higher than those of NIC [n=5, median 57 (47-67)\%]. As a result, the percentages of propionate were lower in HC [median 14 (11-20)\%] than in NIC [median 18 (15-20)\%], and the percentages of butyrate were also lower in HC [median 12 (6-19)\%] than NIC [median 21 (12-24)\%].

In addition, when comparing HI and NIC, HI was observed to have higher percentages of acetate (p<0.0001), and lower percentages of propionate (p=0.007), butyrate (p=0.005) and iso/valerate [median 0.1 (0-0.8)\%, p=0.021].

Despite having no mucosal inflammation, the percentages of SCFA in NIC are different to those of healthy children thus would suggest that they are not an ideal control group for comparison with IBD patients.
4.3 SCFA Profiles in Inflammatory Bowel Disease Patients

4.3.1 Total SCFA Concentrations

Total SCFA concentrations were initially analysed (Figure 4.13) and compared with HC and NIC patients. The total SCFA concentrations for IBD patients were correlated with clinical markers then divided into groups depending on disease activity. Active disease was defined as wPCDAI score of more than 12.5, PUCAI of more than 10 or ESR of more than 20 mm/hr. These indices are the best available method of classifying disease activity although does not reflect mucosal healing (Turner JPGN 2017).
As this was a prospective study, samples were collected opportunistically and longitudinally (1-14 months) thus there were multiple samples from some individual patients. For the subsequent analysis, 1 sample per patient was used. In addition, the patient with CD and Coeliac Disease was excluded.

Comparing the total SCFA concentrations for each patient group, there were no significant differences seen between HC [median 457 (227-1264)µmol/g], NIC patients [median 583 (467-1000)µmol/g] and CD patients, whether in active disease [n=7, median 332 (260-1449)µmol/g] or remission [n=6, median 529 (203-869)µmol/g] ($x^2(2)=9.996$, p=0.1248).

Similarly, the total SCFA concentrations in UC patients in active disease [n=3, median 681 (486-780)µmol/g]. Total SCFA concentrations in UC remission [n=2, mean 404 (291-518)µmol/g] and active IBDU [n=2, mean 230 (141-320)µmol/g] were too few to analyse.

![Figure 4. 13: Total SCFA Concentrations Inflammatory Bowel Disease](image)

**Figure 4. 13: Total SCFA Concentrations Inflammatory Bowel Disease**

Total SCFA concentrations of HC (n=11) and NIC (n=5) were compared with total SCFA of Active CD (n=7), CD in Remission (n=6), Active UC (n=3), UC in Remission (n=2) and IBDU (n=2). SCFA were measured using gas chromatography and comparison were performed using Kruskal-Wallis and Dunn’s Multiple Comparison tests with $p<0.05$ as significant. Data are presented in µmol/g and shown as median and standard error of median.
4.3.3.2 Concentrations and Percentages of Acetate in IBD

Acetate concentrations in IBD patients were compared with HC [median 345 (169-906)µmol/g] and NIC patients [median 312 (305-578)µmol/g] (Figure 4.14A) \((x^2(2)=9.563, p=0.1443)\). Whether in active disease or in remission, patients with CD [active disease: n=7, median 248 (160-837)µmol/g; remission: n=6, median 346 (131-673)µmol/g] and UC [active disease: n=3, median 494 (397-582)µmol/g; remission: n=2, mean 321 (246-396)µmol/g] had comparable acetate concentrations when compared to HC. IBDU patients in active disease appeared to have lower concentrations of acetate [n=2, mean 182 (117-247)µmol/g] than HC but this was not statistically significant.

As the SCFA concentrations varied, in order to compare changes in individual SCFA, each SCFA were also analysed as percentages of the total SCFA concentrations. Firstly, comparing the percentage of acetate percentages between the groups, HC [median 71 (64-75)%] had higher percentage of acetate than NIC patients [median 57 (47-67)%] but lower than UC patients in active disease [n=3, median 82 (63-86)%] and in remission [n=2, mean 81 (84-77)%] \((x^2(2)=16.87, p=0.0098)\) (Figure 4.14B). HC also had lower percentages of acetate than IBDU patients [n=2 mean 80 (77-83)%]. CD patients, on the other hand, had similar percentages of acetate to HC [active CD: n=7 median 67 (58-76)%; CD remission: n=6 median 68 (65-78)%].

Compared to NIC, percentages of acetate were higher in patients with UC in remission \((p=0.038)\) and IBDU \((p=0.038)\). With direct comparison between 2 groups using Mann-Whitney test, percentages of acetate in NIC were significantly lower than HC \((p=0.016)\), CD in remission \((p=0.032)\) and active UC \((p=0.007)\) (data not shown). Using multiple samples per patients and Mann-Whitney analysis, significant differences were also seen between HC and UC (active \(p=0.0307,\) remission \(p=0.0050\)) and IBDU \(p=0.0127\) groups (data not shown).
Figure 4. 14: Concentrations and Percentages of Acetate in Inflammatory Bowel Disease

Stool acetate concentrations (A) and percentages (B) of HC (n=11) and NIC (n=5) were compared with Active CD (n=7), CD in Remission (n=6), Active UC (n=3), UC in Remission (n=2) and IBDU (n=2). Acetate was measured using gas chromatography and the percentage of total SCFA was calculated. Comparisons were performed using Kruskal-Wallis and Dunn’s Multiple Comparison tests with p<0.05 as significant. Data are presented in µmol/g and shown as median and standard error of median.

4.3.3.3 Concentrations and Percentages of Propionate in IBD

Comparison of the groups resulted in a Kruskal-Wallis score of 13.32 (p=0.0383) but no significant differences were seen with Dunn’s Multiple Comparison test. CD patients were found to have comparable propionate concentrations when compared to HC [median 59 (32-177)µmol/g] and NIC patients [median 102 (81-166)µmol/g], irrespective of whether they were in active disease [n=7, median 66 (31-364)µmol/g] or in remission [n=6, median 111 (44-167)µmol/g] (Figure 4.15A). On the other hand, UC patients in active disease [n=3, median 34 (3-81) µmol/g] and in remission [n=2, mean 34 (16-52)µmol/] were found to have lower propionate concentrations than HC. Likewise, IBDU patients [n=3, median 14 (7-27)µmol/g] also had lower propionate concentrations than HC. Statistical significance was achieved with multiple samples per patient and Mann-Whitney test when comparing HC to UC (active n=6, p=0.0138; remission n=4, p=0.0157) and IBDU (n=3, p=0.0127) groups (data not shown).
When comparing the IBD groups, CD patients in active disease appeared to have higher propionate concentrations than UC patients in active disease. Indeed when comparison of both groups using multiple samples per patients and Mann-Whitney test was performed, a significant difference was observed (p=0.0076) (data not shown). Similarly, a difference was seen in patients in remission, with CD patients having higher propionate concentrations than UC patients (p=0.0121) (data not shown).

As with propionate concentrations, comparison of propionate percentages resulted in a Kruskal-Wallis score of 22.37 (p=0.0010) with no significant differences seen following Dunn’s Multiple Comparison test. Percentages of propionate in HC were correspondingly lower than NIC (as reported in Section 4.3.1.2) as well as CD patients in active disease [n=7, median 24 (10-28) %] and in remission [n=6, median 20 (13-32)%] (Figure 4.15B). Propionate percentages were lower still in patients with UC in active disease [n=3, median 7 (0.4-10.4)%] and in remission [n=2, mean 8 (5-10)%] as well as patients with IBDU [n=2, mean 7 (5-8)%].

When multiple samples per patient were used and comparison between 2 groups performed with Mann-Whitney test, HC was significantly lower than NIC (p=0.0092), active CD (n=12, p=0.0035), active UC (n=6, p=0.0011), UC in remission (n=4, p=0.0050) and IBDU (n=3, p=0.0127). Differences in propionate percentages were also seen between CD and UC patients. In active disease, CD patients had higher propionate percentages than UC patients (p=0.0009). This was also the case when patients were in remission (p=0.0061).
Figure 4.15: Concentrations and Percentages of Propionate in Inflammatory Bowel Disease

Propionate concentrations (A) and percentages (B) of HC (n=11) and NIC (n=5) were compared with Active CD (n=7), CD in Remission (n=6), Active UC (n=3), UC in Remission (n=2) and IBDU (n=2). Propionate was measured using gas chromatography and the percentage of total SCFA was calculated. Comparisons were performed using Kruskal-Wallis and Dunn’s Multiple Comparison tests with p<0.05 as significant. Data are presented in µmol/g and shown as median and standard error of median.

4.3.3.4 Concentrations and Percentages of Butyrate in IBD

When comparing HC [median 39 (14-165) µmol/g] with CD and UC patients, there were no significant differences in butyrate concentrations ($x^2(2)=12.35$, p=0.0547) but differences were observed in butyrate percentages ($x^2(2)=13.70$, p=0.0332) (Figures 4.16A, 4.16B). Although NIC patients [median 140 (54-219)µmol/g] appeared to have higher butyrate concentrations than HC, this was not statistically significant.

Interestingly, NIC patients appeared to have higher butyrate concentrations when compared to patients with active CD [n=7, median 23 (19-221)µmol/g] and UC [n=3, median 93 (48-184) µmol/g], but these butyrate concentrations in active disease were higher when compared to patients in remission with CD [n=6, median 40 (6-70)µmol/g] and UC [n=2, mean 41 (23-60)µmol/g] as well as IBDU patients [n=2, mean 26 (7-45)µmol/g].

If multiple samples per patient and Mann-Whitney test were applied, butyrate concentrations in NIC were significantly higher than CD remission (p=0.0101), UC remission (p=0.0317) and IBDU (p=0.0357) (data not shown).

A similar pattern was seen when comparing percentages of butyrate (Figure 4.16B). NIC patients had higher percentages of butyrate [median 21 (12-24)\%] when compared to CD patients in remission [n=6, median 8 (1-10)\%, p=0.032]. Although not statistically significant, percentages of butyrate in NIC were also higher than in HC [median 12 (6-19)\%], active CD [n=7, median 7 (5-15)\%], active UC [n=3, median 14 (10-24)\%], UC remission [n=2, mean 10 (8-12)\%] and IBDU [n=2, mean 10 (5-14)\%].
Using Mann-Whitney analysis of 2 groups, significant differences were observed between NIC and HC (p=0.025) and active CD (p=0.005). Using Mann-Whitney test as well as multiple samples per patient, significant differences were also seen between NIC and CD remission (p=0.0051) and UC remission (p=0.0159) (data not shown). As with butyrate concentrations, there was a trend for higher percentages of butyrate in active IBD but no significant differences were seen in CD or UC patients compared to HC.

**Figure 4. 16: Concentrations and Percentages of Butyrate in Inflammatory Bowel Disease**

Butyrate concentrations (A) and percentages (B) of HC (n=11) and NIC (n=5) were compared with Active CD (n=7 samples), CD in Remission (n=6 samples), Active UC (n=3 samples), UC in Remission (n=2 samples) and mild IBDU (n=2 samples). Butyrate was measured using gas chromatography and the percentage of total SCFA was calculated. Comparisons were performed using Kruskal-Wallis and Dunn’s Multiple Comparison tests with p<0.05 as significant. Data are presented in µmol/g and shown as median and standard error of median.
4.3.3.5 Ratios of Acetate:Propionate:Butyrate in IBD

In NIC patients, the ratio of acetate:propionate:butyrate (56:18:20) was similar to that in adults (57:22:21) (236) (Table 4.5). The HC from this study had more propionate than butyrate but much higher acetate percentage (70:14:11).

Comparing the SCFA ratios in the IBD groups, CD patients (67:20:10) had a similar profile to HC but UC (83:6:10) and IBDU (80:6:10) patients had more acetate thus proportionately less propionate and butyrate. It is also worth noting that UC and IBDU patients have nearly twice as much butyrate than propionate and no differences were seen between IBD patients in active disease (CD: 65:21:11, UC 82:6:11) and in remission (CD: 71:18:8, UC: 83:7:8). The ratios are represented in Figure 4.17 as medians of acetate, propionate and butyrate for ease of comparison.

<table>
<thead>
<tr>
<th>Patient Groups</th>
<th>Acetate median (range)</th>
<th>Propionate median (range)</th>
<th>Butyrate median (range)</th>
<th>Ratio</th>
<th>n=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Controls</td>
<td>70 (64-75)</td>
<td>14 (11-20)</td>
<td>11 (6-19)</td>
<td>70:14:11</td>
<td>11</td>
</tr>
<tr>
<td>NIC Controls</td>
<td>56 (47-67)</td>
<td>18 (15-20)</td>
<td>20 (11-24)</td>
<td>56:18:20</td>
<td>5</td>
</tr>
<tr>
<td>CD overall</td>
<td>67 (58-78)</td>
<td>21 (10-32)</td>
<td>8 (1-15)</td>
<td>67:21:08</td>
<td>13</td>
</tr>
<tr>
<td>Active CD</td>
<td>65 (58-76)</td>
<td>24 (11-29)</td>
<td>7 (5-15)</td>
<td>65:24:07</td>
<td>7</td>
</tr>
<tr>
<td>CD - remission</td>
<td>68 (65-78)</td>
<td>20 (13-32)</td>
<td>8 (1-10)</td>
<td>68:20:08</td>
<td>6</td>
</tr>
<tr>
<td>UC overall</td>
<td>83 (63-92)</td>
<td>6 (0-10)</td>
<td>10 (4-24)</td>
<td>83:06:10</td>
<td>5</td>
</tr>
<tr>
<td>Active UC</td>
<td>82 (63-86)</td>
<td>7 (0-10)</td>
<td>14 (10-24)</td>
<td>82:07:14</td>
<td>3</td>
</tr>
<tr>
<td>UC - remission</td>
<td>81 (77-84)</td>
<td>8 (5-10)</td>
<td>10 (8-12)</td>
<td>81:08:10</td>
<td>2</td>
</tr>
<tr>
<td>IBDU</td>
<td>80 (77-83)</td>
<td>7 (5-8)</td>
<td>10 (5-14)</td>
<td>80:07:10</td>
<td>2</td>
</tr>
</tbody>
</table>

*Table 4.5: Ratios of Acetate:Propionate:Butyrate in Control and IBD Groups*
Figure 4.17: Percentages of SCFA in IBD and Control Groups
Median acetate, propionate and butyrate from Table 4.3.1 are represented in this figure to demonstrate the differences in ratios of acetate:propionate:butyrate in active IBD [CD (n=7) and UC (n=3)] compared to HC (n=11) and NIC (n=5).

4.3.3.6 Concentrations of Valerate and Caproate in IBD

Concentrations of stool valerate were found to be lower than acetate, propionate and butyrate in all groups (Figure 4.18). Valerate concentrations in HC [median 14 (5-28)µmol/g] were similar to NIC patients [median 25 (4-48)µmol/g] as well as CD patients in active disease [n=7, median 7 (0-14)µmol/g] and in remission [n=6, median 6 (2-14)µmol/g]. Lower valerate concentrations were seen in UC patients in active disease [n=3, median 4 (1-11)µmol/g, p=0.0022] and in remission [n=2, mean 3.7 (2.7-4.6)µmol/g], as well as IBDU patients [n=2, mean 2.5 (0.6-4.4)µmol/g] compared to HC. Using Mann-Whitney test, concentrations of valerate were higher in HC than in active UC (p=0.0293) (data not shown).
Figure 4. 18: Concentrations of Valerate in Inflammatory Bowel Disease
Valerate concentrations of HC (n=11) and NIC (n=5) were compared with Active CD (n=7), CD in Remission (n=6), Active UC (n=3), UC in Remission (n=2) and mild IBDU (n=2). Data are presented in µmol/g and shown as median and standard error of median.

Finally, concentrations of caproate were too low in all the groups [HC median 1.7 (0.6-4.6) µmol/g; NIC median 0.6 (0.5-1.9) µmol/g; active CD n=7, median (0.6-7) µmol/g; CD in remission (n=6, median 0.5 (0-1.9) µmol/g; active UC n=3, median 0.6 (0-0.8) µmol/g; UC in remission n=2, mean 0.3 (0-0.6) µmol/g; IBDU n=2, mean 0.6 (0-1.2 µmol/g], thus were unsuitable for further meaningful analysis as demonstrated in Figure 4.19.
Figure 4.19: Concentrations of Caproate in Inflammatory Bowel Disease Patients

Caproate concentrations of HC (n=11) and NIC (n=5) were compared with Active CD (n=7), CD in Remission (n=6), Active UC (n=3), UC in Remission (n=2) and mild IBDU (n=2). Data are presented in µmol/g and shown as median and standard error of median.

4.3.4 Correlation of Total and Individual SCFA with Clinical Parameters in Inflammatory Bowel Disease

Total SCFA concentration and percentages of acetate, propionate and butyrate were then correlated with clinical markers and disease scores.

C-reactive protein (CRP, normal <5 mg/dL) and erythrocyte sedimentation rate (ESR, normal <10 mm/hr) were used as markers of systemic inflammation, and stool calprotectin concentrations (normal <50 µg/g) were used as marker of localised intestinal inflammation. In the management and assessment of CD and UC, weighted Paediatric Crohn’s Disease Activity Index (wPCDAI, remission if <12.5) and Paediatric Ulcerative Colitis Activity Index (PUCAI, remission if <10) are used as a measure of disease activity.

4.3.4.1 SCFA Concentrations and Percentages with Clinical Parameters in Crohn’s Disease

For this analysis, each data point represents paired stool SCFA and calprotectin concentrations measured in the same stool sample. As longitudinal data was available for some patients, paired data on each patient in active disease (red data point) and remission (blue data point) are also presented, with the arrow pointing to the second sample obtained.

In CD, no significant correlations were observed between concentrations and percentages of acetate, propionate and butyrate, with CRP (n=14), ESR (n=13) or PCDAI scores (n=16), nor were there any significant differences seen between active disease and remission (data not shown).

CD patients, including a patient with fistulating CD, were also analysed individually, taking into consideration the SCFA concentrations and percentages, clinical parameters.
and treatments (Appendices 4.1-4.3). From these analyses, individual treatments did not alter SCFA concentrations except perhaps a course of Trimethoprim in a patient, which resulted in reduction in butyrate concentration and percentage. There was also the suggestion of increased butyrate and propionate percentages preceding clinical improvement, however, with the limited samples and small patient cohorts, firm conclusions cannot be drawn from this.

4.3.4.2 SCFA Concentrations and Percentages with Clinical Parameters in Ulcerative Colitis

The same analysis was performed with total SCFA concentrations and percentages of acetate, propionate and butyrate of UC patients (n=4). No associations were observed between SCFA and PUCAI scores. Similarly, there were no associations between stool calprotectin concentrations and total SCFA concentrations ($R^2=0.2996$, $p=0.3396$), nor with percentages of acetate ($R^2=0.02117$, $p=0.8154$), propionate ($R^2=0.04461$, $p=0.7331$) and butyrate ($R^2=0.3735$, $p=0.2735$) (data not shown).

SCFA concentrations and percentages of UC patients, including a patient with severe UC on Sirolimus, were also analysed individually incorporating clinical parameters and treatments (Appendix 4.5). No significant changes were seen but butyrate percentages were found to be higher than propionate percentages in these patients.

4.3.5 Changes in Stool SCFA with Treatment for IBD

Stool SCFA percentages were also compared in individuals receiving treatment. Individual SCFA percentages pre- and post-treatment were compared for EEN in CD (n=2), Infliximab in CD (n=3) and Prednisolone in UC (n=2). Comparison was also made for a UC patient who received Sirolimus at 2 and 13 months, and an IBDU patient who received Infliximab at 6 weeks and 4 months.

4.3.5.1 Exclusive Enteral Nutrition in Crohn’s Disease

Patients A15 and A17 received EEN (Figure 4.20). SCFA prior to and 3 months after starting treatment were analysed. Patient A15 responded to treatment, with the CRP
improving from 23 to 5 mg/dL. The total SCFA increased from 297 to 575 µmol/g, and with this, decreased acetate (76-70%), similar propionate (10-13%) and increased butyrate (7-15%) were observed. In Patient A17, there was improvement in ESR levels from 41 to 22 mm/hr. This was associated with increased total SCFA (332-531 µmol/g), reduced acetate (75-65%) and increased propionate (14-21%) and butyrate (6-10%).

**Figure 4.20: SCFA Changes with Exclusive Enteral Nutrition in CD**

SCFA percentages in 2 patients, Patient A15 and Patient A17, were compared before and after treatment with EEN. Acetate (blue), propionate (green) and butyrate (orange) concentrations were measured using gas chromatography and the percentages of total SCFA were calculated. Data are presented as percentages pre- and post-treatment.

4.3.5.2 Infliximab in Crohn’s Disease

In addition, SCFA percentages were also compared in 3 CD patients who received Infliximab, Patients A25, D2 and D4 (Figure 4.21). Patient A25 received Infliximab for fistulating disease. Only the wPCDAI scores were available for comparison before and after treatment. Stool calprotectin was 2404 µg/g before treatment and the ESR was 18 mm/hr 7 months following treatment. Total SCFA concentration decreased (from 1449 to 869 µmol/g) at 7 months. Percentages of acetate increased (58-78%), propionate decreased (25-13%) and butyrate decreased (15-8%) after treatment with Infliximab.

Treatment for Patient D2 was escalated to Infliximab. The ESR was similar before and after treatment (8-11 mm/hr) but the total SCFA significantly reduced (from 1010 to 446 µmol/g) 3 months after treatment. This was associated with decreased acetate (58-48%), similar propionate (23-20%) and increased butyrate (14-16%).
Similarly, Patient D4 also had escalation of treatment with Infliximab. This patient did not respond to treatment, with the ESR increasing (30 to 64 mm/hr) 5 months after treatment. During this period, the total SCFA increased (260 to 743 μmol/g), acetate remained similar (62-59%), propionate was also similar (25-21%) and butyrate increased (13-19%).

**Figure 4. 21: Changes with Infliximab in CD**

Differences in SCFA percentages were analysed for CD patients (n=3) receiving Infliximab. Pre- and post-treatment percentages were compared for Patient A25 (pre-treatment, 7 months), D2 (pre-treatment, 3 months) and D4 (pre-treatment and 5 months). Acetate (blue), propionate (green) and butyrate (orange) concentrations were measured using gas chromatography and the percentages of total SCFA were calculated. Data are presented as percentages pre- and post-treatment.

### 4.3.5.3 Prednisolone in Ulcerative Colitis

Patient A21 responded well to induction of remission with Prednisolone (ESR 41 to 8 mm/hr). SCFA percentages pre-treatment and 3 months post-treatment were analysed (Figure 4.22). The total SCFA decreased (780-518 μmol/g) while acetate increased (63-77%), propionate remained stable (10%) and butyrate decreased (24-12%).
Changes in SCFA percentages following treatment with Prednisolone was assessed in a patient with UC, Patient A21. Pre-treatment and 3 months post-treatment acetate (blue), propionate (green) and butyrate (orange) concentrations were measured using gas chromatography and the percentages of total SCFA were calculated. Data are presented as percentages for the 3 time points.

4.3.5.4 Sirolimus in Ulcerative Colitis

Patient C1 was diagnosed with UC and commenced first line treatment but the disease progressed despite escalating treatment. Colonic resection was being considered but in a last bid to avoid this, the patient was commenced on Sirolimus a month post-diagnosis. The first stool sample obtained for SCFA was 1 months after starting Sirolimus. The patient initially responded to Sirolimus (ESR 15, 10mm/hr; PUCAI 15, 10) but the raised calprotectin (2361, 1446µg/g) warned of the subsequent relapse (PUCAI 30). From month 1 to 13, the total SCFA concentration increased (291-198-411-681µmol/g) while acetate remained similar (84-83-92-86%), propionate reduced (5-9-3-0.4%) and butyrate increased (8-5-4-14%)(Figure 4.23).
4.3.5.5 Infliximab in IBD-Unclassified

Patient D7 was commenced on Infliximab for on-going active disease despite unremarkable ESR. SCFA percentages at week 6 and month 4 of treatment were analysed. During this period, ESR remained unremarkable (12-16 mm/hr) as did the total SCFA concentrations (175-141 µg/g), acetate (21-23%) and propionate (4-5%) (Figure 4.24). Butyrate, however, was reduced (10-5%).
4.3.6 SCFA Profile in Ileostomy Samples

Ileal samples were collected from 2 patients with ileostomies, a patient with VEOIBD [n=5, median 297 (161-567) µmol/g] and another with IL-10R mutation [n=2, average 163 (87-239)µmol/g].

The total SCFA concentrations of ileostomy samples [n=7, median 238 (65-567)µmol/g] were significantly lower when compared with HC [median 457 (227-1264)µmol/g, p=0.0185](Figure 4.25A).

In all the ileostomy samples collected, acetate accounted for more than 85% [n=7, median 91 (85-98)]% of total SCFA, which was significantly higher than HC (p=0.0006), although the actual acetate concentrations [median 233 (55-508)µmol/g] were similar to or lower than HC (Figure 4.25B).

The propionate and butyrate percentages were thus correspondingly lower. Propionate percentages in ileostomy samples [n=7, median 5.1 (0.6-9.7)%] were significantly lower than HC [median 14 (11-20)%], p=0.0006. Similarly, the butyrate percentages [n=7, median 2.8 (0.2-9.0)]% were significantly lower compared to HC [median 12 (6-19)%, p=0.0021].

![Figure 4.25: Total SCFA Concentrations and Individual SCFA of Ileal Samples](image)

**Figure 4. 25: Total SCFA Concentrations and Individual SCFA of Ileal Samples**

Total SCFA concentrations (A) and percentages of acetate, propionate and butyrate (B) of ileal samples (n=7) from 2 patients with VEOIBD and IL-10 mutation were compared with HC (n=11). Comparisons were performed using Mann-Whitney test with p<0.05 as significant. Data are presented in µmol/g and shown as median and standard error of median.
4.3.7 SCFA in Non-Inflammatory Bowel Disease Patients

Other groups of patients with intestinal symptoms were recruited for comparison with the IBD groups. In addition, there were also patients who were recruited to a group but later found to have another diagnosis. SCFA percentages, clinical parameters and treatments of some of these patients are discussed individually (Appendices 4.7, 4.8).

4.3.7.1 SCFA Profiles of Allergic and Healthy Infants

Patients were recruited to assess the effects of cow's milk protein allergy and its treatment. As it is known that the intestinal microbiota in infants are different when compared to children over the age of 3 years (166), this group of infants was compared to healthy infants (HI). Comparing the 2 allergic, older infants with 5 younger HI, one allergic infant was found to have lower total SCFA concentration (265µmol/g) but the other infant had similar total SCFA concentration (800 µmol/g) to the HI [n=5, median 591 (475-896)µmol/g] (Figure 4.26A).

Allergic infants fed Neocate [n=2, total SCFA mean 533 (265-800)µmol/g, acetate mean 418 (212-624)µmol/g, propionate mean 68 (14-122)µmol/g, butyrate mean 42 (35-50)µmol/g] were compared to healthy breast-fed [n=3, total SCFA median 591 (475-676)µmol/g, acetate median 525 (349-629)µmol/g, propionate median 47 (39-88)µmol/g, butyrate median 16 (5-28)µmol/g] and mixed-fed infants [n=2, total SCFA mean 733 (570-896)µmol/g, acetate mean 623 (566-681)µmol/g, propionate mean 25 (3-47) µmol/g, butyrate mean 67 (0-134)µmol/g] (Figure 4.26B). Given that there were no differences in total SCFA and the main SCFAs (acetate, propionate and butyrate) between the allergic and non-allergic infants, they were subsequently studied as a group.

Infants born vaginally and by caesarean section had comparable SCFA profiles [infants born by normal vaginal delivery: n=4, total SCFA median 580 (475-800)µmol/g, acetate median 545 (349-624)µmol/g, propionate median 67 (3-122)µmol/g, butyrate median 22 (0.2-49.7)µmol/g; infants born by caesarean section, n=3, total SCFA median 676 (265-896)µmol/g, acetate median 629 (212-681)µmol/g, propionate median 39 (14-47)µmol/g, butyrate median 35 (5-134)µmol/g] (Figure 4.26C).

Boys [n=3, total SCFA median 591 (475-676)µmol/g, acetate median 525 (349-629)µmol/g, propionate median 47 (39-88)µmol/g, butyrate median 16 (5-28)µmol/g] had
similar SCFA concentrations to that of girls \( n=4, \) total SCFA median 685 (265-896)µmol/g, acetate median 595 (212-681)µmol/g, propionate median 31 (3-122)µmol/g, butyrate median 42 (0.2-134.4)µmol/g \( \) (Figure 4.26D).
Figure 4. 26: Total SCFA, Acetate, Propionate and Butyrate Concentrations in Healthy and Allergic Infants

**Figure 4.26A:** Total SCFA concentrations in allergic infants (n=2) and healthy infants (n=5).

**Figure 4. 26B:** Comparison of SCFA concentrations between infants who were breastfed (n=3), mixed-fed (n=2) and fed Neocate (n=2). Data are presented in µmol/g with median and standard error of median.

**Figure 4. 26C:** SCFA concentrations of infants born by normal vaginal delivery (n=4) and Caesarean section (n=3). Data are presented in µmol/g with median and standard error of median.

**Figure 4. 26D:** Concentrations of stool SCFA in boys (n=3) and girls (n=4). Data are presented in µmol/g with median and standard error of median.

### 4.3.7.2 SCFA in a Patient with Crohn’s Disease and Coeliac Disease

Patient D2 was recruited to the study with known Coeliac Disease and Crohn’s Disease prior to commencing Infliximab. The patient was initially diagnosed with Coeliac Disease at less than a year of age, and CD was diagnosed at the age of 9 years.

In this patient with Coeliac Disease, total SCFA concentrations [n=8, median 586 (252-1300)µmol/g] were higher than the average patient with IBD, thus was excluded from the CD group for further analysis. There were, however, no differences seen in the total SCFA when compared to HC [n=10, median 565 (227-1264)µmol/g] and other patient groups (Figure 4.27A). Individual SCFA percentages [n=8, acetate median 59 (48-87)%, propionate median 20 (9-23)%, butyrate median 13 (3-22)]% showed no significant differences when compared to other patient groups (Figure 4.27B, Table 4.6).

### 4.3.7.3 SCFA in a Patient with Granulomatosis Polyangiitis

Patient A11 presented with symptoms suggestive of CD thus was managed as such. The disease course was atypical thus the patient was further investigated and subsequently
diagnosed with Granulomatosis polyangiitis, a vasculitic disease affecting multiple systems.

The patient remained in active disease during the study period despite several medications and treatment escalation. The total SCFA concentrations [n=6, median 534 (305-922)µmol/g] fluctuated throughout the study period and were similar to HC and NIC patients [median 652 (467-1000)µmol/g] (Figure 4.27A). SCFA percentages for this patient [n=6, acetate median 62 (45-69)%, propionate median 18 (9-36)%, butyrate median 18 (15-22)%) were also comparable to the other patient groups (Figure 4.27B, Table 4.6).

4.3.7.4 SCFA in Patients with CAPS

As comparison to the IBD groups, 2 patients with CAPS, an inflammatory condition which includes gastrointestinal symptoms, were recruited. Samples were collected at the start of treatment with Cankinumab, a human monoclonal IL-1β antibody, and subsequently after. With limited samples, it was not possible to make any firm conclusions but no significant differences were seen in the total SCFA concentrations [E1 n=3, median 410 (393-643)µmol/g; E2 n=2, mean 213 (201-225)µmol/g] (Figure 4.27A) or percentages of acetate [E1 n=3, median 69 (68-71)%; E2 n=2, mean 72 (69-75)%), propionate [E1 n=3, median 16 (13-17)%; E2 n=2, mean 10.9 (10.6-11.2)%] and butyrate [E1 n=3, median 11 (8-12)%; E2 n=2, mean 9 (7-11)%] when compared to HC (Figure 4.27B, Table 4.6).
Figure 4.27: Total SCFA Concentrations and Percentages of Acetate, Propionate and Butyrate in Patients with Non-Inflammatory Bowel Disease

Total SCFA concentrations (A) and percentages of acetate, propionate and butyrate (B) of HC (n=11) and NIC patients (n=5) were compared with a patient with CD and Coeliac Disease (n=8 samples), a patient with Granulomatosis polyangiitis (n=6 samples) and 2 patients with CAPS (n=5 samples). Comparison made using Kruskal-Wallis and Dunn’s Multiple Comparison tests with p<0.05 as significant. Data are presented in µmol/g and shown as median and standard error of median.

<table>
<thead>
<tr>
<th>Patient Groups</th>
<th>Acetate median (range)</th>
<th>Propionate median (range)</th>
<th>Butyrate median (range)</th>
<th>Ratio</th>
<th>n=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Children</td>
<td>70 (64-75)</td>
<td>14 (11-20)</td>
<td>11 (6-19)</td>
<td>70:14:11</td>
<td>10</td>
</tr>
<tr>
<td>NIC Controls</td>
<td>56 (47-67)</td>
<td>18 (15-20)</td>
<td>20 (11-24)</td>
<td>56:18:20</td>
<td>5</td>
</tr>
<tr>
<td>CD and Coeliac Disease</td>
<td>60 (48-87)</td>
<td>19 (9-23)</td>
<td>13 (3-22)</td>
<td>60:19:13</td>
<td>8</td>
</tr>
<tr>
<td>Granulomatosis polyangiitis</td>
<td>58 (45-69)</td>
<td>19 (9-36)</td>
<td>18 (15-22)</td>
<td>58:19:18</td>
<td>6</td>
</tr>
<tr>
<td>CAPS</td>
<td>70 (69-71)</td>
<td>12 (11-13)</td>
<td>10 (11-12)</td>
<td>70:12:10</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 4.6: Acetate:Propionate:Butyrate Ratios of Healthy Children and Patients with Non-Inflammatory Bowel Disease (NIC)

4.3.8 Summary of Results

Analyses were performed comparing stool SCFA concentrations and percentages in infants, HC, NIC, IBD and non-IBD patients. A summary of the findings is as follows;

Control Groups

- Total SCFA concentrations of HI, HC and NIC were similar
- Infants had higher percentages of acetate than HC
- NIC patients had higher propionate and butyrate percentages, and lower acetate percentages than HC
- NIC patients had higher butyrate concentrations and percentages than IBD patients
In patients with CD (n=8):
- Compared to HC, there were no differences in total SCFA concentrations, acetate or butyrate
- Compared to HC, there were no differences in propionate concentrations but propionate percentages were increased in CD (active and remission)
- Compared to NIC, CD patients in active disease and in remission had lower butyrate concentrations and percentages (vs. CD remission p=0.032)
- There was a suggestion of increased butyrate and propionate percentages preceding clinical improvement (Appendix 4)
- There was a positive trend of association between percentages of butyrate and raised calprotectin concentrations

In patients with UC (n=3):
- Compared to HC, total SCFA concentrations and acetate concentrations and percentages were similar
- Compared to NIC, percentages of acetate were higher
- Propionate concentrations and percentages were lower compared to HC, NIC and CD patients
- Butyrate concentrations and percentages were similar to those of HC but were reduced in UC remission compared to NIC
- Percentages of propionate were lower than butyrate
- Valerate concentrations in active disease and in remission were lower than HC
- There were no significant correlations with clinical parameters

In patients with IBDU (n=2):
- Compared to HC, total SCFA concentrations were decreased
- Percentages of acetate were lower compared to HC and NIC (p=0.038) (Figure 4.3.5)
- Propionate concentrations and percentages in IBDU patients were lower than in HC, NIC and CD patients
- Butyrate concentrations and percentages were similar to those of HC but were reduced compared to NIC
- Percentages of propionate were lower than butyrate, similar to UC
- Valerate concentrations were lower than HC
CD versus UC:
- Propionate concentrations and percentages in CD were higher than in UC
- There was a trend for increased butyrate concentrations and percentages in active disease compared to in remission in both patient groups but this was not statistically significant
- CD patients had higher propionate percentages than butyrate but this was reversed in UC patients

In ileostomy samples:
- There were significantly lower total SCFA concentrations in ileostomy samples compared to HC (p=0.0185)
- Ileostomy SCFA consists predominantly of acetate (>85%)

HI versus Allergic Infants:
- Comparing allergic and non-allergic infants, no differences were seen with age, sex, mode of delivery and type of feed

Analyses of individual IBD patients:
- Norovirus and a course of Trimethoprim preceded a reduction in butyrate concentration despite maintaining similar total SCFA concentrations (Patient A15, Appendix 4.1)
- A patient with CD and Coeliac Disease was found to have higher SCFA concentrations (Patient D2, Appendix 4.2)

In non-IBD patients:
- There were no significant differences seen in total SCFA concentrations and individual SCFA percentages
- CAPS patients had similar acetate:propionate:butyrate ratios to HC
- The patient with Granulomatosis polyangiits and the patient diagnosed with CD and Coeliac Disease had similar acetate:propionate:butyrate ratio to NIC patients
4.4 Discussion

The role of SCFA has long been known and its importance in gut function appreciated. As knowledge of gut microbiota expands, there is greater understanding of the role of SCFA. Although the numbers are small, the results from this study demonstrate some clear patterns differentiating SCFA profiles between children with disease and those without.

4.4.1 Short Chain Fatty Acids in Control Groups

The acetate:propionate:butyrate molar ratio of 57:22:21 has been widely used in the literature (236). Cummings also combined molar ratios of human stool SCFA from other studies, resulting in a molar ratio of 60:24:16 (698). As these were based on 5 adults (aged 36-89 years of age) and 1 child of 16 years, it was not ideal for comparison in this study on children. In addition, the diet and environmental exposures in 1987 would have been different thus influencing the gut microbiota. It was therefore important to establish the ratio in a suitable control group for this study.

As the gut microbiome evolves with age, it was useful to determine the pattern in early childhood. There are currently no studies on stool SCFA in healthy infants of this age with no family history of atopy nor any exposure to antibiotics. From this study, infants were found to have a ratio of 86:8:5 with higher acetate percentages and lower propionate and butyrate percentages compared to HC and NIC patients.

The higher percentage of acetate in healthy infants suggests that the microbiota contains more acetogens than propionate- or butyrate-producing organisms. In addition, or alternatively, their diet of relatively less plant fibre may favour acetogens as more plant fibres are required for greater propionate and butyrate production. Many of the acetogens are from the phylum Firmicutes, which dominated the profiles of a few of the HI. However, their individual microbial profiles varied much more than HC. Also, with the analysis conducted at phyla level, and given the small cohort, it was not possible to draw any conclusions regarding the possibility of greater abundance of acetogens. Functionally, there is a greater need for acetate than propionate and butyrate in infancy. Little of the acetate produced is metabolised in the colon and mostly absorbed then extracted by the liver, where it is utilised for energy production (727, 728). It is also an energy source for
myocytes and for lipogenesis (701, 750). These are important functions for rapidly growing and developing infants.

From the age of 4 years, the SCFA concentrations and percentages were similar. Although SCFA concentrations were similar, there were differences seen in SCFA percentages between the HC and NIC patients. NIC patients had higher percentages of propionate and butyrate, and lower percentage of acetate. This may also suggests that NIC patients appropriately produce more propionate and butyrate, which has roles in maintaining IEC barrier function, immune defence and inflammatory homeostasis, in response to their GI conditions. These findings are similar to those reported by Treem et al. who found that patients with diarrhoea-predominant irritable bowel syndrome (DP-IBS) (aged 15-58 years) had lower total SCFA, acetate and propionate but higher n-butyrate than HC (aged 12-65 years) (863). This would suggest that HC and NIC patients have different microbial profiles, however, no differences were apparent at phyla level. Further analysis at lower taxa may reveal differences to explain the SCFA findings.

4.4.2 Crohn's Disease

The total SCFA concentrations were variable within the groups; for comparison to be made between patients and control groups, the percentages of each SCFA were analysed.

Interestingly, the ratio for CD patients (67:20:10) were similar to HC, but the butyrate percentages were less than NIC patients. Indeed, in active CD, Bacteroidetes dominated while Bacteroidetes and Firmicutes dominated in remission. Many of the butyrate-producing organisms, such as *Faecalibacterium (F.) prausnitzii* and *Roseburia (R.) species*, belong to the phyla Firmicutes (Table 4.1.1, Section 4.1). It has been well-documented that the dysbiosis in CD includes reduction in these species (272). In addition, CD patients had twice the percentage of propionate than butyrate (Table 4.3.1). Given that Bacteroidetes are also propionate-producers, this would suggest increased relative abundance of Bacteroidetes and/or increased capacity for propionate production, possibly part of a compensatory mechanism.

The percentage of each SCFA was investigated against multiple clinical parameters (CRP, ESR, stool calprotectin, PCDAI scores). No correlations were observed. From analysis of individual patients, a rise in butyrate and propionate percentages were often
followed by a decline in ESR (Appendix 4.1-4.3), suggesting modulation in inflammation and clinical improvement. This would imply an increase in butyrate- and propionate-producing species or increased production from the existing microbial population.

Butyrate plays a major role in gut immune homeostasis including anti-inflammatory effects on macrophages and DCs. It is also involved in the maintenance of IEC barrier functions including regulation of IEC proliferation and differentiation, conversion of pro-IL-18 to IL-18, which is involved in epithelial repair and maintenance, and regulates the production of antimicrobial peptides. These are important functions for mucosal healing thus may explain the decreased ESR levels seen following rises in propionate and butyrate concentrations in CD patients. It may also explain the higher concentrations seen in NIC, which may be an appropriate response to their underlying condition. In addition, these observations may partly explain the trend for higher butyrate percentages in active disease compared to those observed in remission.

### 4.4.3 Ulcerative Colitis and IBDU

The total and individual SCFA profiles for patients with IBDU were similar to the UC patients thus will be discussed together. The total SCFA concentrations were found to be lower in UC patients and in IBDU patients compared to HC and NIC (Figure 4.3.4). Although not statistically significant in this study, it has been shown to be significantly lower in paediatric and adult UC patients than control subjects (386, 837, 864). These studies also demonstrated lower concentrations of propionate and butyrate in UC patients, as seen in this study. This would be in keeping with decreased butyrate-producing *R. hominis* and *F. prausnitzii* reported in UC patients (386).

Although the propionate and butyrate concentrations (and percentages) were lower compared to HC, NIC and CD patients, the butyrate concentrations were relatively higher than the propionate concentrations in UC patients, overall resulting in lower propionate and higher acetate concentrations when compared to the other groups (Table 4.3.1). Interestingly, Treem et al. reported similar findings of butyrate and propionate concentrations in children with IBD (837). In contrast, Machiels et al., Takaishi et al. and Vernia et al. reported higher propionate concentrations than butyrate in adult UC patients, although both metabolites were lower than HC (386, 865, 866). On the other hand, Van Nuenen et al. did not distinguish their IBD subjects but of the 8 patients with colitis (CD n=5, UC n=3), some had higher butyrate than propionate concentrations.
Similarly, Huda *et al.* did not identify the CD and UC patients in the group of 8 IBD patients. They found that butyric acid and propionic acid were lower in the IBD group compared to the HC (n=50), with the propionic acid being lower than butyric acid. This was thought to be related to the rice-based diet influencing the intestinal bacterial composition (836). Indeed differences in dietary intake and the resulting microbial profiles, as well as differences in methodology may explain these contradicting findings. In addition, these studies grouped the IBD patients together irrespective of disease activity, which is another factor that may have influenced their findings.

Results from this study revealed dominance of Firmicutes with lower Bacteroidetes in active UC than in remission and greater abundance of Proteobacteria than in CD. Vascularisation plays a major role in UC, increasing oxygen delivery to the tissues. This disadvantages Bacteroidetes, allowing the facultative anaerobic Firmicutes and Proteobacteria to proliferate. As mentioned above, many of the butyrate-producers are from the phylum Firmicutes thus offering an explanation for these findings.

The higher butyrate concentrations would suggest greater production and/or reduced utilisation. Roediger *et al.* demonstrated that colonocytes of UC patients exhibited impaired butyrate oxidation to CO$_2$ and ketone bodies, instead, glucose and glutamine oxidation was increased (830). The availability of free Coenzyme A (CoA) is a factor for butyrate oxidation (867), and free CoA, but not its precursors, is also reduced in UC mucosa (831). This may partly explain the reduced utilisation of butyrate in some UC patients.

Pantothenate (vitamin B5), which is found in most foods especially meat, vegetables, cereal grains, legumes, eggs and milk, is the key precursor for CoA biosynthesis. Pantothenate uptake into cells is tightly regulated and essential for organisms that are unable to synthesise the vitamin (868). Pantothenate is formed by the ATP-dependent condensation of β-alanine and pantoate by pantothenate synthetase before subsequently being used for CoA biosynthesis or exported from the cell (868). As UC mucosa is known to have reduced free CoA (831), it may be that vitamin B5 deficiency is a factor contributing to the impaired butyrate oxidation in these patients.

Butyrate can also be converted to ketone bodies in the colonic mucosa, less so by distal colonocytes than proximal colonocytes (869). The rate of butyrate absorption is equal per unit area of mucosa in the right and left colon, and CO$_2$ production from butyrate is the same at both sites (870). Together, this suggests that more butyrate enters the TCA
cycle in the distal colon thus butyrate is more important as a respiratory fuel in the distal than proximal colon.

UC mucosa is therefore thought to be in an “energy-deficient state” (830). Diminished SCFA as an energy supply results in impaired Na^+\text{-}\text{-}K^+\text{-}ATPase activity, thus inefficient electrolyte absorption and secretion resulting in the diarrhoea seen clinically. Mucus synthesis, an active process requiring energy, is also effected thus goblet cell depletion is seen histologically. Without adequate energy, there is also impaired maturation of undifferentiated crypt cells resulting in crypt abscesses. Given that distal colonocytes are more reliant on butyrate, this may explain the characteristic distribution of more severe disease in the distal colon. Therefore, this would suggest that the increased butyrate concentration seen in UC is due to under-utilisation of available butyrate rather than insufficient production of it. However, there is no way of knowing how much butyrate was actually produced thus the increased butyrate could in fact reflect increased butyrate production with only the “leftover” butyrate measured. Given the findings of this study, the increased and dominance of Firmicutes in active UC would support the increased butyrate production. This increase may still be insufficient to reverse the “energy-deficient” state. However, the RCT conducted by Breuer et al. would suggest that sufficient and sustained SCFA enemas can result in clinical and histological improvements (846).

UC patients are also thought to harbour greater abundance of sulphate-reducing microbes. Many of these belong to the phylum Firmicutes and the class Deltaproteobacteria from the phylum Proteobacteria (833, 834). Further analysis of lower taxa would be needed to investigate this aspect further.

**4.4.4 Differences between CD and UC Patients**

CD patients were found to have higher propionate concentrations and percentages compared to UC and IBDU patients. This is likely to reflect the microbial dysbiosis discussed above. In addition, the valerate concentrations were low but were lower still in patients with CD, UC and IBDU compared to HC and NIC patients. Of the patients with IBD, CD patients had higher valerate concentrations than UC patients but as valerate functions in humans are unknown, the significance of this finding cannot be determined.
4.4.5 Ileostomy SCFA

Ileostomy samples received from patients with VEOIBD and IL-10R mutation were analysed. The SCFA profiles of patients with IL-10 mutations have not been reported. This study found that the total SCFA concentrations were significantly less compared to HC (with more than 85% of the SCFA being acetate. This has been previously reported (871, 872). Although the ileum is capable of absorbing SCFA (873), all SCFA except acetate are produced by anaerobic bacteria only. The higher oxygen concentration in an ileostomy would favour the facultative anaerobic organisms such as those from the phylum of Proteobacteria and impair the growth and function of strict anaerobes. Indeed Proteobacteria dominated the stool microbial profiles in these patients, in addition to Firmicutes in the patient with VEOIBD. This would explain the low concentrations of propionate and butyrate seen but then raises further questions.

Most butyrate functions take place in the colon but it is also involved in immune regulation, and acts via the GPR43 in several organs. In addition, propionate is predominantly absorbed and extracted by hepatocytes for gluconeogenesis. It is also involved in regulating immunity, fatty acid and glucose metabolism. Both SCFA promotes satiety. The long-term effects of low levels of these SCFAs in patients post-colectomy are not well-known or understood. There is however, evidence that butyrate can be increased in ileostomy effluent by increasing resistant starch in the diet (871, 872) but whether this is even necessary remains to be determined.

4.4.6 The Possible Effects of Trimethoprim on SCFA concentrations

The effects of antibiotics on the gut microbiota have been well documented (56). Following a course of Trimethoprim for urinary tract infection in Patient A15, there was a decrease in butyrate concentration associated with a rise in propionate and a small increase in total SCFA. The butyrate level increased subsequently but this suggests that the butyrate-producing bacteria in Patient A15 were particularly sensitive to antibiotics. Prior to the urinary tract infection, this patient also suffered with Norovirus gastroenteritis, which has been reported to cause dysbiosis, increasing the presence of Proteobacteria (874). Therefore, either the Trimethoprim or Norovirus, or both of these, could have caused the decreased Bacteroidetes and increased Proteobacteria observed, providing an explanation for the decreased butyrate measured in this patient.
4.6.7 The effect of Concurrent Coeliac Disease

Patient D2 was diagnosed with Coeliac Disease and CD. Her total SCFA concentrations were often higher than those of other CD patients and HC. Her stool microbial profile also differed from other CD patients and more characteristic of Coeliac Disease, with Firmicutes dominating. It is interesting to note that this was the dominant feature in this patient, despite reporting strict adherence to a gluten-free diet. Although dysbiosis in Coeliac Disease can be attenuated after long-term gluten-free diet, the microbiota is not completely restored (574) thus may explain the persistently higher SCFA concentrations seen in this patient.

4.6.8 Patients with Cryopyrin-Associated Periodic Syndrome (CAPS)

There was a unique opportunity to include 2 patients with CAPS, a condition affecting the IL-1β pathway. These patients were commenced on treatment with Canakinumab for their disease. Despite having GI symptoms, these patients had SCFA profiles similar to HC. This is in keeping with their stool microbial profiles, which were also similar to HC.

4.6.9 SCFA Concentrations in Allergic Infants

Two older, allergic infants were compared with 5 younger healthy infants. One allergic infant (age 16 months) had lower total SCFA but the other (age 12 months) had similar SCFA concentrations to the healthy infants. The older allergic infant with lower total SCFA concentration had presented earlier with rectal bleeding at 5 weeks of age following birth by caesarean section. This is similar to the findings of Sandin et al. who reported lower concentrations of all SCFA in allergic children compared to HC (854). On the other hand, the younger allergic infant with the higher SCFA concentration was born by vaginal delivery then presented with less severe symptoms later. The stool microbial profile was available for the younger allergic infant, in whom Bacteroidetes dominated and low bacterial diversity found.

No pattern of association or differences in SCFA concentrations were seen when comparing infant sexes, mode of delivery and the type of feed. Due to the small numbers, no definitive conclusion could be drawn.
4.6.10 Clinical Implications

In IBD, the lower SCFA concentrations reported has prompted its experimental usage with variable success (721, 875). It is unclear why some responded favourable and others not. A possible reason may be due to the rectal administration of the drug that is felt to be unacceptable for some thus affecting treatment compliance. An alternative method of increasing intraluminal SCFA is by increasing dietary intake of indigestible plant fibres such as pectin, starch, oat and wheat bran (695, 699). Prebiotics is an area of active research and also has the potential to increase luminal SCFA concentrations.

As discussed in Section 4.1.3.4, there is evidence for SCFA enemas for the treatment of distal UC, and also refractory distal UC. Given the low SCFA concentrations, especially butyrate and propionate, seen in the UC patients in this study, addressing diminished SCFA availability may be an effective treatment strategy.

The role of butyrate as a major colonocyte fuel is well recognised. Many patients post-colectomy with remaining colon and/or rectum suffer with diversion colitis. SCFA or butyrate enemas have been shown to effectively reduce diversion colitis if applied consistently twice a day for a minimum of 6 weeks (846). It thus raises the question of why this is not routinely offered to patients with diversion colitis post-colectomy. The route of administration is sometimes seen as a barrier but on balance, patients may benefit from symptomatic relief.

Another way of indirectly increasing SCFA is by increasing intake of fruit and vegetables, thus providing more indigestible plant fibre for fermentation to produce the SCFA. IBD patients are known to reduce or avoid dietary fibre as it can exacerbate gut symptoms such as diarrhoea. Although understandable, this should be discouraged and patients counselled on the benefits of higher/normal dietary fibre intake (377, 378). IBD patients have regular dietetic input but more frequent and intensive involvement may be needed for those requiring a change in dietary habits, which is known to be challenging.

As mentioned earlier, increased butyrate and propionate were followed by decreased ESR levels in some CD patients. This would suggest clinical improvement and treatment response. Although measuring SCFA levels for monitoring treatment may be an attractive idea, the practicalities of this would exclude its use clinically.
4.6.11 Study limitations

Firstly, it is recognised that approximately 5% of the SCFA produced is being measured in the stool. On the other hand, it is easily obtained, unlike blood and intestinal biopsies. In addition, once absorbed into the systemic circulation, the molar ratio of acetate, propionate and butyrate alters from 57:22:21 in the caecum, distal colon and stool to 71:21:8 in portal veins and 81:12:7 in hepatic veins (1). Thus, given the study aim of assessing the relationship between gut microbiota and the SCFA concentrations being produced, the same stool samples were analysed for both microbiota and SCFA concentrations. Although it is possible to analyse SCFA in blood and urine, stool samples were thus felt to be the most practical and suitable for this analysis.

It is also worth bearing in mind that:

1. SCFA are volatile compounds with short half-lives
2. Fermentation rates are not uniform throughout the colon thus SCFA concentrations decreases from caecum (70-140 mM) to rectum (20-70 mM)(695)
3. The absorptive capability of gut mucosa varies within the colon
4. Approximately 80-95% of SCFA are metabolised by bacteria or absorbed by colonocytes (876)
5. A significant percentage of butyrate is metabolised by colonocytes thus influencing its luminal concentration and the ratio
6. Water absorption and loss of digestive material alter the availability of SCFAs in the distal colon independent of rates of production

Therefore, stool SCFA concentration provides little information about actual intestinal SCFA metabolism. However, it could, suggest its availability in the gut and the metabolic function of the gut microbiota.

The small patient numbers in each group was a limitation in this study. This, however, enabled detailed analyses of each individual longitudinally, revealing some trends of SCFA changes with treatment and clinical status that would require greater numbers for confirmation.
4.6.12 Conclusion

There is no doubt that SCFA are important functional metabolites of gut microbiota. Despite the limitations in their measurements, the SCFA concentrations and percentages have been insightful, as a reflection of the intestinal microbial composition and the dynamics occurring at the mucosal surface.

Differences in SCFA concentrations and percentages have been demonstrated between the control and diseased groups, as have changes in SCFA profiles with age. Combining the SCFA results with clinical data helped with the understanding of microbial and SCFA alterations occurring with inflammation. There were no specific changes in SCFA seen with treatment but in some CD patients, increased propionate and butyrate concentrations were followed by reduced systemic inflammation. It is recognised that dietary intake of fibre may partly contribute to these findings.

Therefore, despite the limitations highlighted, evidence for a potential correlation between SCFA with clinical status was identified. These observations, however, warrant further investigation in a larger cohort of IBD patients.
5. Chapter 5

Systemic Cytokines and Vascular Biomarkers

5.1 Introduction

IBD is triggered in at risk individuals resulting in an exaggerated immune responses to luminal microbes. There is failure of the intestinal mucosal barrier that protects the host from commensal, pathogenic and opportunistic microbes. In addition, immune dysfunction in susceptible individuals leads to inflammatory processes resulting in the manifestations seen in IBD.

In this study, the immune responses in IBD were investigated and compared to HC and NIC patients. Blood samples were collected prospectively, thus longitudinal data in the IBD patients with disease activity and treatment were analysed.

5.1.1 The Role of Gastrointestinal Mucosal Homeostasis in Health

The intestinal immune system has the challenge of defending the host against pathogen invasion whilst promoting tolerance to resident commensal microbes. In addition, the mucosal barrier protects the host while enabling absorption of water, electrolytes and nutrient through the mucosa. Active monitoring and antimicrobial mechanisms of the innate and adaptive immune systems are employed to regulate intestinal luminal microbes in a bid to maintain immune homeostasis.
5.1.1.1 Mucosal Barrier

A physical barrier exists between luminal contents and the intestinal host immune system, consisting of mucus produced by goblet cells, intestinal epithelial cells (IEC), specialised epithelial M cells, dendritic cells (DC) and Paneth cells. Paneth cells and DC are considered as immune cells thus discussed in Section 5.1.1.2.

A thick layer of mucus overlying IECs forms the first line of host defence, preventing microorganisms from reaching the epithelial surface. It consists of high molecular weight glycoproteins called mucins, trefoil peptides, and resistin-like molecule β (RELMβ) produced by goblet cells (Abraham 13-16). In the microbially heavily-populated colon, 2 mucus layers protect the epithelial surface. The inner, relatively sterile layer is composed of tightly-packed mucin polymers, and an outer layer colonised by microbes which consists of looser mucin polymers that are broken down by proteolysis (877). The mucus viscosity increases throughout the colon, selectively excluding pathogens proximally and completely distally (878).

IEC protects the host against pathogenic organisms and limit commensal bacterial penetration. They also function as antigen presenting cells (APC) in response to inflammation, expressing class I, class II and non-classical major histocompatibility (MHC) molecules on their surfaces, (879). With stimulation of pattern recognition receptors (PRR), such as toll-like receptors (TLR) and nucleotide-binding oligomerisation domain 2 (NOD2) expressed on IEC, cytokines and antimicrobial proteins are secreted.

Although IEC are non-immune cells, they are also involved in maintaining immune homeostasis by sampling luminal contents and removing pathogens. Specialised epithelial microfold (M) cells found adjacent to Peyer’s patches and lymphoid follicles transcytose luminal contents to be presented to professional APC. Peyer’s patches are aggregates of lymphoid tissues, which are important parts of the gut associated lymphoid tissue (GALT). They are located in the submucosal layer and extends into the mucosal layer of the distal jejunum and ileum.

There are other mechanisms that contribute to maintaining and protecting physical barrier. Desmosomes provide strong intercellular adhesion between adjacent epithelial cells with gap junctions forming pores that provide intercellular connection between these adjacent cells (877). Tight junctions (TJs) are composed of a network of strands that seal the paracellular space but can selectively allow passage of small molecules and water. In IBD, TJ at sites of inflammation are disrupted, resulting in paracellular permeability.
and enables bacterial translocation through the epithelial barrier (880). Interestingly, mice with defective TJs do not exhibit colitis, suggesting that a dysregulated immune system is also necessary to initiate inflammation (881).

At the mucosal surface, acyloxyacyl hydrolase (AOAH), produced by neutrophils, dendritic cells, renal cortical epithelial cells, and macrophages, alters the structure of lipid A moiety of LPS. This attenuates TLR4 stimulation and inflammatory responses (882). Similarly, alkaline phosphatase dephosphorylates the lipid A, thereby modifying the inflammatory responses stimulated by bacteria and contributes to development of mucosal tolerance to commensal bacteria (883).

The ability to repair and regenerate damaged epithelium is an important part of maintaining intestinal barrier integrity. This function depends on factors such as growth factors like trefoil factor (884, 885), innate signals (886) from cytokines such as IL-18 and IL-22 (887, 888), and regulation of endoplasmic reticulum stress (889). In IBD, there is increased epithelial cell turnover however the regenerated IEC are poorly-differentiated (890).

5.1.1.2 Innate immune system

The intestinal mucosal innate immune system is maintained in a state of hypo-responsiveness, having attenuated responses to pathogens and tolerant of commensal bacteria and food antigens. Cells of the innate immune system include Paneth cells, DC and macrophages.

Paneth cells are key players in intestinal innate immunity found at the bottom of small intestinal crypts. They produce antimicrobial proteins (AMPs), such as defensins, lectins and lysozymes, stored in secretory granules and released with bacterial stimulation. They kill Gram negative and Gram positive bacteria, enveloped viruses and fungi (884, 885, 891, 892).

Dendritic cells (DC) are characterised based on their cell surface markers (e.g. CD103), chemokine receptors, cytokine production, differentiation capacity (Th1, Th17, Treg) and defence functions. They sense luminal antigens that have been transported into the lamina propria (LP) and through directly sampling luminal contents with its dendrites, which increase in number in the presence of invasive pathogenic bacteria (33, 893, 894).
Luminal antigens are transcytosed by M cells or through apoptosis of epithelial cells. DC then deliver these antigens to mesenteric lymph nodes (MLN) where they interact with B and T cells. Commensal bacterial antigens initiate tolerogenic responses, promoting peripheral T regulatory cell production which suppresses effector T cells. Thus, DC maintain tolerance towards commensal microbiota and food antigens but generates immune responses to protect the host against pathogens.

Macrophages in the LP do not function as professional APC. Although they phagocytose invading microbes, they do not always initiate an immune response. They have low expression of receptors for IgA, IgG, complements and integrin (895, 896), and do not produce inflammatory cytokines to microbe-associated molecular patterns (MAMPs) or phagocytosed necrotic cells (897). Epithelial cells, subepithelial myofibroblasts, fibroblasts, lamina propria lymphocytes, and intraepithelial lymphocytes produce anti-inflammatory regulatory TGF-β and IL-10 that drive LP macrophages to promote tolerance by inducing T cells to differentiate into Tregs or become anergic (228, 898, 899). They are also able to influence the adaptive immune responses towards Th1, Th2 or Th17 (900). Invading microbes that breach the epithelial barrier are phagocytosed and killed by macrophages which produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) (901-903).

Cells of the innate immune system recognise the carbohydrate and lipid motifs of pathogens, called MAMPs, utilising pattern recognition receptors (PRR) including the family of TLRs, NODs, C-type lectins, nucleotide binding domain and leucine-rich repeat-containing receptors (NLRs), and retinoic acid inducible gene I-like receptors (904). Given the variety of options, it is thought that a deficient or dysfunctional pathway can be compensated by another (890).

Pattern recognition receptors (PRR), expressed on immune and non-immune cells, detect the presence of microbes. TLR activation leads to signalling cascades that result in the production of cytokine milieu that promotes inflammation and augments downstream innate and initiate adaptive immune responses. The inflammatory responses can be modulated by PRR expression level, distribution and responsiveness. An example is TLR5 and TLR9 expression on intestinal epithelium, which is limited to the basolateral surface of the IEC, thus limiting overt activation to luminal contents in health (905, 906). On the other hand, TLR9-deficient mice have increased susceptibility to colonic injury, and delayed and defective epithelial repair. This correlated with increased leukocyte infiltration and delayed resolution of inflammation, which may be related to the reduced expression of VEGF in the colon (907).
NOD2 is an intracellular PRR expressed by haematopoietic cells (T cells, B cells, macrophages, CD and mast cells) and non-haematopoietic cells (Paneth cells, stem cells, goblet cells and enterocytes). It is stimulated by ligand muramyl dipeptide, leading to activation of pro-inflammatory pathways e.g. NF-κB, MAPK and Caspase-1. This conserved ligand of gram-positive and gram-negative bacteria thus enables the host to detect a wide variety of bacteria (908).

Autophagy is another important mechanism of removing bacteria, and genetic mutations are implicated in the pathogenesis of CD (909-911) (See Section 5.1.3.4).

5.1.1.3 Adaptive Immune system

The adaptive immune responses can be mediated by antibodies produced by B lymphocytes, known as humoral immunity, and T lymphocytes, known as cell-mediated immunity.

B cells
Naïve or memory B cells are activated to proliferate and differentiate into antibody-secreting effector cell. These antibodies are involved in direct killing of bacteria and opsonisation for microbial phagocytosis. B cells differentiate into plasma cells that secrete immunoglobulins M, G, A and E, which mediate a variety of immune functions. Secretory IgA plays a crucial role at the intestinal surfaces. Increased bacteria load triggers B cell recruitment to the LP and stimulate IgA secretion into the lumen (912, 913). They are important in defending against pathogens and regulate commensal bacteria (914).

T cells
In cell-mediated immunity, CD4 T cells are capable of differentiating into the main groups Th1, Th2, Th9, Th17 and Tregs (Figure 5.1). T cell lineages are defined by the cytokines produced and their functions. Th1 protects the host against viral and bacterial pathogens, with IFN-γ being a key cytokine, while Th2 responses protect the host against helminths and other parasites, with the main cytokines being IL-4, IL-5 and IL-13. Th17 responses, on the other hand, are generally pro-inflammatory, protecting the host against infections. Regulatory T cells (Treg), producing IL-10 and TGF-β, suppress immune responses thus maintaining immune homeostasis and self-tolerance. In maintaining immune
homeostasis, the balance between effector cells and Treg is crucial. Adaptive immune responses are indirectly and directly regulated by the innate immune pathways.

Figure 5.1: The Role of T cells in IBD

Naïve T cells differentiate into Th1, Th2, Th17 and Treg. CD is predominantly mediated by Th1 while UC is predominantly a Th2-mediated disease. Th17 is involved in both CD and UC. Treg play important roles in regulating immune responses via the actions of IL-10 and TGF-β.

5.1.1.4 The Role of Intestinal Microbiota

Intestinal immune tolerance of luminal contents is possible through a number of mechanisms, with the microbiota being a factor themselves. These microbes are involved in the development and maturation of the intestinal immune system. Germ-free (GF) mice or mice with defective microbe recognition pathways have less CD4+ T cells and exhibit abnormal development of lymphoid follicles, function of the IEC, IgA production, T-cell differentiation, and regulatory mechanisms that down-regulate intestinal inflammation (886, 906, 915-918). In addition, PRR expression and signalling pathways require exposure to microbiota for optimal function (919). Also, the number of NK cells is less in GF mice, resulting in reduced production of IL-22, which has a protective role in host defence (920). Despite the importance of the microbiota for
immune development and function, mechanisms are in place to minimise microbial exposure and immune responses are down-regulated to maintain homeostasis.

Dysbiotic intestinal microbiota is able to modulate diseases such as IBD. On the other hand, host factors such as genetic mutations and conditions such as diet affect intestinal microbiota composition, contributing to disease risk.

5.1.2 The Role of Gastrointestinal Immunity in Inflammatory Bowel Disease

The dysregulated innate and adaptive immune systems in IBD lead to uncontrolled immune reactions to pathogenic and benign stimuli, as observed and reported in IBD patients and animal models.

5.1.2.1 Genetic Susceptibility in IBD

Genetic mutations identified in IBD patients indicate that defective barrier function, immune responses, recognition and handling of bacteria are key in the pathogenesis of IBD (921).

Epithelial Barrier function
Gene defects affecting epithelial barrier function, namely ECM1, CDH1, LAMB1, HNF4A, GNA12, have been identified in UC patients but not in CD (43). Other genetic mutations effecting barrier function has been implicated in IBD such as disordered production of mucin with Mucin 2 (MUC2) and MUC19 deletions in CD (891, 910). Murine studies have also implicated Occludin, Claudin-1 and Claudin-6 deletions affecting TJ proteins thus epithelial permeability (922).

Innate Immunity - NOD2 Mutation
Caspase recruitment domain containing protein 15 (CARD15), which encodes NOD2, was the first genetic mutation associated with IBD. NOD2, which is expressed on various cell populations including DCs, macrophages, IECs, and endothelial cells, regulates immune defence and tolerance in the intestine. The peptidoglycan of Gram-positive and gram-negative bacteria stimulates NOD2 resulting in activation of the NF-κB pathway. NOD2 also functions as a viral PRR thus also plays a role in antiviral defence (923).
NOD2 signalling leads to increased production of pro-inflammatory cytokines, AMPs and ROS in addition to enhanced bacterial killing and APC maturation to activate T cells (924). NOD2 expressed on T cells appear to regulate cytokine production and pathogen clearance (925). Indeed neutrophil recruitment to injured sites is impaired in CD, leading to defective clearance of pathogenic organisms and subsequently leading to inflammation (911).

NOD2 also promotes Treg survival thus contributing to down-regulation of inflammatory responses (926). In mice with NOD2 deficiency, increased intestinal microbiota in the terminal ileum and increased susceptibility to intestinal pathogens were observed (919, 924, 927). Despite these reports, individuals with NOD2 polymorphism does not necessarily develop CD, indicating other contributing factors are at play.

**Autophagy**

Autophagy is a cellular process with a role in structural remodelling, nutrient and energy production, degradation of cellular debris and protection against invading pathogens and foreign materials. It plays an important role in innate immunity, transporting foreign antigens to cells for immune recognition and triggering the adaptive immune system. Defects in this processes result in impaired clearance and sustained activation of monocytes and compensatory induction of adaptive immune responses leading to inflammation (911).

NOD2 recruits ATG16L1 to the site of bacterial entry, initiating autophagy via a process which includes ATG5. ATG16L1, a component of a large protein complex essential for autophagy, is expressed by IECs, APCs, CD4+, CD8+, and CD19+ T cells (928). CD patients with ATG16L1 risk allele were found to have reduced autophagy function and decreased AMP secretion by Paneth cells. Macrophages of mice deficient in ATG16L1 were found to produce high amounts of IL-1β and IL-18 when stimulated with LPS making these mice more susceptible to DSS-induced colitis (929).

Immunity Related GTPase M, more commonly known as IRGM, is expressed in intestinal tissues, peripheral blood leukocytes, and monocytes, and encodes interferon-inducible guanosine triphosphatases. It is important in eliminating intracellular pathogens such as *Toxoplasma gondii, Listeria monocytogenes* (930), mycobacterial (931) and *Salmonella typhimurium* load (932). It is a common target of RNA viruses (933), and in IBD plays a major role in initiating and modulating autophagic responses in CD (934).
**Adaptive Immunity**

In both CD and UC, genetic variations affecting the IL-23-Th17 pathway have also been reported. These include IL-27, IL-12p40, STAT3, JAK2, CCR6, TNFSF15 and Tyk2 (910, 935). In addition, variants in ARPC2, PLA2G2E, and IL-22 (33, 936) are specific to UC.

5.1.2.2 Innate Immunity in IBD

In IBD, there is defective and/or dysregulated immune responses to otherwise benign stimuli, or pathogenic bacteria that evades the protective defences (921).

Defective IEC responses may lead to inappropriate signals to DCs, as seen with IEC isolated from CD patients (937). This causes the DCs to release IL-12, which then drives a Th1 response (937). In addition, DCs of patients with mutated NOD2 are unable to adequately induce IL-17 expression in response to bacterial stimuli (938). Activated DCs influence subsequent immune responses, determining a Th1, Th2 or Th17 response (939). Chemokines and addressins are upregulated, attracting DCs to accumulate at inflammatory sites, causing a reduction of peripheral DCs in IBD patients with active disease (940).

In UC, there were increased expression of TLR2, TLR4 and TLR9 in colonic epithelium when none were detected in HC. This correlated with disease activity index, macroscopic and histological findings (941). DC isolated from blood of UC patients also revealed defective DC function including reduced ability to stimulate T cell responses, which was restored when treated with probiotic *Lactobacillus casei* Shirota (942).

Dysregulated intestinal macrophage responses to bacterial antigen occur in IBD patients and animal models (943). The usually attenuated responses of LP macrophages are also exaggerated in IBD. The number of macrophages increases at sites of mucosal inflammation, partly due to the increased circulating monocytes that migrate to the intestine and mount a more rapid response than LP macrophages. Macrophages of CD patients with autophagy gene mutations are also unable to effectively eradicate intracellular bacteria (944). In addition, in the absence of IL-10, macrophages differentiate into pro-inflammatory subsets, producing IL-12 and IL-23 (943).

Another innate immune cell, Paneth cells, was found to be reduced in the ileum of CD patients. This correlated with decreased production α-defensins at the site (945).
5.1.2.3 Adaptive Immunity in IBD

In both CD and UC, increased production of chemoattractants in the inflamed mucosa results in heavy infiltration of CD4 T cells. These mucosal CD4 T cells have enhanced cell cycling (946) and resistant to apoptosis. Thus it follows that blocking IL-6 results in enhanced T cell apoptosis and inhibits mucosal inflammation (947).

Immune responses in CD patients are predominantly of Th1 (cytokines IFN-γ, IL-6 and TNF-α) but also Th17 (cytokines IL-17) (948). In UC, however, there is marked increased in Th17 cytokines (IL-17) and predominance of Th2 cytokines (948, 949). Thus, Th17 has a role in both CD and UC, exhibiting polymorphic variants in its functions in both (910).

IL-17 and IL-22 initiate and amplify inflammatory responses while activating counter-regulatory mechanisms (950). IL-22 is a member of the IL-10 cytokine family and also involved in regulating intestinal barrier function and resolution of inflammation (951, 952). The other receptor subunit for IL-22, IL-22R1, is expressed only on surfaces of nonhaematopoietic cells e.g. epithelial cells, hepatocytes and keratinocytes, targeting innate cells in the respective organs (953). This results in the production of antimicrobial proteins β-defensin-2, β-defensin-3, S100A7, S100A8, S100A9, RegIIIβ, RegIIIγ, and lipocalin-2 (954, 955).

In CD, IL-12 and IL-18 produced by APCs and macrophages influences CD4 differentiation towards Th1 lymphocytes resulting in increased pro-inflammatory cytokine production e.g. IFN-γ and TNF-α. These Th1 cytokines stimulate further secretion of inflammatory cytokines IL-1, IL-6, IL-8, IL-12 and IL-18, maintaining ongoing inflammation (956).

IL-12 and IL-23 are related cytokines that share the subunit IL-12p40. IL-12p40 activates T and NK cells, and is involved in Th1 responses, while IL-23 is involved in Th17 responses (957). Intestinal macrophages and DCs produce IL-23 which activates STAT-4 in memory T lymphocytes. This stimulates IFN-γ production resulting in further production of pro-inflammatory cytokines exacerbating inflammation (958).

IL-23 has been reported to promote T-cell-mediated colitis in IL-10-deficient mice via IL-17 and IL-6 (959-961). As IL-12p40 is a subunit of IL-23, IL-12 is also recognised to contribute to the inflammation. As IL-10-/ and MyD88-/ double knockout mice do not develop colitis, MyD88 signalling is also thought to be involved in colitis (962-964).
In UC, although there is Th1 involvement, responses are predominantly Th2-mediated with increased production of IL-4, IL-5, and IL-9 (965, 966). It is however, IL-13 that is mainly responsible for the inflammation and chronicity of the inflammation in UC (967). The expression of PU.1 transcription factor regulates IL-9 production and expression of TJ proteins. When defective, these enable bacterial translocation and subsequent immune activation and mucosal inflammation, as seen in UC patients (965).

**Regulatory T cells**

The unregulated inflammatory responses seen in IBD is at least partly due to regulatory T cell dysfunction. IL-10 is a known pleotropic regulatory cytokine that maintains optimal Treg activity during inflammation (896). It is produced by several cell types including macrophages and Tregs, which can also suppress the differentiation and function of Th1 and Th2 cells. IL-10 knock-out mice models have been used extensively to investigate IBD. IEC from IL-10-deficient mice were found to have defective responses to endoplasmic reticulum (ER) stress, energy metabolism and apoptosis (968).

In the human GI tract, dysregulated IL-10 expression and function is associated with IBD (969-973). NOD2 mutations in CD patients were found to have PBMCs with significantly lower IL-10-producing capacity compared to HC or wild-type NOD2 CD patients (974). There have been no reports of NOD1 involvement in IBD (975).

In murine models, IL-10- and TGF-β- deficiency are known to result in spontaneous colitis (976). IL-10, TGF-β and retinoic acid produced by LP DC and macrophages result in the differentiation and maintenance of forkhead box P3 (Foxp3)+ Tregs (897, 977, 978). Dysregulation of these tolerogenic mechanisms result in decreased LP Tregs and increased Th1 and Th17 cells (895). TGF-β regulates mucosal immune responses. Its impaired signalling in T cells and DC results in spontaneous colitis in mice. In IBD patients, its signalling is also impaired. TGF-β production can also be mediated by intestinal bacteria in maintaining intestinal immune homeostasis (979).
5.1.3 The Role of Intestinal Vasculature in Inflammatory Bowel Disease

After leukocytes, the microvasculature and its endothelial lining play the most important role in inflammation (Elie Matchnikoff, 1908 Nobel Laureate).

5.1.3.1 Macrovascular and Microvascular Complications in IBD

Intestinal and extra-intestinal vasculature appears to play a significant role in IBD. Increased CRP with inflammation is known to predict cardiovascular events (980). IBD patients are estimated to have 1.7-5.9 fold higher risk of developing venous thromboembolism (VTE), such as deep venous thrombosis, pulmonary emboli, portal vein thrombosis and cerebral venous sinus thrombosis (981), with the risk being higher in the first year after diagnosis (982-984) and a recurrence rate of 2.5 times higher than the general population (985). There is also increased risk of coronary artery disease and myocardial infarction in these patients (986, 987). In addition, IBD is associated with mesenteric ischaemia (988). These complications of cerebrovascular and Superior Mesenteric Artery or portal vein generally occur in young patients under the age of 50 years (989).

These thromboembolic events more commonly occur during active disease, with a higher rate of fistulising disease in VTE events (990). A third of these complications, however, affected patients in remission, suggesting that there is increased pro-coagulant tendency in IBD, even when in remission (989). The lower incidence of IBD in patients with haemophilia or von Willebrand’s disease indirectly supports this (991) with Factor V Leiden being the most prevalent thrombophilia in IBD (992).

These vascular complications vary between CD and UC, indicating potentially different roles in the pathogenesis and significance between the two IBD entities. From a study of 774 admissions of IBD patients, arterial thromboemboli, MI and strokes were more common in UC than CD (993, 994), with increased risk in men and older patients, similar to the general population (982, 983).

Examining histopathology samples of inflamed small intestine and colon revealed intestinal microvascular changes in IBD patients. Vasodilatation, venocongestion, oedema, infiltration of large inflammatory cells, erosions and ulcerations were seen,
suggesting intestinal immune dysregulation activates intestinal microvascular endothelium and leukocyte recruitment to inflamed intestinal tissues (995).

5.1.3.2 Microvascular Functions in IBD

Intestinal endothelial cells have a significant role in initiating and regulating inflammation (Figure 5.2). The abnormal pro-inflammatory cytokine production in IBD sustains endothelial cell activation, perpetuating the inflammatory process (Papa 2008). These are achieved through mechanisms that promote leukocyte recruitment, angiogenesis and inflammatory responses.

5.1.3.2.1 Leukocyte Recruitment

In the intestine, microvascular endothelial cells are activated by cytokines and bacterial products. This leads to increased expression of adhesion cell molecules such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM). In IBD, leukocyte adhesion is enhanced compared to control individuals. This greater adhesion was found to be higher in inflamed sites compared to uninflamed sites of the same patients (996, 997). The adhesion molecules promote leukocyte interaction and recruitment of inflammatory cells and mediators to the site of inflammation (998), with the endothelium influencing the degree and leukocyte population recruited (662). Macrophage numbers swell in the inflamed mucosa, partly with the influx of circulating macrophages (662). The usually attenuated responses of intestinal macrophages to phagocytosed antigens become exaggerated in IBD.

5.1.3.2.2 Angiogenesis

With chronic intestinal inflammation, angiogenesis occurs to facilitate the inflammatory process and to support the increased metabolic requirement of inflamed tissues. In IBD, however, the pathological neovascularisation potentially contributes to the dysregulated leukocyte recruitment (998).

There are many known angiogenic mediators including vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS) and various inflammatory cytokines involved in angiogenic responses e.g. IL-8 and IL-15 (999, 1000).
As a consequence, the dysfunctional intestinal microvasculature results in impaired perfusion. Human and animal model studies have demonstrated compromised perfusion of chronically inflamed intestinal segments of CD patients (1001-1004). The relative ischaemia seen is thought to be partly due to impaired nitric oxide generation resulting in microvascular dysfunction (1004-1006). This relative tissue ischaemia is another significant stimulus for angiogenesis.

**Figure 5.2: The inflammatory Process in IBD**

A defective barrier enables antigens (including microbes) to invade the mucosal tissue via leaky epithelial layer (1). Stimulated dendritic cells (DC) activate T cell differentiation and secretion of pro-inflammatory cytokines (2). Leucocyte adhesion and migration occurs, facilitated by chemokines, pro-inflammatory markers (3). The endothelium
interacts with neutrophils in inflammation (4). Angiogenesis occurs (5). In addition, there is pathological neovascularisation and increased metabolic demands with inflammation, resulting in relative ischaemia (6). Adapted from Baumgart et al. and Cromer et al. (663, 1007).

5.1.3.2.3 Endothelial Cell Participation in Intestinal Inflammation

Hypoxia activates endothelial cells, initiating a cascade of reactions involving neutrophils and smooth muscle cells (1008).

Endothelial cells are able to modulate inflammation through their interactions with neutrophils. This occurs through 3 sequential steps;

1. Rolling adhesion. Mediated by selectins, leukocytes at the endothelial cell surface slow down during this initial phase.
2. Tight adhesion. Firm adherence of leukocytes, facilitated by adhesion molecules such as ICAM-1 occurs next.
3. Transmigration. A chemotactic gradient encourages transmigration through the endothelium.

Endothelial cell also regulates endothelial leakiness, and determines leukocyte number and type migrating through the endothelium (1009-1012). One such mediator is vascular endothelial growth factor-A (VEGF-A), which is produced by endothelial cell in inflammation (1012). In addition to inducing angiogenesis of intestinal microvascular endothelial cell, VEGF-A can also induce an inflammatory phenotype and neutrophil adherence to human intestinal endothelium in vitro. VEGF-A overexpression exacerbates DSS-induced colitis (1012). In addition, VEGF-A promotes increased vessel permeability and vascular leak. The oedema resulting from increased interstitial fluid contributes to the stiffness of inflamed intestinal wall (1013). This potentially exacerbates the impaired perfusion and relative ischaemia (1014, 1015). In UC, the degree of endothelial dysfunction is associated with disease severity (1016).

In addition, hypoxia also triggers smooth muscle cell proliferation. PGF-2α and basic fibroblast growth factor (bFGF) in acute hypoxia, in addition to platelet-derived growth factor-B (PDGF-B) and endothelin-1 (ET-1) in chronic hypoxia mediate this response (1017, 1018). The increased production of these mitogens in addition to endothelial NO
synthase suppression accelerate smooth muscle cell growth and induce vascular remodelling (1008).

5.1.3.3 Microbial Effects on Microvasculature

The intestinal microbiota appears to play a significant role in the development of intestinal capillary network, as demonstrated in germ-free mice models (229). However, in IBD, the higher circulating LPS levels can be detrimental (1019-1021). In UC, it was shown to correlate with disease activity (1022). In addition, TLR4 is expressed by endothelial cells and can be down-regulated for example by docosahexaenoic acid (DHA) in human intestinal microvascular endothelial cells (HIMEC) (1023). In fact, endothelial TLR2 and TLR4 mediate atherogenesis (1024), with TLR4 polymorphism reported to decrease atherosclerosis risk in patients with TLR4 polymorphisms (1025).

The increased circulating LPS in IBD triggers exaggerated immune responses, leading to increased pro-inflammatory cytokine production. In addition, the ineffective neutrophil recruitment and association of NOD2 mutation in CD suggests impaired microbial clearance as a contributing factor to the intestinal inflammation, and potentially a contributing factor to the pathogenesis (911, 1026, 1027).

TNF-like ligand 1A (TL1A) is a member of the TNF family and also known as TNFSF15. It is expressed by monocytes, macrophages, DC and endothelium of inflamed intestines in CD (928, 1028, 1029), and binds to death domain receptor 3 (DR3), also known as TNFRSF25, which is expressed mainly by T cells (928). Microbes are also able to induce TL1A in APC, partly via TLR1, 2, 4, and 9 whilst maintaining homeostasis through negative regulation via TLR8 (1029, 1030). It stimulates increased production of IFN-γ (Shih 1), co-stimulate NK T cell production of IL-13 (1031) and enhances IL-17 production (1032).

5.1.3.4 Genetic Mutation affecting Microvasculature in IBD

NKX2-3 gene mutation has been associated with CD and UC (1033, 1034). Murine studies have reported its role in epithelial proliferation, splenic development, and regulation of leukocyte homing co-receptor mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression in specialised visceral endothelial cells (1035, 1036).
5.1.4 Medications in Inflammatory Bowel Disease

Medical treatment in IBD is generally focused on halting the inflammatory process to enable mucosal healing with the aim of achieving clinical remission (Figure 5.3).

5.1.4.1 Treatments Modulating Host Immunity

The treatments discussed below have been demonstrated to modulate the inflammatory processes occurring in IBD. Exclusive enteral nutrition (EEN), corticosteroids and biological therapies are administered to induce remission as well as to maintain remission.

5.1.4.1.1 Exclusive Enteral Nutrition

Exclusive enteral nutrition (EEN) treatments in the UK include polymeric formula (Modulen) or elemental feed such as E028. They are thought to induce remission by acting directly on epithelial cells to reduce pro-inflammatory cytokine release and maintain barrier function. In addition, the 6-8 week course significantly alters the intestinal microbiota, usually within a few weeks of commencement (Discussed in Section 3.1.4.1).

In vitro models of colonic epithelial cells have elegantly demonstrated reduction of IL-8, paracellular permeability and maintenance of intestinal barrier function with EEN (1037, 1038). In an IL-10 KO murine model, Helicobacter-induced colitis was reversed with EEN. This was associated with restored intestinal barrier function, maintained barrier integrity and reduced bacterial load (1039). EEN was reported to reduce TNF-α-induced ICAM-1 expression and increase NF-κB inhibition, in addition to reduce production and circulation of IL-1, IL-6, IL-8 and TNF-α (1040).

In a study of 29 children with CD, clinical remission following EEN treatment was associated with reduced ileal and colonic IL-1β, reduced ileal IFN-γ and increased TGF-β, and decreased colonic IL-8 (1041). It thus appear that EEN directly favourably alter epithelial responses to inflammation.
Figure 5.3: Medical Therapies in IBD

Exclusive enteral nutrition (EEN) reduces the luminal microbial load thereby reducing the host immune stimulation. Infliximab, Adalimumab and Golimumab are TNF-α antibodies thus reducing its pro-inflammatory effects. Ustekinumab inhibits IL-12p40, therefore inhibiting the production of IL-12 and IL-23. Tofacitinib is a JAK1 and JAK3 inhibitor, consequently interfering with the JAK-STAT signalling pathway. Natalizumab, Vedolizumab and Etrolizumab inhibit the α4β7 integrin which is an adhesion molecule.
that facilitates T cell migration to intestinal tissues. Glucocorticoids increase the synthesis of IκBα, which inhibits IKK (IkB kinase). IKK phosphorylates IκBα, resulting in dissociation of IκBα from NF-κB and freeing it to migrate into the nucleus. IκBα therefore decreases activated NF-κB. Adapted from Danese et al. (1042).

5.1.4.1.2 Glucocorticosteroids

Glucocorticosteroids (GC) are widely used in the treatment of inflammatory condition including IBD. These include first generation GC, namely Prednisolone, Methylprednisolone and Hydrocortisone. More recently, Budesonide, a second generation GC is used with similar effectiveness and fewer side effects.

Exogenous GC is lipophilic thus able to passively diffuse through cell membranes and bind to cytosolic glucocorticoid receptors (GR) (1043, 1044). It inactivates pro-inflammatory proteins NF-κB, Activator Protein 1 (AP-1) (1045) and MAPK signalling pathway (1046) thereby suppressing inflammatory responses. Expression of pro-inflammatory cytokines IL-1α, IL-1β and IL-8 (1047, 1048), and chemokines (106, 107) are down-regulated, while IL-10 expression is upregulated (1049). In fact, GC and IL-10 act synergistically (1050). GC also inhibits B cell (1051) and T cell proliferation (1052), and promote macrophage tolerance (1053).

5.1.4.1.3 Biological Therapy

Currently, five biologic agents are approved by FDA for the treatment of IBD: Adalimumab, Infliximab, Golimumab, Certolizumab pegol, and Vedolizumab.

Anti-TNF-α was first available as Infliximab, a chimeric monoclonal IgG1 antibody consisting of a segment of the native mouse protein containing the binding site for the TNF-α and a portion of human immunoglobulin responsible for the effector function of the antibody molecule (1054-1056). It binds to TNF-α, blocking its interaction with their surface receptors p55 and p75, thereby inducing apoptosis of these inflammatory cells (1057). Infliximab has been effective in reducing disease severity and surgical interventions in IBD patients (1058-1060). In patients unresponsive to Infliximab, Adalimumab, a fully human IgG1 monoclonal antibody, is an alternative.
Certolizumab, a pegylated and fully humanized monoclonal anti-TNF antibody fragment, and Golimumab, a human IgG1 TNF-α antagonist monoclonal antibody, have been proven as more effective therapies while being less immunogenic than Infliximab and Adalimumab (1061-1065). Anti-TNF agents, however, have not been effective in treating all patients, thus is another area of interest worth exploring.

Integrins aid cell attachment to the extracellular matrix. They also facilitate signal transduction through the cell membrane in addition to immune monitoring and cell migration. They are composed of an α and a β subunits. The α4β7 is an important homing integrin expressed on memory T cells that preferentially migrate to the GI tract. Activated α4β7 are increased in CD and UC patients (1066) and currently, a humanized monoclonal antibody which binds to the β7 subunit of the integrins α4β7 and αEβ7 is available as Etrolizumab for the treatment of moderate to severe UC (1067, 1068).

Janus kinases are intracellular tyrosine kinases consisting of JAK1, JAK2, JAK3 and TYK2. Activated cytokine receptor activates JAK which then leads to phosphorylation, recruitment and activation of signal transducers and activators of transcription (STATs). The 2 phosphorylated STAT combine, translocate into the nucleus and regulate expression of various target genes (1069). JAK inhibitors, also known as small molecule drug (SMD) have shown promise in UC. Tofacitinib, a small molecule inhibiting JAK1 and JAK3, inhibits phosphorylation and activation of JAK. It has also been trialled and found to be effective as an induction and maintenance therapy in moderate to severe UC (1070, 1071).

Ustekinumab, a monoclonal antibody against IL-12p40 has been effective in severe CD patients previously treated with Infliximab (1072, 1073) (See Section 5.1.2.3). IL-10 has been explored as a potential therapeutic target but with variable success and currently not being further developed further (1074) (Section 5.1.4).

Long-term use of biological therapy can cause immunogenicity by generating antibodies. Immunogenicity can vary depending on the structure and origin of the drug, administration route, dosing schedule and individuals (1075, 1076). The generated antibodies can in turn cause acute and delayed drug reactions, and reduce treatment response. As a result, concomitant immunosuppression is added to reduce immunogenicity and improve therapeutic control.

With the expiry of drug patents, biosimilars have emerged to replace anti-TNFα therapies, resulting in significant cost reduction. Biosimilars contain the same amino acid
sequences and a very similar glycosylation pattern compared with the original product. Its long-term efficacy, safety and immunogenicity are still to be evaluated (1077, 1078).

5.1.4.2 Medications Modulating Intestinal Vasculature

IBD patients have been found to have decreased anti-thrombin III and reduced fibrinolysis (1079, 1080). In addition, in active UC, prothrombin fragments, thrombin-anti-thrombin complexes, fibrinogen, activated factor XI, IX, VIII and V are elevated (1081). Anticoagulation is therefore recommended in acute severe UC when one or more risk factors are present, given the higher relative risk of VTE during this period (25).

IBD treatment may exacerbate the risk of vascular complications. The increased risk of VTE with steroid use may be due to the increased platelet function, hypercoagulability and hypo-fibrinolysis associated with steroid use (1082). Anti-TNF-α, on the other hand, reduces coagulation and activates fibrinolysis (992). In addition, it reverses the pro-atherogenic effects of TNF-α, improving endothelial dysfunction (1083). These may partly account for its effective use in IBD and the low occurrence of VTE complications in patients receiving anti-TNFα (1084).

Selectively blocking adhesion molecules with anti-α4 integrin monoclonal antibodies Natalizumab and Vedolizumab has been effective in treating moderate to severe CD (21, 1061, 1085). In addition, anti-α4β7 antibody Vedolizumab resulted in remission in 33-50% of UC patients (1086-1088).

Another potential therapeutic agent is Alicaforsen, an oligodeoxynucleotide which inhibits ICAM-1, thus decreasing leukocyte recruitment to inflamed sites (1085, 1089-1091). It has been trialled as an infusion with clinical improvement seen in CD patients (1092). It has also been trialled as a topical treatment administered rectally in left-sided UC and ulcerative proctitis (1093). The 6-week daily course of enemas was reported as safe and effective although the relapse rate was high, perhaps due to compliance or the need for regular treatment.

VEGF-A is increased in IBD. It is a known mediator of angiogenesis and upregulation of intercellular adhesion molecule-1 (ICAM-1), interacting with VEGFR2 which is the key receptor for VEGF-A. VEGFR2 thus represents a potential therapeutic target not just for IBD patients but for other inflammatory conditions (1012).
During leukocyte recruitment to inflammatory sites, activated platelets express adhesion proteins e.g. P-selectin, that may facilitate leukocyte rolling on platelets adherent to endothelial surface (1094). Platelet-leukocyte aggregates form a surface for coagulation and inflammation. These aggregates have been reported to be sensitive to thiopurine treatment (1095). In addition, increased activated platelets and platelet aggregates in IBD increase thromboembolic risk in these patients (1096). Activated platelets also express CD40, which induces endothelial inflammation and is increased in IBD patients (1097-1099). In mice, inhibition of CD40/CD40L system reduced atherogenesis (1100).

Another antiangiogenic agent that has been successful in refractory CD is thalidomide (1101, 1102). It is also able to inhibit TNF-α production. Curiously, curcumin, which is a constituent of turmeric, was found to inhibit HIMEC growth in vitro (1103). This antiangiogenic property may explain its efficacy in maintaining remission in UC patients in a multicentre RCT (1104).

5.1.5 The Role of Vitamin D in Immune Function

Nutritional status affects health and general wellbeing. Adequate macronutrients and micronutrients are necessary for normal bodily functions. Vitamins and trace elements contribute to cellular immunity, antibody production and maintenance of epithelial barrier (Appendix 5A) (1105). IBD patients, particularly CD patients, can be malnourished due to self-restricted intake or malabsorption. Deficiency in micronutrients can further exacerbate the impaired immune functions in these patients.

Epidemiological studies hinted at the significance of vitamin D. Low vitamin D levels seen in IBD may be due to inadequate dietary intake, nutrient malabsorption or reduced synthesis. In North America and Europe, the incidence of IBD rises with increasing latitude (1106). Canada, which is known to have widespread vitamin D deficiency (generally defined as <20 ng/ml), has the highest prevalence in the world (21, 1107).

The vitamin D axis consists of vitamin D, its binding protein (VDBP) and its receptor (VDR). VDBP, which is produced by the liver, transports active and inactive vitamin D in plasma (1108). Vitamin D has been shown to regulate the expression of antimicrobial peptides (1109, 1110), mucosal barrier function, and innate immunity (1111), thereby influencing the composition and functions of intestinal bacterial communities (1112).
Active vitamin D increases the expression of several tight junction and adherent junction proteins (1113). Pre-treatment with 1,25(OH)2D3 before DSS administration was found to protect intestinal epithelial cells (IEC) from increased permeability. This was also demonstrated in VDR KO mice showing increased susceptibility to DSS-induced colitis when compared to their wild-type littermates (1114).

VDR is expressed in a variety of cell types. In mice models, VDR KO mice were found to have ineffective autophagy resulting in dysbiosis, with depletion of fecal Lactobacillus and Bacteroides (1115). In fact, intact VDR pathway is needed for probiotic to be effective against colitis (1116). VDR KO mice administered probiotics did not respond to probiotics such as Lactobacillus rhamnosus strain GG (LGG) and Lactobacillus plantarum (LP) and had worse Salmonella-induced colitis compared to littermates. In comparison, the wild-type mice were able to increase VDR expression and its transcriptional activity, increase antimicrobial peptide expression and were relatively protected from Salmonella-induced colitis (1116). In addition, butyrate increased intestinal VDR expression and suppressed inflammation (1115).

In humans, the CD risk is increased with VDR Apal polymorphism and the TaqI tt genotype. In Asians, the VDR FokI polymorphism has been associated with UC susceptibility (1117), perhaps explaining the higher representation of Asians among UC patients locally. In addition, vitamin D signalling may be reduced by inflammation, perhaps by down-regulation of VDR mRNA by TNF-α (1118). In keeping with this, IEC VDR levels were decreased in CD and UC patients (1119, 1120). Interestingly, up-regulation of inflammation-dependent colonic VDR was associated with reduced Parabacteriodes in CD and UC patients compared to HC (1121).

Higher 25D levels appears to significantly reduced CD risk (388). In a retrospective study on 78 children, low vitamin D levels was found to increase the risk of CD (1122). Higher vitamin D levels also correlated with improved quality of life (1119, 1123) and non-significant (possibly due to the small sample size) reduction in Crohn's disease activity index. In addition, vitamin D levels was found to inversely correlate with CRP (1123).

Low 25D levels were associated with increased incidence of hospitalization and surgery in IBD patients, while normal vitamin D levels after supplementation reduced the rates of both (388). In a separate study, vitamin D supplementation was associated with reduced disease-associated medical and laboratory tests, and medication use in US veterans with IBD (1124). In a study involving 505 children with UC and CD, vitamin D supplementation was associated with quiescent disease (1125).
Treatment with vitamin D supplementation requires careful monitoring as excessively high levels can lead to hypercalcaemia. Mild/moderate CD patients supplemented with up to 5000 IU per day for 24 weeks reported decreased symptoms, Crohn's disease activity index and improved quality-of-life scores (1126). In another study of 84 CD patients in remission, 1200 IU/day for 12 months increased serum vitamin D levels and reduced relapse rates (1127). In a RCT trial involving supplementing CD patients in remission with of 2000 IU/ day of vitamin D for 3 months, there was increased serum 25D with associated improved intestinal epithelial barrier permeability (1123).

5.1.6 Study Aims and Objectives

In summary, the pro-inflammatory state in IBD is the result of exaggerated responses to microbial pathogens from a dysregulated immune system in genetically susceptible individuals. The intestinal bacterial profile is also a factor, as is the dysregulated intestinal microvascular system.

IBD and the treatments administered modulate host immunity. There are few published studies reporting alterations of plasma cytokine and vascular mediators with response to treatment in children with IBD (Appendix 5B), and limited publications on the potential association of these mediators with SCFA. Therefore, this study aimed to:

1. Characterise the immune and vascular profiles of IBD patients with clinical status,
2. Identify alterations in immune and vascular mediators with treatment and
3. Investigate the potential association between immune and vascular mediators with SCFA.
5.2 Materials and Methods

5.2.1 Identifying a Suitable Method

Experiments were first conducted to ascertain the best method for investigating immune profiles. These experiments were conducted using blood from healthy adult volunteers thus preserving patient samples for the study. The experiments are summarised below (data not shown).

Given that IL-10 was a specific cytokine of interest, its production from whole blood and peripheral blood mononuclear cells (PBMC) of 3 healthy adult donors were compared to determine the best source. Supernatants were analysed for IL-10 concentration by Enzyme-linked immunosorbent assay (ELISA) (eBioscience, Hatfield, UK) as per manufacturer's standard protocols.

In addition, experiments were conducted to assess IL-10 production from PBMC and whole blood when stimulated with LPS 100 ng/ml in a time-dependent manner. This could be replicated with patient samples to determine immune response to bacterial stimulus.

IL-10 concentrations were considerably higher following stimulation of PBMC compared to whole blood. IL-10 was detected after 18 hours, and peaked after 28 hours of stimulation. Concentrations then decreased, likely due to subsequent cell death. Experiments with PBMC enabled a standardised number of cells used, thus making it easier to compare results. PBMC was therefore used in subsequent experiments.

In addition to the above, consideration was given to the small volume (3-5 ml) of blood received from the study patients. These small volumes restricted the PBMC yield, and therefore experiments that could be conducted reliably. It was thus felt that plasma cytokine analysis would be more suitable for immune profiling as the samples received would consistently be sufficient for this. Meso Scale Discovery (MSD) Multi-spot Assay System (MSD, USA)(Figure 5.4) was the method of choice. This method offered the possibility of analysing multiple cytokines and biochemical markers using small plasma volumes whilst being easy to use and produce reliable results.
5.2.2 Patient Blood Samples

Blood samples were collected opportunistically from patients recruited to the study, as discussed in Section 2.2. Samples were collected prior to endoscopic investigations, and prospectively after in those diagnosed with IBD. Samples received from those not diagnosed with IBD were used as comparison (non-inflammatory controls). In addition, samples were also collected from IBD patients commencing Infliximab, and HC under another study.

Blood from study patients (2.5-5 ml) was collected alongside clinical samples. Approximately 1.4 ml of blood was transferred into EDTA tubes and a minimum of 2.5 ml was transferred into lithium heparinised tubes. Samples were transferred to the laboratory at room temperature within 30 to 60 minutes.

5.2.3 Plasma Extraction from Patient Blood

Cytokines could be quantified in plasma as well as serum. There are several reports of serum cytokine concentrations varying with age in children (Appendix 5B), however, plasma was chosen for this study as it was the laboratory preference.

Blood samples were transferred from EDTA-containing tubes into 1.5 ml Eppendorf tubes then centrifuged at 10,000 rpm for 10 minutes. Plasma was transferred into
another 1.5 ml Eppendorf tube and centrifuged again with the same settings. Plasma was then transferred into sterile 1 ml Eppendorf tubes in 100-200 µl aliquots before storage at -80°C. The remaining red cell pellets were also frozen at -80°C.

5.2.4 Cytokine quantification using Meso Scale Discovery (MSD) Multi-Spot Assay System

To quantify plasma cytokine levels, Cytokine Panel 1 (human), Proinflammatory Panel 1 (human) and Vascular Injury Panel 2 (human) V-PLEX kits (MSD, USA) were used as per manufacturer’s instructions (Table 5.1).

The assays in the cytokine panels are sandwich immunoassays. Briefly, the plates provided were pre-coated with capture antibodies on defined spots within each well. Analytes in the sample bind to the capture antibodies, then bind to the detection antibodies, which are conjugated with electrochemiluminescent labels during the incubation periods completing the sandwich. The MSD buffer subsequently added provided the appropriate conditions for electrochemiluminescence. In the MSD instrument, voltage through the plate electrodes caused the capture labels to emit light. The intensity of the light was proportional to the amount of analytes present in the sample. The fluorescence was measured by the MSD instrument and a quantitative measure of each analyte in the samples was provided.

Cytokines and biochemical markers included in the panels were:

<table>
<thead>
<tr>
<th>Panel</th>
<th>Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine Panel 1</td>
<td>GM-CSF, IL1α, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF-A</td>
</tr>
<tr>
<td>Proinflammatory Panel 1</td>
<td>IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α</td>
</tr>
<tr>
<td>Vascular Injury Panel 2</td>
<td>CRP, SAA, sICAM-1, sVCAM-1</td>
</tr>
</tbody>
</table>

Table 5.1: Cytokine Panels using MSD Multi-spot Assay System
(See Appendix 5C for functions)

Duplicates of the calibrators were prepared in 7 dilutions. 1000 µl of Diluent 43 was added to the lyophilised calibrator vial provided, mixed by inverting and incubated at room temperature for 15-30 minutes. After a briefly mixing with pulse-vortex, 100 µl was
transferred to another tube containing 300 µl Diluent 43. The four-fold serial dilutions were repeated further to generate 7 calibrators. Diluent 43 was used as the zero calibrator.

The plates were first washed 3 times with 150 µl of Wash Buffer (prepared by diluting 15 ml of MSD Wash Buffer provided at 20x concentration with 285 ml deionised water). Plasma samples were added to the plate and diluted two-fold by adding 25 µl Diluent 43 to 25 µl of plasma. Calibrators were also added and the plates covered and incubated at room temperature with shaking for 2 hours (Stuart SSM5, USA).

The plates were washed 3 times with 150 µl Wash Buffer then 25 µl of detection antibody solution (prepared by adding 2 4000 µl of Diluent 3 to 60 µl of each detection antibody) was added to each well. The plates were then covered and incubated further at room temperature with shaking for 2 hours.

The plates were washed 3 times with 150 µl of Wash Buffer then 150 µl of Read buffer (prepared by diluting 10 ml of Read buffer T provided at 4x concentration with 10 ml of deionised water) was added to each well. The plates were then analysed using the MSD instrument, SECTOR Imager 6000 (MSD, USA).

Plasma samples were analysed using the Vascular Injury Panel 2, with the following variations;

- Samples were diluted by adding 190 µl of Diluent 101 to 10 µl of each sample
- Detection antibodies were prepared by adding 60 µl of each antibody to 2 760 µl of Diluent 101
- Read Buffer was diluted by adding 5 ml of Read Buffer T (at 4x concentration) with 15 ml of deionised water.

### 5.2.5 Analysis

Concentrations of cytokines and biochemical markers for each sample were produced by the MSD instrument in Excel.
5.2.5.1 Sensitivity of Meso Scale Discovery (MSD) Assays Detection of Cytokines

Concentrations of cytokines and biochemical markers are quantified based on the standard curves produced by the MSD calibrators. A standard curve is produced for each cytokine and biochemical marker (Figure 5.5).

The lower limit of detection (LLOD) was calculated as 2.5 standard deviations above the lowest standard (zero calibrator), whilst the lower limit of quantification (LLOQ) provided by MSD was the lowest concentration at which the coefficient of variation (CV) of the calculated concentration was less than 20% and the recovery of the individual analyte was between 80-120% of the known value.

The upper limit of detection (ULOQ) is the highest concentration at which the CV of the calculated concentration was less than 20% and the recovery of the individual analyte was between 80-120% of the known value. Concentrations between LLOQ and ULOQ were quantified. Both LLOQ and ULOQ for each cytokine and biochemical marker were provided for each kit by MSD. For analysis, all concentration values below the LLOD were replaced with half the LLOD value (0.5 x LLOD).

![Figure 5.5: Example of Standard Curve for IFN-γ using the MesoScale Discovery (MSD) Assay](image)

Standard curves were produced from 7 calibrators prepared and analysed as per MSD instructions. Concentrations were calculated based on the intensity of fluorescence detected by SECTOR Imager 6000 instrument (MSD, USA). The detection range was between the LLOD (2.5 standard deviations above the lowest standard) and ULOQ (provided by MSD).
5.2.5.2 Statistical Analysis

Analysis was performed using Excel and GraphPad PRISM Version 5.0. Transformation of the concentrations of cytokine and vascular markers was performed to align the data sets obtained from 2 different batches. This enabled comparison between the data sets and thus patient groups. Correlation between cytokines and with SCFA were assessed using linear regression with 95% confidence intervals. Comparison between patient groups and disease activity groups were analysed using Kruskal-Wallis test. Statistical significance was considered for p values of less than 0.05.
5.3 Results of Cytokine Analysis

Cytokine concentrations of HC (n=21, age median 17.0 years (12.6-18.4), 33% male) were compared to patient samples. There were 16 non-inflammatory control (NIC) patients including those diagnosed with IBS (n=8) and food allergies (n=4). These patients were compared with IBD patients, while the remaining patients (2 with JIA and 2 with other Rheumatology conditions) were not analysed due to the small sample numbers. One blood sample was received from these patients.

The IBD patients recruited were diagnosed with CD (n=8), UC (n=4) and IBDU (n=2). Samples were collected prospectively and opportunistically when venepuncture was performed for clinically-indicated investigations, thus a total of 56 blood samples were analysed for cytokine and vascular profiles. In addition, data on individual patients with variants of IBD (1 patient with VEOIBD and another with Granulomatous Polyangiitis) are presented. An overall total of 93 samples were thus analysed. Longitudinal sample collections are presented in Table 2.3, Chapter 2.

Cytokines IL-1-α, IL-1β, IL-2, IL-4 and IL-13 were mostly undetectable thus excluded from analysis (Table 5.2). For the remaining cytokines, the majority of the samples were above the LLOD thus quantified.

5.3.1 Systemic Cytokines in HC, NIC and IBD Patients

Cytokines of HC and NIC (IBS n=8, food allergies n=4) were compared with IBD patients. Samples from CD, IBDU and UC patients (n=14) were combined then analysed in 2 groups depending on disease activity, Active IBD (n=14) and IBD Remission (n=6), based on disease scores (wPCDAI active >12.5, PUCAI active >10)(Figure 5.6). There were less samples received from patients in remission as some patients were followed up locally. In addition, blood investigations were not clinically indicated for some patients in remission thus opportunistic sample collection for research was not possible. For those with samples collected prospectively, only 1 sample was used in the active IBD and IBD Remission groups. Data is only presented for cytokines with statistically significant differences (Figure 5.6).
Table 5.2: The Number of Samples detected and quantified for Cytokine Analysis by MesoScale Discovery (MSD) Assays
(Total 72 samples excluding HC; LLOD – Lower limit of detection, LLOQ – Lower limit of quantification, ULOD – Upper limit of detection)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LLOD (pg/ml)</th>
<th>Number of Samples between LLOD-LLOQ</th>
<th>LLOQ (pg/ml)</th>
<th>Number of Samples above LLOQ</th>
<th>ULOD (pg/ml)</th>
<th>Number of Samples above ULOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>0.058</td>
<td>25</td>
<td>1.9</td>
<td>46</td>
<td>972</td>
<td>0</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0.059</td>
<td>16</td>
<td>2.85</td>
<td>9</td>
<td>388</td>
<td>0</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.043</td>
<td>0</td>
<td>6.28</td>
<td>72</td>
<td>891</td>
<td>0</td>
</tr>
<tr>
<td>IL-7</td>
<td>0.094</td>
<td>0</td>
<td>1.37</td>
<td>72</td>
<td>836</td>
<td>0</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>0.138</td>
<td>0</td>
<td>5.68</td>
<td>72</td>
<td>3110</td>
<td>0</td>
</tr>
<tr>
<td>IL-15</td>
<td>0.047</td>
<td>0</td>
<td>1.4</td>
<td>72</td>
<td>762</td>
<td>0</td>
</tr>
<tr>
<td>IL-16</td>
<td>1.41</td>
<td>0</td>
<td>19.1</td>
<td>72</td>
<td>2580</td>
<td>0</td>
</tr>
<tr>
<td>IL-17A</td>
<td>0.136</td>
<td>0</td>
<td>9.32</td>
<td>72</td>
<td>5890</td>
<td>0</td>
</tr>
<tr>
<td>TNF-β</td>
<td>0.077</td>
<td>14</td>
<td>1.15</td>
<td>58</td>
<td>625</td>
<td>0</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.412</td>
<td>10</td>
<td>7.47</td>
<td>62</td>
<td>1500</td>
<td>0</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.05</td>
<td>10</td>
<td>2.14</td>
<td>9</td>
<td>496</td>
<td>0</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.091</td>
<td>42</td>
<td>0.89</td>
<td>25</td>
<td>1380</td>
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</tr>
<tr>
<td>IL-4</td>
<td>0.009</td>
<td>57</td>
<td>0.45</td>
<td>11</td>
<td>262</td>
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</tr>
<tr>
<td>IL-6</td>
<td>0.042</td>
<td>2</td>
<td>1.58</td>
<td>68</td>
<td>653</td>
<td>0</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.048</td>
<td>0</td>
<td>1.13</td>
<td>72</td>
<td>575</td>
<td>0</td>
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<tr>
<td>IL-10</td>
<td>0.136</td>
<td>21</td>
<td>0.68</td>
<td>51</td>
<td>312</td>
<td>0</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.042</td>
<td>39</td>
<td>1.22</td>
<td>26</td>
<td>425</td>
<td>0</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.496</td>
<td>46</td>
<td>4.21</td>
<td>7</td>
<td>539</td>
<td>0</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.061</td>
<td>0</td>
<td>0.69</td>
<td>72</td>
<td>327</td>
<td>0</td>
</tr>
<tr>
<td>CRP</td>
<td>4.71</td>
<td>0</td>
<td>27.6</td>
<td>72</td>
<td>19500</td>
<td>0</td>
</tr>
</tbody>
</table>

In HC (n=21), the TNF-α concentrations ranged from 0.03-8.5 pg/ml, with a median of 1.27 pg/ml. Patients with IBS [n=8, median 1.6 (1.2-3.3)pg/ml], food allergies [n=4, median 2.1 (1.4-2.5)pg/ml] and IBD in remission [n=6, median] had similar concentrations but concentrations were higher in active IBD [n=14, median 2.06 (1.20-4.23)pg/ml, p=0.049]. Changes in TNF-α during the course of the disease are shown for 5 CD and 4 UC patients in Figure 5.6 Right column. TNF-α decreased in those who achieved remission (blue points) and in those who improved clinically but still in active disease (blue line, red point) except a UC patient, while increasing in those who continued in active inflammation (red line, red point).
The IL-17 concentrations in HC were 0.07-0.74 pg/ml, and the median 0.07 pg/ml. Concentrations were higher in patients with IBS [median 2.73 (1.88-5.03)pg/ml, p=0.002], active IBD [n=14, median 7.92 (0.73-91.52)pg/ml, p<0.0001] and IBD in remission [n=6, median 4.95 (0.78-11.75)pg/ml, p=0.002]. Although patients with food allergies also had higher IL-17 [median 2.3 (0.9-3.1)pg/ml], this was not statistically significant. Similarly, in individual patients (CD n=5, UC n=4) with samples collected during active disease and remission, decreased IL-17 concentrations were demonstrated in those who subsequently achieved remission or improved, except a UC patient who reported to be better. IL-17 concentrations remained the same or increased in those with ongoing active disease. There was also a CD patient in active disease but had decreased IL-17 concentration. It was noted that a UC patient in active disease had much higher concentrations which continued to increase.

The IL-15 concentrations in HC were 0.02-6.32 pg/ml, with a median of 0.02 pg/ml. As with TNF-α, IL-15 was higher in active IBD [n=14, median 1.67 (1.25-8.60)pg/ml, p=0.001] with no significant differences seen in IBS [median 1.28 (0.99-1.53)pg/ml], food allergies [median 0.94 (0.90-1.05)pg/ml] and IBD remission [n=6, median 1.94 (0.77-2.31)pg/ml]. In contrast, however, analysis of individual patients revealed similar or increased IL-15 in remission while decreasing in active disease despite reported clinical improvement. An exception to this was a CD patient with ongoing active disease and much higher concentrations compared to the other patients.

In HC, GM-CSF concentrations were 1.20-3.81 pg/ml, with a median of 1.2 pg/ml. In comparison, patients with IBS [median 0.11 (0.03-0.190pg/ml, p<0.0001], food allergies [median 0.11 (0.05-0.19)pg/ml, p=0.003], active IBD [n=14, median 0.20 (0.01-0.42)pg/ml, p<0.0001] and IBD in remission [n=6, median 0.12 (0.05-0.30)pg/ml, p=0.001] had lower concentrations. Analysis of individuals revealed increased GM-CSF in those with ongoing active UC but decreased in the UC patient in remission. In CD, however, similar or decreased concentrations were seen in all patients regardless of disease activity.

Concentrations of IL-5 were 0.19-8.42 pg/ml, with a median of 0.57 pg/ml in HC. Similar concentrations were seen in IBS [median 0.35 (0.15-1.81)pg/ml], food allergies [median 0.26 (0.20-0.36)pg/ml] and IBD in remission [n=6, median 0.40 (0.15-0.62)pg/ml]. On the other hand, IL-5 was higher in active IBD [n=14, median 0.57 (0.19-8.42)pg/ml, p=0.002]. Subsequent analysis of individual patients were performed. IL-5 decreased in the UC patient in remission but increased in those with continued inflammation despite improving PUCAI scores. In CD, decreased concentrations were seen in patients in remission and
those with ongoing inflammation, with similar levels seen in those with improvement despite inflammation.

IL-16 has recently been recognised to play a role in IBD (1128). Concentrations were 0.7-185.4 pg/ml, with a median of 105.2 pg/ml in HC. A trend for increased cytokine concentrations was observed with inflammation, namely with IBS [median 130 (59-239)pg/ml] and food allergies [median 170 (146-179)pg/ml], with significantly higher concentrations in active IBD [n=14, median 174 (92-333)pg/ml, p=0.028]. Compared to active IBD, IL-16 was lower in patients in remission [n=6, median 87 (37-151)pg/ml], although this was not statistically significant. Analysis of individual patients revealed decreased concentrations with disease improvement but similar or increased concentrations in those with ongoing inflammation.

With IL-12p70, concentrations in HC were median 0.021 (0.02-0.36) pg/ml, with higher concentrations seen in IBS [median 0.07 (0.021-0.30)pg/ml, p=0.036] and food allergy [median 0.08 (0.05-0.17)pg/ml, p=0.025] patients. Although there was a trend for higher concentrations in both IBD groups, the differences were not significant. Analysis of individuals comparing concentrations with disease activity revealed decreased concentrations in remission and in ongoing active disease, however, increased concentrations were seen in those who reported clinical improvement but remained in active disease.

No significant differences were observed between the groups for concentrations of IL-6, IL-7, IL-8, IL-10, IL-12p40 and TNF-β. IFN-γ was not included in this analysis as it is specific to CD, thus analysed separately in the following section.
Patient Groups and Disease Activity

Healthy Control  IBS  Food Allergy  Active IBD  IBD Remission

TNF-α (pg/ml)

Timepoints

CD Time 1  CD Time 2  UC Time 1  UC Time 2

0
1
2
3
4
5

p=0.049

p=0.002

p<0.0001

p=0.002

IL-17 (pg/ml)

Timepoints

CD Time 1  CD Time 2  UC Time 1  UC Time 2

0
5
10
15
20

Patient Groups and Disease Activity

IL-15 (pg/ml)

Timepoints

CD Time 1  CD Time 2  UC Time 1  UC Time 2

0
2
4
6
8

Patient Groups and Disease Activity

Healthy Control  IBS  Food Allergy  Active IBD  IBD Remission

IL-17 (pg/ml)

Timepoints

CD Time 1  CD Time 2  UC Time 1  UC Time 2

0
5
10
15
20

p=0.002

p=0.001

Patient Groups and Disease Activity

Healthy Control  IBS  Food Allergy  Active IBD  IBD Remission

IL-15 (pg/ml)

Timepoints

CD Time 1  CD Time 2  UC Time 1  UC Time 2

0
2
4
6
8

362
Concentrations of TNF-α, IL-17, IL-15, GM-CSF, IL-5, IL-16 and IL-12p70 in HC (n=21), IBS (n=8), food allergies (n=4), active IBD (n=14) and IBD in remission (n=6) as quantified with MSD kits V-Plex Cytokine Panel 1 and Proinflammatory Panel 1 (left column), with disease activity classified based on PUCAI or wPCDAI scores. Data are presented as median (pg/ml) and p values following Kruskal-Wallis and Dunn’s Multiple Comparison tests. Analysis was also performed on individual IBD patients comparing cytokine concentrations in active disease and remission (red line represents ongoing inflammation, blue line presents disease improvement, red points represent active disease and blue points represent remission).

5.3.2 Systemic Cytokines in Crohn’s Disease, Ulcerative Colitis and IBD

Undetermined

Given the differences in immuno-pathogenesis, patients with CD (n=8) and UC (n=4) in active disease (CD n=8, UC n=4) and remission (CD n=3, UC n=3) were also analysed separately (Figure 5.7). Disease activity was defined based on PUCAI (active >10) or wPCDAI scores (active >12.5).

Comparison for IL-17 was first made between HC [median 0.07 (0.07-0.74)pg/ml] and the IBD groups (Figure 5.7A). Concentrations were higher in active CD [n=8, median 6.09 (0.73-26.66)pg/ml, p<0.0001] and active UC [n=4, median 8.81 (3.84-91.52)pg/ml,
In addition, IL-17 was also raised in CD remission \([n=3, \text{median 7.86 (1.78-11.55)pg/ml, } p=0.042]\). Although there was a trend for higher concentrations in UC remission \([n=3, \text{median 2.03 (0.78-11.75)pg/ml, } p=0.001]\), this was not statistically significant.

Subsequently, analysis was performed to investigate the possibility of association/correlation between the pro-inflammatory cytokine and the anti-inflammatory cytokine IL-10 (Figure 5.7B). In CD, IL-17 positively correlated with IL-10 \((R^2=0.4914, p=0.0162, 95\% \text{ CI: 5.187-39.34})\), as supported by samples of individual patients in active disease (red points) and remission (blue points). In UC, however, no trends were observed \((R^2=0.0236, p=0.7422)\).

Given this positive correlation, IL-17 was then analysed as a ratio to IL-10 (Figure 5.7C). Significant differences were observed between the medians \((x^2(2)=30.30, p<0.0001)\). Compared to HC \([\text{median 0.30 (0.02-18.41)}]\), the ratios were higher in inflammation including IBS \([n=8, \text{median 10.08 (2.01-25.61), } p=0.010]\) and CD remission \([n=3, \text{median 20.99 (12.36-44.18), } p=0.024]\). IL-17:IL-10 was higher also in active CD \([n=8, \text{median 14.49 (1.31-51.55), } p=0.001]\) and active UC \([n=4, \text{median 23.72 (6.43-206.89), } p=0.018]\). Although there was a trend for higher ratios in UC remission \([n=3, \text{median 13.13 (0.90-15.26)}]\), this was not statistically significant.

Differences were also seen with GM-CSF (Figure 5.7D). Compared to HC \([\text{median 1.2 (1.20-3.81) pg/ml}]\), GM-CSF concentrations were lower in all IBD groups. This was statistically significant in active CD \([n=8, \text{median 0.22 (0.01-0.29)pg/ml, } p<0.0001]\), CD remission \([n=3, \text{median 0.15 (0.08-0.30)pg/ml, } p=0.039]\) and UC remission \([n=3, \text{median 0.10 (0.05-0.21)pg/ml, } p=0.008]\) but not active UC \([n=4, \text{median 0.42 (0.11-0.29)pg/ml, } p=0.090]\).

IL-15 concentrations of HC \([\text{median 0.02 (0.02-7.67)pg/ml}]\) were compared with the IBD groups (Figure 5.7E). The IBD groups appeared to have higher concentrations than HC \([\text{CD remission } n=3, \text{median 2.10 (1.79-2.31)pg/ml; active UC } n=4, \text{median 1.49 (1.45-1.64)pg/ml, UC remission } n=3, \text{median 1.40 (0.77-2.02)pg/ml}]\), however, statistical significance was seen only in active CD \([n=8, \text{median 2.34 (1.25-8.60)pg/ml, } p=0.021]\). There were no correlations observed between IL-15 and IL-10 in CD or UC, although a positive trend was seen in UC \((R^2=0.4527, p=0.0977, 95\% \text{ CI: 0.235-2.021})\) but not CD \((R^2=0.1586, p=0.2251, 95\% \text{ CI: -3.199-11.88})\) (Figure 5.7F). On analysis of samples from individual patients in active disease and remission, no clear patterns were observed.
Concentrations of IL-5 were 0.02-6.32 pg/ml, with a median of 0.02 pg/ml, in HC (Figure 5.7G). IL-5 was higher in active UC [n=4, median 1.24 (0.26-1.49)pg/ml], and significantly higher in active CD [n=8, median 0.57 (0.19-8.42)pg/ml, p=0.036]. The concentrations in CD remission [n=3, median 0.55 (0.18-0.62)pg/ml] and UC remission [n=3, median 0.31 (0.15-0.50)pg/ml] were similar to HC. Despite IL-5 being a Th2 cytokine, there was no correlation or association seen in UC but a trend for positive association was observed for CD (R²=0.3138, p=0.0731, 95% CI: -0.7041-12.95)(Figure 5.7H). However, in the analysis of active disease and remission in 2 UC patients, there was decreased IL-5 in remission. In general, IL-5 concentrations were low thus these may not be true trends.

Comparison of TNF-α concentrations between the groups suggested differences in the medians (χ²(2)=8.799, p=0.0663), with higher concentrations in the IBD groups (Figure 5.7I). Subsequent post-test Dunn’s Multiple Comparison Test, however, did not reveal any statistically significant differences between the groups. Similarly, for IL-16, the medians varied significantly (χ²(2)=10.19, p=0.0374) but no statistical differences were seen between the groups following post-test Dunn’s Multiple Comparison Test (Figure 5.7J).

A. IBD

B. Crohn’s Disease

Ulcerative Colitis

\[ R^2 = 0.4914, \quad p = 0.0162 \]

\[ R^2 = 0.0236, \quad p = 0.7422 \]
C. IBD

![IL-17:IL-10 Ratio Graph]

Patient Groups and Disease Activity

D. IBD

![GM-CSF (pg/ml) Graph]

Patient Groups and Disease Activity

E. IBD

![IL-15 (pg/ml) Graph]

Patient Groups and Disease Activity
F. Crohn's Disease

\[ R^2 = 0.1586, \quad p = 0.2251 \]

G. IBD

\[ p = 0.036 \]

H. Crohn's Disease

\[ R^2 = 0.3138, \quad p = 0.0731 \]
I. IBD

Figure 5. 7: Systemic Cytokines in CD and UC
Concentrations of IL-17, GM-CSF, IL-15, IL-5, TNF-α and IL-16 in HC (n=21), CD (n=8) and UC (n=4) patients. IBD patients were classified depending on disease activity defined by PUCAI (active >10) and wPCDAI (active >12.5) scores. Cytokines in active disease (CD n=8, UC n=4) were compared with those in remission (CD n=3, UC n=3). Data are presented as pg/ml and shown with median and p values following Kruskal-Wallis and Dunn’s Multiple Comparison tests. Linear regression was performed for IL-17, IL-15 and IL-5 against IL-10 with 95% confidence interval, and $R^2$ and p values are presented (B). The red points represent active disease and the blue points represent disease remission (connected by blue arrow pointing to the second timepoint).
IFN-γ, being a Th1 cytokine, is known to play a significant role in CD. Comparison of IFN-γ concentrations was made between of HC [median of 0.21 (0.21-35.16) pg/ml] and the IBD groups (Figure 5.8A). As expected, higher concentrations were seen in CD with a significant difference seen in active CD [n=8, median 8.34 (2.12-109.78) pg/ml, p=0.003], but not CD remission [n=3, median 8.06 (1.58-15.03) pg/ml]. On the other hand, IFN-γ in UC was comparable to HC [active UC n=4, median 0.86 (0.42-6.29) pg/ml; UC remission n=3, median 0.62 (0.43-3.60) pg/ml]. Linear correlation with the anti-inflammatory cytokine IL-10 was also assessed in CD but no significant association was seen between the two cytokines (R²=0.05332, p=0.4945, 95% CI: -65-125) (Figure 5.8B).

In 2 out of 3 individual patients, samples collected during active disease and remission demonstrated reduced IFN-γ and IL-10 in remission.

Analysis was also performed on IFN-γ as a ratio with IL-10 (Figure 5.8C). Significant differences between the medians were suggested (X²(2)=15.69, p=0.0078), however, compared to HC [median 2.69 (0.59-55.76)] active CD had significantly higher median [n=8, median 33.81 (2.43-172.53), p=0.023] by the Dunn’s Multiple Comparison test. The ratios were also higher in CD remission compared to HC [n=3, median 30.83 (10.98-40.11), p=0.302] and UC remission [median 4.97 (1.92-13.21)]. In UC remission [n=3, median 4.02 (0.50-4.68)].

Although CD is predominantly a Th1-mediated disease, Th17 responses have also been implicated (1129). Analysis was thus performed on IL-17 as a ratio to IFN-γ (Figure 5.8D). The Kruskal-Wallis test revealed significant differences between the medians (X²(2)=36.05, p<0.0001) with differences seen between HC [median 0.330 (0.002-0.705)] and patients. Ratios in IBS [median 2.55 (0.57-4.31), p<0.0001], active CD [n=8, median 0.35 (0.08-10.60)] and CD remission [n=3, median 1.13 (0.52-1.43)] were higher than HC with significantly higher ratios seen in active UC [n=4, median 12.55 (4.92-14.54), p<0.0001] and UC remission [n=3, median 3.26 (1.80-3.27), p=0.045]. IL-17: IFN-γ ratios did not, however, statistically discriminate between active CD and UC patients.
Patient Groups and Disease Activity

A. IFN-γ (pg/ml)

B. IFN-γ vs. IL-10 (pg/ml)

C. IFN-γ: IL-10 Ratio

D. IL-17: IFN-γ Ratio

\[ p = 0.003 \]

\[ R^2 = 0.05332, \quad p = 0.4945 \]

\[ p = 0.023 \]

\[ p = 0.045 \]

\[ p < 0.0001 \]
Figure 5.8: IFN-γ in CD
Concentrations of IFN-γ, IL-10 and IL-17 in HC (n=21), IBS (n=8), CD (n=8) and UC (n=4). IBD patients in active disease (CD n=8, UC n=4) or disease remission (CD n=3, UC n=3) were compared and linear regression was performed between IL-10 and IL-17 with 95% confidence interval and presented with $R^2$ and $p$ values. The red points represent active disease and the blue points represent disease remission (connected by blue arrow pointing to the second time point). Data are presented as pg/ml and shown with median and $p$ values following Kruskal-Wallis and Dunn’s Multiple Comparison tests.

5.4.3.2 IFN-γ in IBDU Patients
As IFN-γ discriminates CD from UC, IFN-γ concentrations, IFN-γ:IL-10 and IL-17: IFN-γ ratios of IBDU patients (n=2) were compared to those of CD and UC (Table 5.3). Patient IBDU 1 was in active disease during blood sample collection (ESR 34 mm/hr) while Patient IBDU 2 was in remission (ESR 12 mm/hr). The IFN-γ profiles of Patient IBDU 1 were more similar to CD than UC, while the profile of Patient IBDU 2 could be either CD or UC.

<table>
<thead>
<tr>
<th>Disease</th>
<th>No of Patients</th>
<th>IFN-γ median/ratio</th>
<th>range</th>
<th>IFN-γ:IL-10 median/ratio</th>
<th>range</th>
<th>IL-17:IFN-γ median/ratio</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active CD</td>
<td>8</td>
<td>8.34</td>
<td>2.12-109.78</td>
<td>33.81</td>
<td>2.43-172.53</td>
<td>0.35</td>
<td>0.08-10.60</td>
</tr>
<tr>
<td>CD Remission</td>
<td>3</td>
<td>8.06</td>
<td>1.58-15.03</td>
<td>30.83</td>
<td>10.98-40.11</td>
<td>1.13</td>
<td>0.52-1.43</td>
</tr>
<tr>
<td>Active UC</td>
<td>4</td>
<td>0.86</td>
<td>0.42-6.29</td>
<td>2.31</td>
<td>0.57-14.22</td>
<td>12.55</td>
<td>4.92-14.54</td>
</tr>
<tr>
<td>UC Remission</td>
<td>3</td>
<td>0.62</td>
<td>0.43-3.60</td>
<td>4.02</td>
<td>0.50-4.68</td>
<td>3.26</td>
<td>1.80-3.27</td>
</tr>
<tr>
<td>IBDU 1</td>
<td>1</td>
<td>10.72</td>
<td>-</td>
<td>28.07</td>
<td>-</td>
<td>0.69</td>
<td>-</td>
</tr>
<tr>
<td>IBDU 2</td>
<td>1</td>
<td>3.56</td>
<td>-</td>
<td>11.77</td>
<td>-</td>
<td>3.11</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.3: Plasma IFN-γ Concentration, IFN-γ:IL-10 and IL-17: IFN-γ Ratios of CD, UC and IBDU
5.3.3 Alterations in Cytokines with IBD Treatment

Cytokine concentrations of IBD patients were further analysed before and after treatment (Figure 5.9). CD patients who were treatment-naïve and had EEN (n=2), and Infliximab (n=3) for on-going disease were analysed utilising ESR (mm/hr) or CRP (mg/dL) as a marker of inflammation. Similarly, UC patients who were treatment-naïve and received Prednisolone (n=2), and Infliximab (n=1) for continuing colitis were also analysed. Increased ESR was seen in a CD (D2, ESR 8 to 11 to 15 mm/hr, although still < 20 mm/hr) and UC (D5, ESR 10 to 11 to 36 mm/hr) patients receiving Infliximab, while a patient with CD (A14, ESR 67 to 36 mm/hr) and UC (A8, ESR 60 to 41 mm/hr) patients had ESR that improved but remained elevated. The remaining patients responded to treatment with the inflammatory markers returning to normal levels post-treatment. Cytokine concentrations pre-treatment, following induction and post-treatment or during maintenance are detailed in Table 5.4.

With improving ESR or CRP, TNF-α, IL-17 and IL-12p40 concentrations decreased in CD and UC patients irrespective of the treatment received. IL-5 concentrations were high in 2 CD patients starting Infliximab, then decreased with reduction in ESR. Minimal changes were seen in CD patients on EEN and UC patients except a modest increase in the UC patient with ongoing elevated ESR.

Minimal changes were seen in IL-8 in CD except an increase in Patient D2, in whom the ESR increased slightly after Infliximab induction. High IL-8 concentration in a UC patient decreased after a course of Prednisolone. A lower concentration and smaller reduction was seen in Patient A8, who remained in active disease. Modest reduction in GM-CSF concentrations with inflammatory markers were observed but increased concentrations were detected in all patients with inflammatory markers that remained elevated.

As with GM-CSF, IL-7, IL-15 and IL-16 concentrations increased with on-going inflammation despite the reduction in inflammatory markers. Minimal changes in IL-7 concentrations were detected in CD patients unless ESR remained elevated, in which case IL-7 increased. In contrast, in UC, IL-7 concentrations decreased with on-going inflammation.

IL-15 concentrations were higher in CD patients starting Infliximab. Reduction was seen with improved inflammatory markers, while an increase was seen with ongoing inflammation. In contrast, modest changes were seen in UC patients, with a small reduction seen in the patient with ongoing active disease. IL-16 concentrations generally
reduced with decreased inflammatory markers but increased in CD Patients A14, who had ongoing elevated ESR, and Patient D2, whose CRP was mildly raised.

As expected, IFN-γ concentrations were higher in CD patients and decreased with improving inflammatory markers. In Patient A14 (ESR 67 to 36 mm/hr), IFN-γ increased.

**Crohn’s Disease versus Ulcerative Colitis**

<table>
<thead>
<tr>
<th>CD Pt</th>
<th>Pre-treatment ESR (mm/hr)</th>
<th>Induction</th>
<th>Post-treatment ESR (mm/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A14</td>
<td>67</td>
<td>NA</td>
<td>36</td>
</tr>
<tr>
<td>A15</td>
<td>23 *</td>
<td>NA</td>
<td>5 *</td>
</tr>
<tr>
<td>D2</td>
<td>8</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>D4</td>
<td>38</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>D6</td>
<td>2</td>
<td>N</td>
<td>2</td>
</tr>
</tbody>
</table>

*CRP (mg/dL)

<table>
<thead>
<tr>
<th>UC Pt</th>
<th>Pre-treatment ESR (mm/hr)</th>
<th>Induction ESR (mm/hr)</th>
<th>Post-treatment ESR (mm/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8</td>
<td>60</td>
<td>NA</td>
<td>41</td>
</tr>
<tr>
<td>A21</td>
<td>41</td>
<td>NA</td>
<td>8</td>
</tr>
<tr>
<td>D5</td>
<td>10</td>
<td>11</td>
<td>36</td>
</tr>
</tbody>
</table>

**TGF-β (pg/ml)**

<table>
<thead>
<tr>
<th>CD Pt</th>
<th>Pre-treatment</th>
<th>Induction</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A14</td>
<td>A15, Mth0,4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A15</td>
<td>A15, Mth0,2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>D2, Wk2, Mth3,14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D4</td>
<td>D4, Wk0,2, Mth12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D6</td>
<td>D6, Mth0,3,7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Treatment and Time (weeks, months)**

**IL-17 (pg/ml)**

<table>
<thead>
<tr>
<th>CD Pt</th>
<th>Pre-treatment</th>
<th>Induction</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A14</td>
<td>A15, Mth0,4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A15</td>
<td>A15, Mth0,2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>D2, Wk2, Mth3,14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D4</td>
<td>D4, Wk0,2, Mth12</td>
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<tr>
<td>D6</td>
<td>D6, Mth0,3,7</td>
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**Treatment and Time (months)**
<table>
<thead>
<tr>
<th>EEN</th>
<th>Prednisolone</th>
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<tbody>
<tr>
<td><strong>Pre-treatment</strong></td>
<td>Infliximab</td>
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<tr>
<td><strong>Induction</strong></td>
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<tr>
<td><strong>Post-treatment</strong></td>
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<table>
<thead>
<tr>
<th>IL-7 (pg/ml)</th>
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<tbody>
<tr>
<td>A14, Mth0,4</td>
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<tr>
<td>D2, Wk2,Mth3,14</td>
</tr>
<tr>
<td>D4, Wk0,2,Mth12</td>
</tr>
<tr>
<td>D6, Mth0,3,7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment and Time (weeks, months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEN</td>
</tr>
<tr>
<td><strong>Pre-treatment</strong></td>
</tr>
<tr>
<td><strong>Induction</strong></td>
</tr>
<tr>
<td><strong>Post-treatment</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IL-15 (pg/ml)</th>
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<tbody>
<tr>
<td>A8, Mth0,6</td>
</tr>
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<td>A21, Mth0,1</td>
</tr>
<tr>
<td>D5, Mth3,7</td>
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<table>
<thead>
<tr>
<th>Treatment and Time (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEN</td>
</tr>
<tr>
<td><strong>Pre-treatment</strong></td>
</tr>
<tr>
<td><strong>Induction</strong></td>
</tr>
<tr>
<td><strong>Post-treatment</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IL-16 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A14, Mth0,4</td>
</tr>
<tr>
<td>A15, Mth0,2</td>
</tr>
<tr>
<td>D2, Wk2,Mth3,14</td>
</tr>
<tr>
<td>D4, Wk0,2,Mth12</td>
</tr>
<tr>
<td>D6, Mth0,3,7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment and Time (weeks, months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEN</td>
</tr>
<tr>
<td><strong>Pre-treatment</strong></td>
</tr>
<tr>
<td><strong>Induction</strong></td>
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<tr>
<td><strong>Post-treatment</strong></td>
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<th>IFN-γ (pg/ml)</th>
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<td>D5, Mth3,7</td>
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<table>
<thead>
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<th>Treatment and Time (months)</th>
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<td>EEN</td>
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<td><strong>Induction</strong></td>
</tr>
<tr>
<td><strong>Post-treatment</strong></td>
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<th>IFN-γ (pg/ml)</th>
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<tr>
<td>A21, Mth0,1</td>
</tr>
<tr>
<td>D5, Mth3,7</td>
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</table>
Figure 5.9: The Effect of Treatment on Systemic Cytokines in CD and UC

CD patients (left) received EEN (n=2) and Infliximab (n=3) whilst UC patients (right) received Prednisolone (n=2) and Infliximab (n=1). Responses to treatment are indicated by changes in inflammatory markers (ESR or CRP), as presented in the table. Cytokine concentrations are shown pre-treatment, post-induction and post-treatment as pg/ml.
<table>
<thead>
<tr>
<th>Cytokine/Time</th>
<th>CD Patients</th>
<th>UC Patients</th>
</tr>
</thead>
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<td></td>
<td>Infliximab</td>
<td>Prednisolone</td>
</tr>
<tr>
<td></td>
<td>EEN</td>
<td>A14, Mth0.2</td>
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<tr>
<td>TNF-α</td>
<td></td>
<td>A15, Mth0.2</td>
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<td>Pre-treatment</td>
<td>3.26 4.23</td>
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<td>2.34 2.69</td>
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<td>1.27 1.59 1.40</td>
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<td>- - -</td>
<td>3.02 1.01 1.48</td>
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<tr>
<td>Maintenance</td>
<td>- - -</td>
<td>1.91 1.31 1.51</td>
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**IL-17**

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<td>EEN</td>
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<td>Pre-treatment</td>
<td>8.60 18.88</td>
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<td>3.79 26.66 0.73</td>
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<td>9.47 23.80 1.36</td>
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**IL-12p40**

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<tr>
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<td>EEN</td>
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<tr>
<td>Pre-treatment</td>
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<td>Post-treatment</td>
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<td>187.90 125.11 65.68</td>
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<td>Induction</td>
<td>- - -</td>
<td>257.12 139.98 75.92</td>
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<tr>
<td>Maintenance</td>
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<td>241.03 103.56 29.46</td>
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**IL-5**

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<tr>
<td></td>
<td>EEN</td>
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<td>Pre-treatment</td>
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<td>8.42 5.88 0.19</td>
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<td>0.53 5.35 0.32</td>
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**IL-8**

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<td></td>
<td>EEN</td>
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<tr>
<td>Pre-treatment</td>
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<tr>
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<td>- - -</td>
<td>6.61 7.56 6.75</td>
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<td>22.68 7.11 4.64</td>
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<td>13.33 4.01 1.81</td>
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**GM-CSF**

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<td>EEN</td>
<td>A14, Mth0.2</td>
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<tr>
<td>Pre-treatment</td>
<td>0.24 0.29</td>
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</tr>
<tr>
<td>Post-treatment</td>
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<tr>
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<td>0.20 0.26 0.10</td>
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<td>0.17 0.20 0.09</td>
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<td>0.10 0.20 0.08</td>
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**IL-7**

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<tr>
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**IL-15**

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<td>A14, Mth0.2</td>
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<tr>
<td>Pre-treatment</td>
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<tr>
<td>Post-treatment</td>
<td>3.60 1.79</td>
<td>- - -</td>
</tr>
<tr>
<td>Pre-treatment</td>
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<td>5.95 2.24 2.10</td>
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**IL-16**

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<td>Prednisolone</td>
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<tr>
<td></td>
<td>EEN</td>
<td>A14, Mth0.2</td>
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<td>Pre-treatment</td>
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<td>Pre-treatment</td>
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<td>95.01 104.43 101.36</td>
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<tr>
<td>Induction</td>
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<td>118.64 136.51 114.29</td>
</tr>
<tr>
<td>Maintenance</td>
<td>- - -</td>
<td>123.01 89.53 45.76</td>
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Table 5.4: Cytokine Concentrations with Treatment

Cytokine concentrations, as measured with MSD kits V-Plex Cytokine Panel 1 and Proinflammatory Panel 1, for CD patients receiving EEN (n=2) and Infliximab (n=3), and UC patients receiving Prednisolone (n=2) and Infliximab (n=1) are listed. Concentrations are presented as pg/ml.

5.3.4 Cytokines and Short Chain Fatty Acids in IBD Patients

To ascertain any potential correlation or associations between microbial metabolite SCFA and immune status, linear regression analysis was performed on stool SCFA (n=28) and plasma cytokines from IBD (n=16) and non-inflammatory control (NIC) (n=5) patients at the same time points. The blood and stool samples were collected concurrently on the same day.

All cytokines quantified were analysed with acetate, propionate and butyrate percentages of the total SCFA concentrations. Significant results or those of interest are presented. For some patients, data was available from samples collected during active disease (presented as red points) and in remission (presented as blue points), with the results for individual patients (n=3) connected by an arrow pointing to the second time point. For other patients, 1 time point was used for each patient.

5.3.4.1 Systemic TNF-β and Short Chain Fatty Acids

TNF-β concentrations and acetate percentages for IBD patients were analysed (Figure 5.10A). A negative correlation was observed (R^2=0.5797, p=0.0006, 95% CI: -0.0215-0.0074). From the analysis of individual patients in active disease and remission, active disease was associated with lower acetate (53-67%) in 2 patients as well as higher TNF-β (0.28 pg/ml, 0.75 pg/ml) than in remission (acetate 58-80%, TNF-β 0.20-0.57 pg/ml).

In contrast to acetate, propionate percentages positively correlated with TNF-β concentrations (R^2=0.3314, p=0.0196, 95% CI: 0.0023-0.0229) (Figure 5.10B). Active
disease in the 2 individuals was associated with higher propionate (10%, 32%), and higher TNF-β (as above) in 2 patients.

Although there were no significant correlation between TNF-β concentrations and butyrate percentages ($R^2=0.1212$, $p=0.1863$, 95% CI: -0.0067-0.0315)(Figure 5.10C), a clear trend was seen with higher butyrate (14-24%) and TNF-β (0.28-0.75 pg/ml) in active disease than in remission (butyrate 1-14%, TNF-β (0.20-0.57 pg/ml) all 3 patients.

Similarly, a strong trend of positive association was seen between TNF-β concentrations and butyrate percentages in UC patients (n=3, 4 samples, $R^2=0.9824$, $p=0.0848$, 95% CI: -0.0027-0.0102)(Figure 5.10D). There was only 1 pair of samples for this cohort, which also revealed higher butyrate (23.7%) and TNF-β (0.28 pg/ml) in active disease than in remission (butyrate 8.8%, TNF-β 0.20 pg/ml).

NIC patients (n=5) also demonstrated a positive trend of association between TNF-β concentrations (0.10-0.26 pg/ml) and butyrate percentages (2-24%) ($R^2=0.7512$, $p=0.0572$, 95% CI: -0.0004-0.0145)(Figure 5.10E). Inflammatory markers were normal for 3 of the patients, with no CRP or ESR measurements available for the remaining 2 patients. Given the small cohort, these results are cautiously presented as trends of association, which would benefit from confirmation with greater numbers.

There were no particular trends of associations seen with TNF-α concentrations with SCFA percentages.
Figure 5. 10: Relationship between Systemic TNF-β and Short Chain Fatty Acids in IBD and NIC

A, B and C show the relationship between TNF-β concentrations and acetate (A), propionate (B) and butyrate (C) in IBD patients (n=16 samples). Separate analysis was also performed on UC (D) (n=4 samples) and NIC (E) (n=5) patients. The red points represent active disease and the blue points represent disease remission in individual patients (connected by blue arrows pointing to the second time point). Data are presented with $R^2$ and p values.
5.3.4.2 Systemic Cytokines and Propionate in Crohn’s Disease and Ulcerative Colitis

There were no associations observed between cytokines of IBD patients collectively with propionate. As longitudinal data was available for 2 CD patients, this was analysed separately and trends of associations were observed. In addition, there were also trends seen in the small UC cohort (n=3, 4 samples).

5.3.4.2.1 Systemic Cytokines and Propionate in CD

Systemic cytokines for Patient A15 (n=4 samples) and D2 (n=5 samples) were analysed for potential associations with SCFA. Patient A15 received EEN and Patient D2 received Infliximab. Trends of associations were seen between IL-12p40 and IL-16 concentrations with propionate percentages.

In both patients, a negative trend was observed with IL-12p40 (A15 $R^2$=-0.576, $p=0.2414$, 95% CI: -10.42-4.65; D2 $R^2$=-0.225, $p=0.4200$, 95% CI: -9.936-5.433) (data not shown). Similarly, a negative trend was seen with IL-16 (A15 $R^2$=-0.791, $p=0.1105$, 95% CI: -2.893-0.636; D2 $R^2$=-0.322, $p=0.3183$, 95% CI: -5.741-2.608) (data not shown).

5.3.4.2.2 Systemic Cytokines and Propionate in Ulcerative Colitis

IL-17 concentrations and propionate percentages were assessed for possible association. In UC, a negative trend of association was observed (n=3, $R^2$=-0.9763, $p=0.0984$, 95% CI: -21.52-7.07) (Figure 5.11A). Despite the statistical significance, given the small cohort, this result is presented as a trend of association. Samples from an individual patient, obtained during active disease (red) and in remission (blue), were included. The differences between the 2 samples were small but active disease appeared to be associated with higher IL-17 (4.8 pg/ml) and propionate (10.4%) than in remission (IL-17: 0.8 pg/ml, propionate 9.6%).

As IFN-$\gamma$ is a Th1 cytokine, it was thus surprising to find a negative association between IFN-$\gamma$ and propionate in UC (n=3, $R^2$=-0.9996, $p=0.0122$, 95% CI: -0.5783-(-0.3637)) (Figure 5.11B). Similar to IL-17, active disease appeared to be associated with higher propionate (10.4%) but IFN-$\gamma$ was similar (0.42, 0.43 pg/ml).
5.3.4.3 Systemic Cytokines and Butyrate in IBD and NIC

Butyrate, for its many intestinal functions, is of particular interest. Cytokines were analysed for potential associations with butyrate percentages.

5.3.4.3.1 Systemic IL-5 and Butyrate in IBD

A positive correlation was seen between IL-5 and butyrate percentages \((R^2=0.327, \ p=0.0207, \ 95\% \ CI: \ 0.0065-0.0664)\) in IBD patients \((n=16)\)(Figure 5.12). Samples from 3 patients in active disease and remission revealed higher butyrate percentages in active disease (red points) \((10-24\%)\) than in remission (blue points) \((3-9\%)\). IL-5 was also higher in active disease in 2 patients \((0.51 \ pg/ml, 1.49 \ pg/ml)\).
IL-5 concentration and butyrate percentages were analysed for potential correlation in IBD patients (n=16 samples). The red points represent active disease and the blue points represent disease remission in an individual patient, with the arrows pointing to the second sample. Data are presented with $R^2$ and $p$ values.

**Figure 5.12: Systemic IL-5 Concentrations and Butyrate Percentages in IBD**

In the IBD cohort, when a particularly high IL-8 concentration was excluded, a statistically insignificant (n=15, $R^2=0.04422$, $p=0.4519$, 95% CI: -1.340-0.632) relationship was found between IL-8 and butyrate (Figure 5.13A). In 2 individual patients, higher butyrate percentages in active disease (14%, 18%) than in remission (1%, 3%) was observed, as described in Chapter 4.

Further analysis of IL-8 concentrations with butyrate percentages in UC demonstrated a positive trend of association (n=3, $R^2=0.9224$, $p=0.1797$, 95% CI: -24.15-42.14)(Figure 5.13B). In a UC patient, active disease was associated with higher IL-8 concentration (127 pg/ml) and butyrate percentage (24%) than in remission (IL-8: 21 pg/ml, butyrate 9%).
5.3.4.3.3 Systemic IL-10 and Butyrate in IBD, Crohn’s Disease and Non-inflammatory Controls

Although there were no statistically significant relationship between IL-10 concentrations and butyrate percentages, data are presented to demonstrate the apparent trends.

A trend of positive association was seen between IL-10 concentration and butyrate percentages in IBD patients ($R^2=0.2204$, $p=0.0666$, 95% CI: -0.0013-0.0355) (Figure 5.14). In the 3 individual patients, consistent with previous findings, active disease was associated with higher butyrate (14-24%) than in remission (1-14%). In 2 of the patients, active disease was also associated with higher IL-10 concentrations (0.52 pg/ml, 0.57 pg/ml). However, these are admittedly small values.

There were no particular associations seen in IL-10 concentrations and butyrate percentages in UC or the 2 individual CD patients (data not shown). As NIC patients were found to have higher butyrate percentages (Section 4.3.2.4, Chapter 4), analysis was performed to identify potential correlation with cytokines, however, no association was apparent between butyrate (2-24%) and any of the cytokines (data not shown).
IL-10 concentrations were analysed for correlation with butyrate percentages, as measured by gas chromatography, in IBD patients (n=16 samples). Concentrations are presented as pg/ml, and butyrate percentages are calculated from total SCFA concentrations. Data points are red to represent concurrent active disease and blue to represent remission, with the arrows pointing to the second sample. Linear correlation with 95% confidence interval was performed, and results presented with $R^2$ and $p$ values.

### 5.3.5 Systemic Cytokines of IBD Subgroups

There were 3 patients whose results were separated from the IBD groups, Patients B3, A11 and C3 (Figure 5.15). This heatmap illustrates cytokine concentrations as a percentage of the highest concentration measured for each cytokine, with the darker colours representing higher concentrations. Data for the other patient and control groups are also presented here for comparison.

At the time of cytokine measurements for Patient B3, diagnosed with IL-10RB mutation, the ESR was 50 mm/hr. TNF-α (4.03 pg/ml), IL-12p40 (327.64 pg/ml) and IL-16 (104.67 pg/ml) were high, with IL-7 (1.30 pg/ml), IL-8 (3.22 pg/ml) and IL-17 (4.39 pg/ml) being moderately high.

Patient A11 was diagnosed with CD then subsequently diagnosed with Granulomatosis polyangiitis as well. The patient remained in active disease throughout the study period,
with measured ESR 73, 66, 83 and 55 mm/hr at pre-treatment, months 3, 6 and 11 respectively. The cytokine concentrations increased with higher ESR (IL-6: 0.7, 0.2, 1.9, 0.6; TNF-α: 5.6, 1.4, 5.4, 3.5; IFN-γ: 1.1, 0.3, 3.4, 0.9; IL-10: 0.5, 0.1, 1.5, 0.6; IL-12p40: 357, 70, 217, 156; IL-16: 233, 58, 203, 171; TNF-β: 0.4, 9.1, 0.9, 0.3 pg/ml). An exception was IL-17 (23, 90, 72, 52 pg/ml), which increased with reducing ESR, and GM-CSF (0.4, 0.4, 0.2 0.2 pg/ml), which remained unchanged (Figure 5.15). In addition, IL-5 (0.99, 1.51, 1.84, 3.04 pg/ml), IL-7 (6.1, 1.0, 2.2, 5.7 pg/ml), IL-8 (43, 8, 10, 26 pg/ml) and IL-15 (1.5, 0.7, 2.3, 2.0 pg/ml) reduced then increased further in month 11, when the ESR was lowest (55 ml/hr).

Patient C3, diagnosed with VEIOBD, had surgery for refractory IBD. Cytokines were measured at 1, 3 and 5 months post-operatively, when ESR levels were 18, 30 and 41 mm/hr respectively. Cytokine concentrations were found to increase with ESR values (IL-6: 0.2, 0.4, 0.4; TNFα: 2.1, 4.0, 4.1; IFN-γ: 1.4, 3.5, 4.2; IL-10: 0.2, 0.5, 0.5; IL-5: 0.6, 0.9, 0.9; IL-7: 3.3, 4.4, 2.8; IL-15: 1.6, 1.7, 2.0; TNF-β: 0.1, 0.3, 0.5; GM-CSF: 0.1, 0.8, 0.6; IL-1α: 0.03, 56.18, 0.13; IL-8: 25, 36, 19; IL-12p40: 148, 420, 560; IL-16: 116, 180, 211; IL-17: 3, 17, 17 pg/ml). IL-1α levels were predominantly undetectable in other patients (64% of the total samples) with 35% of the samples quantified (maximum 0.5 pg/ml). At month 3, the IL-1α concentration for this patient was 56 pg/ml.
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<td>A15b 3/11</td>
<td>Active CD</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A9</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>D7a 29/4</td>
<td>Remission</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC1</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC2</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC4</td>
<td>Active CD</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC5</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC6</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC7</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC8</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC9</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC10</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC11</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC12</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC13</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC14</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC15</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC16</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC17</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC18</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC19</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC20</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>JHC21</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A6</td>
<td>Remission</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A1</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A3</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A4</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A10</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A13</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A20</td>
<td>Active UC</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A24</td>
<td>Healthy Control</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A5, 6yr</td>
<td>Food Allergies</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A16</td>
<td>Rheumatoid Arthritis</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A23</td>
<td>Auto-immune</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A17</td>
<td>IBD</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A19</td>
<td>IBD</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
<tr>
<td>A24</td>
<td>Auto-immune</td>
<td>TNF-α 0.01, IL-15 0.02, IL-17 0.05, IL-5 0.14</td>
</tr>
</tbody>
</table>

**Notes:**
- **IL-12 p 40**: Interleukin-12 p40 expression.
- **IFN-γ**: Interferon-γ expression.
- **IL-6**: Interleukin-6 expression.
- **TNF-α**: Tumor necrosis factor-alpha expression.
- **IL-15**: Interleukin-15 expression.
- **IL-17**: Interleukin-17 expression.
- **IL-5**: Interleukin-5 expression.
- **TNF-β**: Tumor necrosis factor-beta expression.
- **IL-10**: Interleukin-10 expression.
- **GM-CSF**: Granulocyte-macrophage colony-stimulating factor expression.
- **SAA**: Sarcoidosis-associated antigen expression.
- **VEGF**: Vascular endothelial growth factor expression.
- **ICAM-1**: Intercellular adhesion molecule-1 expression.
- **VCAM-1**: Vascular cell adhesion molecule-1 expression.
Figure 5.15: Heatmap of Cytokine Concentrations

IL-12p40, IFN-γ, IL-6, TNF-α, IL-15, IL-16, IL-7, IL-17, IL-5, IL-8, TNF-β, IL-10, GM-CSF, SAA, VEGF, ICAM-1 and VCAM-1 concentrations for patients with active UC (n=4), UC in remission (n=3), active CD (n=8), CD in remission (n=3), active IBDU (n=2), HC (n=21), IBS (n=8), food allergies (n=4), Rheumatology disorders (n=4) as well as Patients B3, A11 and C3 are presented as percentages of the highest cytokine concentration for each cytokine measured. Each cell is coloured depending on the percentage, with the higher concentrations being darker in colour.

5.3.6 Results Summary

Analyses of plasma cytokine concentrations of HC, NIC, IBD and other patients with GI inflammation were performed. Data were interpreted in the context of active disease, remission and in response to treatment. In addition, trends of associations with stool SCFA were assessed. The findings are summarised below;

Cytokines of IBD Patients in comparison to HC and NIC

Significant results of the comparison between IBD patients (n=14) in active disease (n=14) and remission (n=6) with HC (n=21) and NIC (n=8) are as follows;

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Active IBD vs. HC (p value)</th>
<th>IBD Remission vs. HC (p value)</th>
<th>NIC vs. HC (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>↑ (*)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-17</td>
<td>↑ (***)</td>
<td>↑ (**)</td>
<td>↑ IBS (**)</td>
</tr>
<tr>
<td>IL-15</td>
<td>↑ (***)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>↓ (***</td>
<td>↓ (**)</td>
<td>↓ IBS (***</td>
</tr>
<tr>
<td>IL-5</td>
<td>↑ (**)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-16</td>
<td>↑ (*)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>-</td>
<td>-</td>
<td>↑ IBS (**)</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.001, *** p<0.0005
Systemic Cytokines in CD and UC Patients

When CD (active n=8, remission n=3) and UC (active n=4, remission n=3) patients were analysed separately, significantly differences were seen for the following cytokines in comparison to HC;

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Kruskal-Wallis Test (p value)</th>
<th>Active CD (p value)</th>
<th>CD Remission (p value)</th>
<th>Active UC (p value)</th>
<th>UC Remission (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17</td>
<td>$x^2(2)=32.21$, p&lt;0.0001</td>
<td>↑ (**), ↑ (*)</td>
<td>↑ (*)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>$x^2(2)=29.94$, p&lt;0.0001</td>
<td>↓ (**), ↓ (*)</td>
<td>-</td>
<td>↓ (**), ↓ (*)</td>
<td>-</td>
</tr>
<tr>
<td>IL-15</td>
<td>$x^2(2)=12.25$, p=0.0156</td>
<td>↑ (*)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-5</td>
<td>$x^2(2)=12.36$, p=0.0148</td>
<td>↑ (*)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TNF-α</td>
<td>$x^2(2)=8.799$, p=0.0663</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-16</td>
<td>$x^2(2)=10.19$, p=0.0374</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>$x^2(2)=15.38$, p=0.0004</td>
<td>↑ (*)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*p<0.05,**p<0.001,***p<0.0005

Cytokine Ratios

Pro-inflammatory cytokines were also analysed for correlation or association with linear regression.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Disease</th>
<th>Coefficient of Determination ($R^2$)</th>
<th>Statistical Significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17 and IL-10</td>
<td>CD</td>
<td>0.4914</td>
<td>0.0162*</td>
</tr>
<tr>
<td>IL-17 and IL-10</td>
<td>UC</td>
<td>0.0236</td>
<td>0.7422</td>
</tr>
<tr>
<td>IL-15 and IL-10</td>
<td>CD</td>
<td>0.1586</td>
<td>0.2251</td>
</tr>
<tr>
<td>IL-15 and IL-10</td>
<td>UC</td>
<td>0.4527</td>
<td>0.0977</td>
</tr>
<tr>
<td>IL-5 and IL-10</td>
<td>CD</td>
<td>0.3138</td>
<td>0.0731</td>
</tr>
<tr>
<td>IFN-γ and IL-10</td>
<td>CD</td>
<td>0.05332</td>
<td>0.4945</td>
</tr>
</tbody>
</table>
Subsequently, IL-17 was analysed further as a ratio to the anti-inflammatory cytokine IL-10. In addition, the Th1 to Th17 balance was assessed as a ratio of IL17:IFN-γ.

<table>
<thead>
<tr>
<th>Ratio</th>
<th>IBS (n=8)</th>
<th>Active CD (n=8)</th>
<th>CD in Remission (n=3)</th>
<th>Active UC (n=4)</th>
<th>UC in Remission (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17: IL-10</td>
<td>↑ (*)</td>
<td>↑ (*)</td>
<td>↑ (*)</td>
<td>↑ (*)</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ:IL-10</td>
<td>-</td>
<td>↑ (*)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-17: IFN-γ</td>
<td>↑ (*)</td>
<td>-</td>
<td>-</td>
<td>↑ (*)</td>
<td>↑ (*)</td>
</tr>
</tbody>
</table>

*p<0.05

**Systemic Cytokines with IBD Treatment**

Immune responses were investigated following IBD treatment. With decreasing ESR or CRP, concentrations of these cytokines altered as follows:

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>EEN in CD (n=2)</th>
<th>Infliximab in CD (n=3)</th>
<th>Prednisolone in UC (n=2)</th>
<th>Infliximab in UC (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>IL-17</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>IL-5</td>
<td>→ Variable</td>
<td>↓ *</td>
<td>→</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>→</td>
<td>↓</td>
<td>↓</td>
<td>→</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>→ *</td>
<td>→</td>
<td>↓ *</td>
<td>→</td>
</tr>
<tr>
<td>IL-7</td>
<td>→ *</td>
<td>→ *</td>
<td>→ **</td>
<td>↑</td>
</tr>
<tr>
<td>IL-15</td>
<td>→ *</td>
<td>↓</td>
<td>↓ *</td>
<td>→</td>
</tr>
<tr>
<td>IL-16</td>
<td>↓ *</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>↓ *</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

* = Increased if inflammatory markers remain elevated

** = Decreased if inflammatory markers remain elevated
Associations and Correlations between Systemic Cytokines and SCFA

Trends of associations and correlations were observed between SCFA with the following cytokines:

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Cohort</th>
<th>Acetate (R², p values)</th>
<th>Propionate (R², p values)</th>
<th>Butyrate (R², p values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-β</td>
<td>IBD</td>
<td>Negative (R²=0.452, p=0.0043)</td>
<td>Positive (R²=0.285, p=0.0332)</td>
<td>Positive (R²=0.089, p=0.2622)</td>
</tr>
<tr>
<td>TNF-β</td>
<td>UC</td>
<td>-</td>
<td>-</td>
<td>Positive (R²=0.9009, p=0.0508)</td>
</tr>
<tr>
<td>TNF-β</td>
<td>NIC</td>
<td>-</td>
<td>-</td>
<td>Positive (R²=0.7512, p=0.0572)</td>
</tr>
<tr>
<td>IL-17</td>
<td>UC</td>
<td>-</td>
<td>Negative (R²=0.9754, p=0.0124)</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>UC</td>
<td>-</td>
<td>Negative (R²=0.9944, p=0.0028)</td>
<td>-</td>
</tr>
<tr>
<td>IL-5</td>
<td>IBD</td>
<td>-</td>
<td>-</td>
<td>Positive (R²=0.327, p=0.0207)</td>
</tr>
<tr>
<td>IL-10</td>
<td>IBD</td>
<td>-</td>
<td>-</td>
<td>Positive (R²=0.165, p=0.1188)</td>
</tr>
</tbody>
</table>
5.4 Results of Vascular Markers Analysis

Concentrations of systemic vascular markers of HC (n=21, age median 17.0 years (12.6-18.4), 33% male) were compared to 16 NIC [IBS (n=8), food allergies (n=4), JIA n=2, other Rheumatology conditions n=2] and IBD [CD (n=8), UC (n=4), IBDU (n=2)] patients. As mentioned in Section 5.3, a total of 56 blood samples were analysed for vascular profiles. Additional data on individual patients (1 patient with VEOIBD, 1 with Granulomatous Polyangiitis) are also presented. A total of 93 samples were thus analysed.

Concentrations of vascular markers utilising standard curves generated by the MesoScale Discovery (MSD) V-PLEX Vascular Injury Panel 2 assay kit were analysed (Table 5.5).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LLOD (pg/ml)</th>
<th>No of Samples between LLOD- LLOQ (pg/ml)</th>
<th>No of Samples above LLOQ</th>
<th>ULOQ (pg/ml)</th>
<th>No of Samples above ULOQ</th>
<th>ULOD (pg/ml)</th>
<th>No of Samples above ULOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>0.77</td>
<td>0</td>
<td>7.7</td>
<td>72</td>
<td>562</td>
<td>0</td>
<td>1070</td>
</tr>
<tr>
<td>SAA</td>
<td>19.2</td>
<td>0</td>
<td>54</td>
<td>69</td>
<td>138,000</td>
<td>3</td>
<td>218,000</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>10.7</td>
<td>0</td>
<td>37.6</td>
<td>72</td>
<td>32,000</td>
<td>0</td>
<td>51,500</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>2.3</td>
<td>0</td>
<td>15</td>
<td>72</td>
<td>32,700</td>
<td>0</td>
<td>61,500</td>
</tr>
</tbody>
</table>

Table 5. 5: The Number of Samples detected and quantified by MesoScale Discovery (MSD) Assay
(Total 72 samples excluding HC; LLOD – Lower limit of detection, LLOQ – Lower limit of quantification, ULOQ – Upper limit of detection)

5.4.1 Vascular Markers in HC, NIC and IBD Patients

Vascular markers of HC (n=21) and NIC (IBS n=8, food allergies n=4) were compared with IBD patients. CD, IBDU and UC patients (n=14) were combined then analysed in 2 groups depending on disease activity, Active IBD (n=14) and IBD Remission (n=6), based on disease scores (Figure 5.16). For those with samples collected prospectively, only 1 sample was used in the active IBD and IBD Remission groups.

In HC, median concentration of VCAM-1 was 0.48 \times 10^6 (0.22 \times 10^6 – 1.07 \times 10^6) pg/ml (Figure 5.16A). VCAM-1 was raised in inflammation, namely IBS [median 0.78 \times 10^6(0.57 \times 10^6 – 1.17 \times 10^6)pg/ml, p=0.004], food allergies [median 1.04 \times 10^6 (0.91 \times 10^6 – 1.31 \times 10^6)].
Subsequently, analysis was performed on samples received from individual patients during active disease and remission (CD n=5, UC n=4) (Figure 5.16A, Right Column). There were patients who achieved remission (blue lines and points), patients with ongoing inflammation (red lines and points) and those who improved but continued in active disease (blue lines, red points). No apparent trends were observed in VCAM-1 concentrations.

Median SAA concentration was 3.55 x10^6 (0.73 x10^6 - 26.80 x10^6) pg/ml in HC (Figure 5.16B). Concentrations were similar in IBS [median 1.78 x10^6 (0.28 x10^6 - 13.10 x10^6) pg/ml], food allergies [median 2.10 x10^6 (0.52 x10^6 - 3.29 x10^6) pg/ml] and IBD remission [n=6, median 0.68 x10^6 (0.11 x10^6 - 5.32 x10^6) pg/ml]. SAA was raised in active IBD [n=14, median 20.75 x10^6 (1.00 x10^6 - 335 x10^6) pg/ml], although this was not statistically significant. There was, however, a significant difference between active IBD and IBD in remission (p=0.005). In analysing samples from individual patients in active disease and remission, a trend of decreased or similar SAA was observed in patients who achieved remission or clinically improved (Figure 5.16B Right Column). In active disease, SAA was seen to increase in 2 patients and decrease in another.

In HC, median VEGF concentration was 149.23 (0.38 - 327.00) pg/ml (Figure 5.16C). In contrast, VEGF was lower in IBS [median 43 (29 - 140) pg/ml], food allergies [median 34 (23 - 170) pg/ml], active IBD [n=14, median 86 (22 - 213) pg/ml] and IBD in remission [n=6, median 42 (6 - 138) pg/ml], none of which were statistically significant although the Kruskal-Wallis score was 11.58 (p=0.0207). The lower concentrations of VEGF with inflammation was unexpected, and perhaps related to the different batches of MSD kits used to analyse the HC and patient samples. In contrast to this result, analysis of individual patients in active disease and remission (Figure 5.16C Right Column) revealed decreased VEGF in patients who clinically improved or achieved remission. In those with ongoing active disease, VEGF increased or remained stable but a small decrease was seen the UC patient.

Median concentration of ICAM-1 in HC was 0.59 x10^6 (0.41 x10^6 - 1.05 x10^6) pg/ml, There were no differences observed between HC and IBS [median 0.71 x10^6 (0.54 x10^6 - 1.04 x10^6) pg/ml], food allergies [median 0.72 x10^6 (0.68 x10^6 - 0.79 x10^6) pg/ml], active IBD [n=14 median 0.72 x10^6 (0.48 x10^6 - 1.06 x10^6) pg/ml] and IBD remission [n=6 median
0.56 x10^6 (0.14 x10^6–1.08 x10^6) pg/ml) (x^2(2)=3.71, p=0.4467). Analysis of individual patients in active disease and remission revealed no particular trends (data not shown).

A.

B.

C.
**Figure 5.16: Systemic Vascular Markers in HC, NIC and IBD Patients**

VCAM-1, SAA and VEGF concentrations in HC (n=21), IBS (n=8), food allergies (n=4), active IBD (n=14) and IBD in remission (n=6) were quantified with MSD kit V-Plex Vascular Injury Panel 2, with disease activity classified based on PUCAI or wPCDAI scores. Data are presented as pg/ml and shown with median and p values following Kruskal-Wallis and Dunn’s Multiple Comparison tests. Analysis was also performed on samples from individual IBD patients comparing vascular markers with disease activity (red line represents ongoing inflammation, blue line presents disease improvement, red points represent active disease and blue points represent remission).

5.4.2 Systemic Vascular Markers in IBD

As with the cytokine analysis, patients with CD (n=8) and UC (n=4) in active disease (CD n=8, UC n=4) and remission (CD n=3, UC n=3) were also analysed separately (Figure 5.17). Differences were still apparent despite the smaller numbers. Significant findings are presented.

In comparison to HC [median 0.48 x10^6 (0.22 x10^6– 1.07 x10^6)pg/ml], median VCAM-1 concentrations were higher in all IBD groups (χ^2(2) 15.19, p=0.0043)(Figure 5.17A). Statistical significance was observed with active CD [n=8, median 0.71 x10^6 (0.57 x10^6– 0.89 x10^6)pg/ml, p=0.017] but not in CD remission [n=3, median 1.02 x10^6 (0.29 x10^6– 1.06 x10^6)pg/ml], active UC [n=4, median 0.73 x10^6 (0.65 x10^6–1.19 x10^6)pg/ml] and UC remission [n=3, median 0.64 x10^6 (0.29 x10^6– 1.00 x10^6)pg/ml], perhaps due to the smaller cohorts.

Consistent with the analysis above, SAA was raised in active disease compared to HC [median 3.55 x10^6 (0.73 x10^6– 26.80 x10^6)pg/ml](Figure 5.17B). Although not significant, increased SAA was seen in active CD [n=8, median 29.4 x10^6 (2.49 x10^6–335 x10^6)pg/ml] and active UC [n=4, median 2.57 x10^6 (1.10 x10^6– 114.0 x10^6)pg/ml]. Concentrations were similar to HC in both groups in remission [CD: n=3 median 0.69 x10^6 (0.11 x10^6–1.10 x10^6)pg/ml; UC: n=3 median 0.68 x10^6 (0.17 x10^6– 5.32 x10^6)pg/ml]. Significant differences was only observed between active CD and CD remission (p=0.008).
There were no significant differences observed between the groups with VEGF \((x^2(2)=9.352, p=0.0529)\) and ICAM-1 \((x^2(2)=4.708, p=0.3186)\).

**Figure 5. 17: Systemic Vascular Markers in CD and UC**

Concentrations of VCAM-1 and SAA in HC \((n=21)\), CD \((n=8)\) and UC \((n=4)\) patients were analysed depending on disease activity defined by PUCAI (active >10) and wPCDAI (active >12.5) scores, comparing active disease (CD \(n=8\), UC \(n=4\)) with disease remission (CD \(n=3\), UC \(n=3\)). Data are presented as pg/ml and shown with median and \(p\) values following Kruskal-Wallis and Dunn’s Multiple Comparison tests.

### 5.4.3 Alterations of Vascular Markers with IBD Treatment

The effects of IBD treatment on vascular markers were subsequently analysed (Figure 5.18). CD patients who were treatment-naïve and received EEN \((n=2)\), and Infliximab \((n=3)\) were analysed utilising ESR (mm/hr) or CRP (mg/dL) as a marker of inflammation. Similarly, UC patients who were treatment-naïve and received Prednisolone \((n=2)\), and Infliximab \((n=1)\) were also analysed. Increased ESR was seen in a CD \((D2-ESR 8 to 11 to 15 \text{ mm/hr}, \text{ although still } <20 \text{ mm/hr})\) and UC \((D5-ESR 10 to 11 to 36 \text{ mm/hr})\) patients receiving Infliximab, and a patient with CD \((A14-ESR 67 to 36 \text{ mm/hr})\) and UC \((A8-ESR 60 to 41 \text{ mm/hr})\) in whom ESR improved but remained elevated. The inflammatory markers returned to normal levels in the remaining patients. Concentrations of vascular markers pre-treatment, following induction and post-treatment or during maintenance are detailed in Table 5.6.
VCAM-1 concentrations were lower with decreased CRP or ESR in both treatment groups in CD (Patients A14–0.70 x10^6 to 0.77 x10^6 pg/ml; A15–0.84 x10^6 to 1.06 x10^6 pg/ml; D2–0.72 x10^6 to 0.76 x10^6 to 0.66 x10^6 pg/ml; D4–0.74 x10^6 to 0.64 x10^6 to 0.59 x10^6 pg/ml; D6–8.95 x10^6 to 0.91 x10^6 to 0.29 x10^6 pg/ml) and UC (Patients A8–1.19 x10^6 to 0.37 x10^6 pg/ml; A2–0.79 x10^6 to 0.64 x10^6 pg/ml; D5–0.66 x10^6 to 0.84 x10^6 to 1.00 x10^6 pg/ml). 

SAA concentrations also decreased in CD (Patients A14–18.99 x10^6 to 15.25 x10^6 pg/ml; A15–36.3 x10^6 to 0.69 x10^6 pg/ml; D2–2.86 x10^6 to 5.14 x10^6 to 3.23 x10^6 pg/ml; D4–54.5 x10^6 to 19.70 x10^6 to 2.54 x10^6 pg/ml; D6–2.49 x10^6 to .057 x10^6 to 0.11 x10^6 pg/ml) and UC groups (Patient A21–2.48 x10^6 to 0.68 x10^6 pg/ml) although increased slightly in Patient A8 (1.10 x10^6 to 1.32 x10^6 pg/ml), whose inflammatory markers improved but remained elevated. In Patient D5, the SAA concentration was higher with increased ESR (2.66 x10^6 to 14.86 x10^6 to 5.32 x10^6 pg/ml).

VEGF concentrations in CD and UC patients were seen to decrease with improving inflammation. Reduction in concentrations were seen in CD patients receiving EEN (Patients A14-42 to 33 pg/ml; A15-143 to 138 pg/ml) and Infliximab (Patients D2-90 to 119 to 158 pg/ml; D4-33 to 52 to 32 pg/ml; D6-83 to 170 to 69 pg/ml). Likewise, UC patients receiving Prednisolone (Patients A8–213 to 22 pg/ml; A21–97 to 56 pg/ml) and Infliximab (Patient D5–24 to 50 to 23 pg/ml) also had decreased VEGF concentrations.

On the other hand, ICAM-1 concentrations decreased in line with ESR levels in UC (Patients A8–0.91 x10^6 to 0.27 x10^6 pg/ml; A21–0.64 x10^6 to 0.38 x10^6 pg/ml; D5–0.48 x10^6 to 0.78 x10^6 to 0.73 x10^6 pg/ml) but varied in CD patients. ICAM-1 concentrations increased with improving ESR and CRP in CD patients receiving EEN (Patients A14–1.05 x10^6 to 1.09 x10^6 pg/ml; A15–0.72 x10^6 to 0.87 x10^6 pg/ml) but decreased in the patients receiving Infliximab (Patients D2–0.86 x10^6 to 0.84 x10^6 to 0.80 x10^6 pg/ml; D4–0.63 x10^6 to 0.65 x10^6 to 0.52 x10^6 pg/ml; D6–0.58 x10^6 to 0.55 x10^6 to 0.14 x10^6 pg/ml).
## Crohn’s Disease versus Ulcerative Colitis

<table>
<thead>
<tr>
<th>CD Pt</th>
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<th>Induction</th>
<th>Post-treatment ESR (mm/hr)</th>
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<tr>
<td>A14</td>
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<td>36</td>
</tr>
<tr>
<td>A15</td>
<td>23 *</td>
<td>NA</td>
<td>5 *</td>
</tr>
<tr>
<td>D2</td>
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<td>11</td>
<td>15</td>
</tr>
<tr>
<td>D4</td>
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<tr>
<td>D6</td>
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</tr>
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</table>

*CRP (mg/dL)

**Graphs:**
- **VCAM-1 (pg/ml)**
  - EEN: Pre-treatment, Post-treatment, Induction, Post-treatment
  - Infliximab: Pre-treatment, Post-treatment, Induction, Post-treatment
  - Treatment and Time (weeks, months)
  - CD Pt: Pre-treatment, Post-treatment, Induction, Post-treatment
  - UC Pt: Pre-treatment, Post-treatment, Induction, Post-treatment

<table>
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<th>UC Pt</th>
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<th>Induction ESR (mm/hr)</th>
<th>Post-treatment ESR (mm/hr)</th>
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<td>NA</td>
<td>41</td>
</tr>
<tr>
<td>A21</td>
<td>41</td>
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<td>8</td>
</tr>
<tr>
<td>D5</td>
<td>10</td>
<td>11</td>
<td>36</td>
</tr>
</tbody>
</table>

**Graphs:**
- **SAA (pg/ml)**
  - EEN: Pre-treatment, Post-treatment, Induction, Post-treatment
  - Infliximab: Pre-treatment, Post-treatment, Induction, Post-treatment
  - Treatment and Time (weeks, months)
  - CD Pt: Pre-treatment, Post-treatment, Induction, Post-treatment
  - UC Pt: Pre-treatment, Post-treatment, Induction, Post-treatment

- **VEGF (pg/ml)**
  - EEN: Pre-treatment, Post-treatment, Induction, Post-treatment
  - Infliximab: Pre-treatment, Post-treatment, Induction, Post-treatment
  - Treatment and Time (weeks, months)
  - CD Pt: Pre-treatment, Post-treatment, Induction, Post-treatment
  - UC Pt: Pre-treatment, Post-treatment, Induction, Post-treatment

- **ESR (mm/hr)**
  - Pre-treatment, Post-treatment, Induction, Post-treatment
  - CD Pt, UC Pt

*Legend*
- A14, Mth0.4
- A15, Mth0.2
- D2, Wk2, Mth3.14
- D4, Wk2, Mth12
- D6, Mth0.3,7
Figure 5. 18: Concentrations of Vascular Markers in CD and UC Patients with Treatment

Concentrations of VCAM-1, SAA, VEGF and ICAM-1 in CD (left) and UC (right) patients were quantified with MSD kits V-Plex Vascular Injury Panel 2. CD patients received EEN (n=2) and Infliximab (n=3) whilst UC patients received Prednisolone (n=2) and Infliximab (n=1). Responses to treatment are indicated by changes in inflammatory markers (ESR or CRP), as presented in the table. Vascular markers are shown pre-treatment, post-induction and post-treatment as pg/ml.

<table>
<thead>
<tr>
<th>Cytokine/Time</th>
<th>EEN CD Patients</th>
<th>Infliximab</th>
<th>UC Patients</th>
<th>Prednisolone</th>
<th>Infliximab</th>
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<tbody>
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<td>VCAM-1</td>
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<td></td>
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</tr>
<tr>
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<td>6.959E+05</td>
<td>8.359E+05</td>
<td>-</td>
<td>-</td>
<td>1.191E+06</td>
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<tr>
<td>Post-treatment</td>
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<td>1.059E+06</td>
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<td>3.671E+05</td>
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<tr>
<td>Induction</td>
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<td>7.417E+05</td>
<td>8.946E+05</td>
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<td>-</td>
<td>6.598E+05</td>
<td>5.950E+05</td>
<td>2.877E+05</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>SAA</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-treatment</td>
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<td>3.630E+07</td>
<td>-</td>
<td>-</td>
<td>1.096E+06</td>
</tr>
<tr>
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<td>-</td>
<td>1.316E+06</td>
</tr>
<tr>
<td>Induction</td>
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<td>2.892E+06</td>
<td>5.450E+07</td>
<td>2.490E+06</td>
</tr>
<tr>
<td>Maintenance</td>
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<td>-</td>
<td>3.229E+05</td>
<td>2.540E+06</td>
<td>1.074E+05</td>
</tr>
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<td></td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-treatment</td>
<td>42</td>
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</tr>
<tr>
<td>Post-treatment</td>
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<tr>
<td>Induction</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>56</td>
</tr>
<tr>
<td>Maintenance</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-treatment</td>
<td>1.049E+06</td>
<td>7.194E+05</td>
<td>-</td>
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<td>9.134E+05</td>
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<tr>
<td>Post-treatment</td>
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<td>-</td>
<td>8.389E+05</td>
<td>6.482E+05</td>
<td>5.485E+05</td>
</tr>
</tbody>
</table>

Table 5. 6: Concentrations of Vascular Markers with Treatment
VCAM-1, SAA, VEGF and ICAM-1 concentrations for CD patients receiving EEN (n=2) and Infliximab (n=3), and UC patients receiving Prednisolone (n=2) and Infliximab (n=1) are listed. Concentrations are presented as pg/ml.

5.4.4 Vascular Markers and Short Chain Fatty Acids

Possible relationship between vascular markers and SCFA were investigated in IBD patients (n=16). Although there were no significant correlations seen between concentrations of vascular markers with acetate, propionate and butyrate percentages, trends of associations observed in IBD, UC and individual CD patients are presented.

5.4.4.1 Systemic VCAM-1 and Butyrate

A negative trend of association between VCAM-1 and butyrate was seen ($R^2=-0.1545$, \(p=0.1321\))(Figure 5.19). Data included 3 pairs of samples from individual patients obtained in active disease (red points) and remission (blue points, arrows point to the second sample). Butyrate was higher in active disease (14-24%) than in remission (1-14%), while VCAM-1 was higher in 2 patients in active disease (0.84 $\times 10^6$ pg/ml, 0.70 $\times 10^6$ pg/ml) than in remission (0.72 $\times 10^6$ pg/ml, 0.64 $\times 10^6$ pg/ml).

![Figure 5.19: Systemic VCAM-1 and Butyrate in IBD](image)

*Figure 5. 19: Systemic VCAM-1 and Butyrate in IBD*

Concentrations of VCAM-1 and stool SCFA from IBD patients (n=16) were analysed for potential correlation or association. The red points represent active disease and the blue points represent disease remission in individual patients (connected by blue arrows pointing to the second time point). Linear regression was performed with 95% confidence interval, with VCAM-1 concentrations (pg/ml), and SCFA percentages of the total SCFA concentrations. Data is presented with $R^2$ and $p$ values.
5.4.4.2 Systemic SAA and Propionate

There were no particular trends of vascular markers and propionate seen in IBD patients. When UC patients (n=3) were analysed separately, a trend was observed (Figure 5.20). There was a negative trend of association with 3 samples from 3 patients in active disease \((R^2=-0.8437, p=0.2588, 95\% \ CI: -6.8 \times 10^6 - 4.7 \times 10^6)\). The trend was strengthened if the fourth sample (a duplicate of a patient but in remission) was included in the analysis \((R^2=-0.8688, p=0.0679, 95\% \ CI: -2.4 \times 10^6 - 0.2 \times 10^6)\). Analysis of the samples from individual patients in active disease and in remission found higher SAA and propionate in active disease than in remission.

![Figure 5.20: Systemic SAA and Propionate in UC](image)

\textit{Figure 5. 20: Systemic SAA and Propionate in UC}

SAA concentrations and propionate percentages in UC patients \((n=3)\) were analysed for correlation with linear regression. The red points represent active disease and the blue points represent disease remission in an individual patient, with the arrow pointing to the second sample. Data is presented as \(R^2\) and \(p\) values.

5.4.4.3 Systemic VEGF and Short Chain Fatty Acids in IBD Patients

5.4.4.3.1 Systemic VEGF and Acetate in IBD Patients

In IBD patients, a negative trend of association was seen between VEGF concentrations and acetate percentage \((n=16, R^2=0.1028, p=0.2259, 95\% \ CI: -4.5-1.0)\) (Figure 5.21A). Analysis of the 3 pairs of samples received during active disease and remission suggested higher VEGF in active disease \((97-188 \text{ pg/ml})\) than in remission \((56-138 \text{ pg/ml})\). In 2 of the patients, active disease was associated with lower acetate \((53\%, 63\%)\) than in remission \((58\%, 80\%)\).
5.4.4.3.2 Systemic VEGF and Propionate in IBD

In contrast to acetate, a positive association was observed between VEGF and propionate \( (n=16, \ R^2=0.1032, \ p=0.2251, \ 95\% \ CI: \ -1.3-5.2) \) (Figure 5.21B). As commented above, VEGF was higher in active disease than in remission. In addition, propionate was higher in 2 patients in active disease (10.4%, 21.5%) than in remission (8.6%, 9.6%).

5.5.4.3.3 Systemic VEGF and Acetate in UC

VEGF in UC patients appear to be negatively associated with acetate \( (R^2=-0.9992, \ p=0.018, \ 95\% \ CI: \ -3.6-(-1.7)) \) (Figure 5.21C). In a patient with samples received in active disease and in remission, VEGF was higher in active disease (97 pg/ml) than in remission (56 pg/ml), while acetate was lower in active disease (63%) than in remission (80%). Including the fourth sample altered the statistical result \( (R^2=-0.940, \ p=0.0304, \ 95\% \ CI: \ -4.3- (-0.6)) \).

5.5.4.3.4 Systemic VEGF and Propionate in Individual Crohn’s Disease Patients

Although there were no trends of associations seen in CD patients as a cohort, analysis of longitudinal samples from 2 CD patients, A15 (n=4) and D2 (n=5) revealed negative trends of association between VEGF and propionate in Patient A15 \( (R^2=-0.1707, \ p=0.4117, \ 95\% \ CI: \ -10.9-8.1) \) and Patient D2 \( (R^2=-0.8901, \ p=0.0160, \ 95\% \ CI: \ -7.0- (-1.5)) \) (Figure 5.21D). Most of the samples received were during active disease. From the few samples received during remission, VEGF was lower (90-149 pg/ml) and propionate was higher in 2 of the samples (22.6%, 31.6%) compared to samples received during active disease (VEGF 105-188 pg/ml; propionate 8.6-21.5).
Concentrations of VEGF and stool SCFA percentages from IBD patients (A) (n=16 samples) were analysed for potential correlation or association. Separate analysis was also conducted on UC (C) (n=3) and individual CD patients (D) (n=2). Patient A15 (n=4) received EEN and Patient D2 (n=5) received Infliximab. The red points represent active disease and the blue points represent disease remission in individual patients (connected by blue arrows pointing to the second time point). Data are presented with $R^2$ and $p$ values.

Figure 5.21: Systemic VEGF and Short Chain Fatty Acids in IBD
5.4.5 Vascular Markers of IBD Subgroups

The 3 patients excluded from the IBD group analysis were Patients B3 (IL-10RB mutation), A11 (Granulomatosis with polyangiitis) and C3 (VEOIBD). The concentrations of their vascular markers are shown in the heat map Figure 5.16 and corresponds to ESR measurements of 50 mm/hr for B3, 73 mm/hr for A11 and 30 mm/hr for C3.

One blood sample was received from Patient B3. During that time, concentrations of VCAM-1 was 1.37 x10^6 pg/ml, SAA 2.00 x10^6 pg/ml, VEGF 18 pg/ml and ICAM-1 0.93 x10^6 pg/ml.

In addition, longitudinal samples for Patients A11 (n=4) and C3 (n=3) were analysed using ESR as a marker of inflammation. In Patient A11, whose ESR levels were 73, 66, 83 and 55 mm/hr at pre-treatment, months 3, 6 and 11, VEGF (58, 18, 64, 68 pg/ml) and SAA (48.94 x10^6, 2.69 x10^6, 357.31 x10^6, 8.33 x10^6 pg/ml) concentrations were directly related to ESR levels. VCAM-1 (1.29 x10^6, 0.51 x10^6, 1.28 x10^6, 1.44 x10^6 pg/ml) and ICAM-1 (2.04 x10^6, 0.62 x10^6, 1.72 x10^6, 10.02 x10^6 pg/ml) concentrations were stable.

Vascular markers for Patient C3 were measured at 1, 3 and 5 months post-operatively when ESR levels were 18, 30 and 41 mm/hr respectively. VEGF (55, 93, 52 pg/ml) and SAA (0.48 x10^6, 2.41 x10^6, 1.12 x10^6 pg/ml) concentrations were seen to increase with raised ESR levels. In contrast, the VCAM-1 (0.94 x10^6, 0.700 x10^6, 0.93 x10^6 pg/ml) and ICAM-1 (0.64 x10^6, 0.53 x10^6, 0.72 x10^6 pg/ml) concentrations appeared to decrease with raised ESR although can arguably be considered stable.

5.4.6 Results Summary

Vascular markers of HC, NIC, IBD and other patients with GI inflammation were compared, and trends of associations with stool SCFA were assessed. Data was interpreted in the context of active disease, remission and in response to treatment. In addition, trends of associations with stool SCFA were assessed. The findings are summarised below;
Vascular Markers in IBD Patients in comparison to HC and NIC

Results of the comparison between HC (n=21) and NIC (n=8) with Active IBD (n=14) and IBD Remission (n=6) are as follows;

<table>
<thead>
<tr>
<th>Vascular Markers</th>
<th>Kruskal-Wallis Test (p value)</th>
<th>Active IBD vs. HC (p value)</th>
<th>IBD Remission vs. HC (p value)</th>
<th>Active IBD vs. IBD Remission (p value)</th>
<th>NIC vs. HC (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1</td>
<td>$x^2(2)=27.16 (0.0001)$</td>
<td>↑ (*)</td>
<td>-</td>
<td>-</td>
<td>↑ IBS (*)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑ Food Allergy (*)</td>
</tr>
<tr>
<td>SAA</td>
<td>$x^2(2)=15.12 (0.0045)$</td>
<td>-</td>
<td>-</td>
<td>↑ (*)</td>
<td>-</td>
</tr>
<tr>
<td>VEGF</td>
<td>$x^2(2)=11.58 (0.0207)$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>$x^2(2)=3.71 (0.4467)$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*p<0.05,**p<0.001,***p<0.0005

Vascular Markers in CD and UC Patients

When CD (active n=8, remission n=3) and UC (active n=4, remission n=3) patients were analysed separately and compared to HC, significantly increased concentrations were still seen for the following vascular markers;

<table>
<thead>
<tr>
<th>Vascular Markers</th>
<th>Kruskal-Wallis Test (p value)</th>
<th>Active CD (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1</td>
<td>$x^2(2)=15.19 (0.0043)$</td>
<td>↑ (*)</td>
</tr>
<tr>
<td>SAA</td>
<td>$x^2(2)=14.94 (0.0048)$</td>
<td>↑ (**)#</td>
</tr>
<tr>
<td>VEGF</td>
<td>$x^2(2)=9.352 (0.0529)$</td>
<td>-</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>$x^2(2)=4.708 (0.3186)$</td>
<td>-</td>
</tr>
</tbody>
</table>

# comparison with CD Remission
Vascular Markers with IBD Treatment

Changes in vascular markers in individual CD and UC patients were analysed following treatment. With decreasing ESR or CRP, concentrations of these vascular markers altered as follows:

*Increased if inflammatory markers remain elevated

**Decreased with increasing ESR

<table>
<thead>
<tr>
<th>Vascular Markers</th>
<th>EEN in CD (n=2)</th>
<th>Infliximab in CD (n=3)</th>
<th>Prednisolone in UC (n=2)</th>
<th>Infliximab in UC (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM-1</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓ **</td>
</tr>
<tr>
<td>SAA</td>
<td>↓</td>
<td>↓</td>
<td>↓ *</td>
<td>↓</td>
</tr>
<tr>
<td>VEGF</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑ **</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

Correlations between Vascular Markers and SCFA

There were no correlations between SCFA and vascular markers, although a negative trend was observed between VEGF and propionate in Patient D2 ($R^2=0.890$, $p=0.0160$).
5.6 Discussion

The role of immune and vascular mediators are not only recognised in IBD but increasingly being manipulated in the treatment of these patients, however, more reports accumulate on “new” mediators as our understanding of IBD pathogenesis expand. This study supports published data and report new perspectives including potential association with SCFA.

5.6.1 Cytokines and Vascular Profiles with Disease Activity

Comparison was first made between HC (n=21) and IBD patients, incorporating CD (n=8), UC (n=3) and IBDU (n=2) patients. Disease activity was determined based on PUCAI and wPCDAI scores. Analysed was also performed using ESR to classify disease activity (ESR >20 mm/hr indicates active disease). As results were similar and wPCDAI scores appeared to be more representative of disease status, only data associated with disease scores were presented.

Subsequently, CD and UC patients were analysed separately. This was not possible for IBDU patients (n=2). Whilst analyses of CD and UC patients were possible, many results did not achieve statistical significance likely due to the small sample numbers. There were, however, similar trends to the cumulative IBD group.

In health, there is immune homeostasis with a balance of pro-inflammatory and anti-inflammatory cytokines and mediators maintaining host defence and function. Analysis was thus performed to assess the balance between specific cytokines. Positive correlation or trends of association between cytokines IL-17 and IL-10 were analysed further as ratios as this is a more practical analysis in the clinical setting.

5.6.1.1 Cytokines Profiles with Disease Activity

Consistent with previous reports, pro-inflammatory cytokines TNF-α and IL-17 were observed to be higher in active IBD (n=14 samples) compared to HC. IL-17 was also raised in IBD patients in remission (n=6). IL-5 and IL-16 were also raised in active IBD while GM-CSF was lower in IBD patients in active disease and in remission compared to HC. These are discussed further below.
TNF-α

TNF-α is a common downstream pro-inflammatory cytokine for both CD and UC thus explains the raised concentrations in active IBD compared to HC. Although TNF-α was increased in active IBD, no statistical differences were seen between HC with active CD or UC. The small patient numbers may explain the non-significant difference seen in UC. In addition, there were a few HC with higher than expected pro-inflammatory cytokines including TNF-α concentrations, likely due to inter-current mild infection. As comparison was made against HC, this would affect the statistical results.

TNF-α: IL-10 ratios were found to be predictive of coronary artery disease, severity of burn injury and non-alcoholic fatty liver disease (1130-1132). However, this ratio was not significantly different between IBD patients and HC (data not shown), probably due to the low concentrations of both cytokines in HC and high concentrations of both cytokines in IBD. IL-10, a major anti-inflammatory cytokines with pleotropic effects and dynamic systemic concentrations, has been reported to increase in active UC (but not CD) and decrease with disease resolution (1133). This study was not adequately powered to capture these changes in concentrations.

IL-17

IL-17 which mediates Th17 responses was raised in active CD (n=8), active UC (n=4) and CD in remission (n=3) in comparison to HC. This has been widely reported and expected given its role in chemoattraction of CD4+ T cells, monocytes and eosinophils, and stimulation of pro-inflammatory cytokine release (1134). A positive correlation between IL-17 and IL-10 in CD was demonstrated with significant differences seen in IL-17:IL-10 ratios of active CD, active UC and CD remission.

IL-5

IL-5 was raised in active CD. In UC, likely due to the smaller numbers, similar trends were seen. Being a Th2 cytokine, raised IL-5 was expected in UC as previously reported but was a surprising result with CD. This is contrary to published reports such as Nemeth et al., who reported IL-5 as a cytokine that differentiated UC from CD (1135, 1136). IL-5 plays a role in differentiation and release of eosinophils, and is a chemotactic agent attracting eosinophils to the intestinal mucosa. As eosinophils play a major role in wound healing, its presence in the mucosa suggests mucosal healing may be taking place (1137). In addition, it also stimulates intestinal smooth muscle contractility (1138). The raised IL-5 observed could be explained by the large proportion of study patients reporting food intolerances/allergies, or that mucosal healing may be occurring. As
increased mucosal expression of IL-5 was reported in diseased sites of patients in early recurrence of CD (1139), this could be another potential explanation.

**IL-16**

Although IL-16 was raised in active IBD, there were no significant differences seen in CD and UC when analysed separately. IL-16 is a chemo-attractant for CD4+ cells and is highly expressed in inflamed colonic mucosa of IBD patients (1128). In addition, it stimulates the production of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β, IL-15) by monocytes (1140), thus explaining the increased concentrations seen in active IBD.

**IFN-γ**

IFN-γ, a key cytokine mediating Th1 responses in CD, was raised in active CD with a trend for higher concentrations in remission. Increased IFN-γ in CD is widely reported, differentiating between Th1 in CD and Th2 in UC (1141, 1142). In addition, IFN-γ is a negative regulator, causing IL-23 inhibition (1143) thus inhibiting Th17 responses.

Using IFN-γ as a Th1 marker, IFN-γ:IL-10 ratios were higher in CD but lower in UC, differentiating CD (active and in remission) from UC (active and in remission) although these differences were not statistically significant. To investigate the dominant T helper subgroup, the Th1:Th17 ratio was attempted utilising IL-17:IFN-γ. The ratios appeared to differentiate CD from UC, with the ratios being higher in UC. It was, however, recognised that the analysis was performed on concentrations which may not necessarily reflect biologically significant values.

As IFN-γ differed between CD and UC, its concentration, IFN-γ:IL-10 and IL-17:IFN-γ ratios of IBDU patients (n=2) were compared to CD and UC patients. The IFN-γ profile of one IBDU patient was more similar to CD than UC, while the other could have been either.

**5.6.1.2 Vascular Profiles with Disease Activity**

Pro-inflammatory cytokine **IL-15** is also pro-angiogenic (999). It was raised in active IBD with a higher trend seen in IBD patients in remission. When analysed separately, IL-15 was also higher in active CD than HC. IL-15 is known to induce T cell recruitment to inflamed sites, their proliferation and pro-inflammatory cytokine production by T cell and macrophages (1144).
**VCAM-1 and ICAM-1**

Adhesion molecules are critical in leukocyte migration into inflamed tissues. Circulating VCAM-1 was reported to be higher in active UC compared to remission in UC, and between active CD and HC, while ICAM-1 was higher in CD compared to HC (1145). In this study, VCAM-1 was increased in active IBD, and when analysed separately, was raised in active CD compared to HC. Interestingly, VCAM-1 but not ICAM-1 had increased expression in the colon of IBD patients in remission who subsequently flared, thus potentially predicting relapse (1146).

**SAA**

SAA production induced by pro-inflammatory cytokines (IL-6, IL-1, TNF and IFN-γ), is also involved in the recruitment of neutrophils and mast cells to inflammatory sites, and the expression of pro-inflammatory and pro-thrombotic mediators ICAM-1, VCAM-1, IL-6 and IL-8. There was a trend of higher SAA in active IBD compared to HC was seen but significant difference was observed between active IBD and remission. Similarly, raised SAA was seen in active CD compared to CD in remission. This confirms previous reports of higher levels of SAA with active disease in CD (1147). In addition, SAA was suggested as a better marker of disease activity, being raised in 70% of patients with normal CRP but active inflammation (1147).

**VEGF**

VEGF is known to induce vascular permeability and stimulate endothelial proliferation. Higher expression of VEGF-A and its receptor VEGFR-2 have been reported in IBD patients and murine model (1012). In contrast, a trend of lower VEGF-A in IBD (in active disease and in remission) was observed in this study. This may be due to transformation of the HC values, performed to enable comparison between samples analysed during different MSD experiments, potentially resulting in untrue VEGF values which may not be reflective of the cohort. On the other hand, Kapsoritakis et al. (1148) reported similar findings, and was supported by Ferrante et al. who also found that VEGF polymorphisms had no effect on IBD susceptibility (1149).

The small patient numbers in this study made it challenging to draw any firm conclusions but the trends observed warrant further investigations with larger cohorts before this could be considered for clinical use.
5.6.2 Effects of Treatment on Cytokines and Vascular Mediators

To achieve mucosal healing, IBD treatment modulates host immunity to cease the inflammatory process and facilitate repair to take place. Longitudinal data was available for treatment-naïve patients receiving exclusive enteral nutrition (EEN) (n=2), Prednisolone (n=2) and Infliximab (CD n=3, UC n=1).

5.6.2.1 Exclusive Enteral Nutrition

Both CD patients improved with EEN although Patient A14 remained in active disease after the EEN course (ESR 67-36 mm/hr). As expected, pro-inflammatory cytokines TNF-α, IL-17 and IL-12p40 in decreased in parallel with reducing ESR or CRP. This was also the trend seen with SAA and VEGF.

Concentrations of IL-5, and IL-8 were low and remained unchanged. IL-5 may not be raised in CD as it is a Th2 cytokine. IL-8, on the other hand, is recognised as an important chemoattractant for neutrophils in CD and UC (1150, 1151). Greater expression was seen in chronic (from CD3+ T cells and macrophages) than early inflammation (from neutrophils) in ileal lesions of CD patients (1152). Perhaps this is not necessarily associated with increased plasma IL-8 levels.

Interestingly, IFN-γ and IL-16 decreased in Patient A15, who achieved remission (CRP 23-5 mg/dL), however increased in Patient A14, who improved but did not achieve clinical remission thus reflecting the continuing inflammation. Similarly, IL-7, IL-15 and GM-CSF remained stable in Patient A15 but increased in Patient A14.

IL-7 is essential for intestinal T cell proliferation and mediates homeostasis to maintain a stable number of native memory T cells (1153). In active UC, decreased colonic expression of IL-7 was seen compared to control and UC patients in remission (1154). Similarly, lower serum concentrations were seen in active CD patients (1155) and in children with active IBD compared to those in remission (1156). However, the raised IL-7 observed in Patient A14 may reflect continuing T cell proliferation with ongoing inflammation. Similarly, IL-15 is essential for T cell homeostasis and induces T cell recruitment to sites of inflammation thus can be detrimental when raised with dysregulation (1157) (Section 5.6.1.1). This may be an explanation for the ongoing increase in IL-15 observed in Patient A14.
**GM-CSF** promotes intestinal homeostasis by maintaining mucosal barrier, facilitating bacterial killing and regulating myelopoiesis. It promotes macrophage, DC and neutrophil recruitment, activation, survival and maturation. In addition, GM-CSF plays a key role in mucosal healing by increasing intestinal epithelial cell proliferation and survival thereby facilitating wound healing (1158). GM-CSF and its receptor CD116 were lower in IBD compared to controls, with the CD116 decrease being more prominent in UC than CD, independent of disease activity and irrespective of treatment (1159). The increased concentration observed in Patient A14 may reflect the inflammatory and/or healing processes taking place.

Interestingly, VCAM-1 and ICAM-1 concentrations were high and increased further despite Patient A15 being in remission. This is unlike the Prednisolone and Infliximab groups suggesting that EEN does not alter the vascular component in the resolution of inflammation, unlike the other treatments. Appreciating the dynamic variations of biomarkers in disease activity and resolution, these adhesion molecules may be the later mediators to normalise in CD, or the first to indicate onset of relapse.

### 5.6.2.2 Prednisolone

In the 2 UC patients who received Prednisolone, pro-inflammatory cytokines TNF-α and IL-17 decreased with response to treatment. This was also the case with IL-12p40, which is a subunit for IL-12 (Th1) and IL-23 (Th17). In addition, concentrations of IL-8 and IL-16 also declined. Similarly vascular mediators VEGF, VCAM-1 and ICAM-1 were seen to decrease in both patients. These suggest that inflammation was resolving with reduced recruitment of immune/inflammatory cells.

Patient A8 improved with Prednisolone but remained in active disease (ESR 60-41 mm/hr). In this patient, IL-5, GM-CSF and SAA continued to rise while a reduction was seen in Patient A21 (ESR 41-8 mm/hr), who achieved clinical remission. This suggests ongoing Th2 inflammatory responses with recruitment of inflammatory cells and the process of mucosal healing taking place.

In contrast, IL-7 remained largely unchanged and IL-15 increased in Patient A21, while both decreased in Patient A8, reflecting dysregulated T cell homeostasis in UC.
5.6.2.3 Infliximab

The effects of Infliximab were somewhat challenging to conclude due to the heterogeneous group. There were 3 CD patients who were treated with Infliximab; Patient D2 did not respond to treatment while Patient D6 initially responded to treatment. In both these patients, disease activity was not reflected in the ESR measurements (D2 ESR 8-15 mm/hr, D6 ESR 2 mm/hr pre- and post-treatment). On the other hand, clinical improvement was seen in Patient D4 (ESR 38-16 mm/hr). The UC patient who received Infliximab, however, remained in active disease (ESR 10-36 mm/hr) but later responded to ongoing treatment. Analysis of this one UC patient on Infliximab was limited.

In all these patients, TNF-α, IL-12p40, IL-17 and IFN-γ were directly related to ESR, reducing as inflammation improved or resolved. GM-CSF was relatively unchanged. In UC, this was also the case with IL-5, IL-8 and IL-15 suggesting that these cytokines did not have a major role in the disease process in these patients. The decreased IL-7 observed perhaps indicates lessening T cell proliferation or reflects disease activity (1153, 1155, 1156). IL-16 increased then decreased, suggesting reduced CD4+ cell recruitment and production of pro-inflammatory cytokines in UC.

In UC, VCAM-1 and VEGF initially increased then decreased despite the rise in ESR. As this patient later achieved clinical remission, this decrease perhaps indicated resolution was taking place. SAA and ICAM-1 increased and then plateaued with ongoing active disease, indicating continuing inflammatory processes with recruitment of inflammatory cells.

In CD, although IL-8 was relatively unchanged, it increased with a rise in ESR in Patient D2, perhaps indicating increased neutrophil recruitment. Contradicting trends were seen between IL-5 and ESR; it was directly related in Patient D4 but inversely related in Patient D2 (who continued in active disease) and unchanged in Patient D6. The role of IL-5 in Patient D2 is unclear here. Similarly, IL-15 remained stable in Patient D4 (with ESR reduction and clinical improvement), decreased in Patient D6 (correlating with clinical improvement reported) and increased with the higher ESR in Patient D2, reflecting changes in T cell recruitment, proliferation and production of pro-inflammatory cytokines.

In addition, IL-7 was generally inversely related to ESR, remaining elevated in Patient D2, who failed to respond to treatment. Perhaps with ongoing inflammation, IL-7 increases with intestinal T cell proliferation. IL-16 was directly related to ESR or clinical
response in CD. The difference in IL-16 changes between CD and UC suggests different roles in disease pathogenesis.

In CD, changes in VEGF, SAA, VCAM-1 and ICAM-1 was directly related to ESR with individual variations seen. This perhaps suggests different degrees of vascular involvement in the disease process of different individuals. Together, these changes reflect the ongoing inflammatory processes taking place during active disease and resolution as inflammation settles.

It thus appears that pro-inflammatory cytokines generally mirror the changes in inflammatory markers. An exception to this was when the inflammatory markers did not reflect true disease activity. This highlights that systemic markers may not always reflect disease activity.

In addition, some cytokines remained elevated or increased further despite reduction in inflammatory markers in patients who had ongoing active disease. These were IL-7, IL-15, IL-16, IFN-γ and GM-CSF in CD patients receiving EEN, and IL-7 in a CD patient receiving Infliximab. In UC, IL-5 remained elevated and GM-CSF increased in Patient A8, who continued to have active disease. Thus, these cytokines may be more reflective of disease activity, indicating ongoing inflammatory responses. These may be the cytokines and mediators to “switch off” later. In addition, these cytokines may allude to the different mechanisms of action between the treatments.

5.6.4 Potential Association between SCFA with Cytokines and Vascular Mediators

It is known that intestinal bacteria interacts with the host, stimulating immune responses. It is less clear if their metabolites, specifically SCFA, also interacts similarly with the host. It was beyond the scope of this study to assess this possibility at molecular level but potential associations were assessed.

Firstly, potential associations were analysed for IBD patients (n=16). A negative correlation was seen between TNF-β and acetate, whilst a positive correlation was seen with propionate and a positive trend with butyrate. TNF-β is known to play a role in cell cytotoxicity and induces the expression of ICAM-1. Paired samples of the same patient in active disease and remission revealed that higher TNF-β concentrations were
associated with active disease as well as lower acetate, but higher propionate and butyrate percentages.

The importance of butyrate in the GI tract is well recognised as discussed in Chapter 4. Here, a positive trend was seen with IL-10, consistent with the observation that butyrate enhance IL-10 production (748). In addition, a positive correlation was observed with IL-5. These cytokines and butyrate are known to be higher in inflammation thus reflecting concurrent inflammatory responses which may or may not be associated with each other.

5.6.4.1 Crohn's Disease

There were no particular trends observed between cytokines and vascular mediators with SCFA in CD. The only significant correlation with vascular mediators was seen between VEGF and propionate in Patient D2, who commenced Infliximab. Higher VEGF would be expected in active disease, and indeed a positive trend was seen in IBD between VEGF and propionate. However, it was not possible to firmly conclude that VEGF was highest during active disease in this patient, given that the same wPCDAI was associated with the lowest and highest VEGF concentrations. Despite this, the wPCDAI rather than inflammatory markers may have been more reflective of disease activity in this patient.

5.6.4.2 Ulcerative Colitis

Despite the smaller numbers, trends of associations were seen in UC patients. A negative trend was seen between VEGF with acetate. Similarly, there was a negative association between IFN-γ and IL-17 with propionate. However, paired samples from a patient in active disease and remission suggested increased propionate and biomarkers with inflammation. In addition, IL-8 appeared to be positively associated with butyrate percentages.

As with the combined IBD group, a positive trend was seen between TNF-β and butyrate. A paired sample from a patient in active disease and in remission demonstrated reduction in butyrate percentages with decrease TNF-β concentrations in remission supporting the trend for higher butyrate in active disease, as discussed in Chapter 4.
Due to the small number, statistically significant p values were not achieved despite $R^2$ above 0.9. At present, it is not possible to conclude that these changes are associated with one another or simply reflecting concurrent inflammatory processes or bacterial population.

### 5.6.6 Cytokine and Vascular Profiles of Non-Inflammatory Control Patients

Although NIC patients did not have IBD, they reported GI symptoms severe enough to warrant endoscopic investigations for the possibility of IBD. Measurements of cytokines and vascular mediators from blood samples obtained prior to endoscopic assessment were available and compared to HC. Statistical analysis was only possible for IBS and food allergies groups, not the JIA group ($n=2$). The trends were similar in the JIA patients, except the markedly raised SAA ($4.82 \times 10^7$, $3.06 \times 10^8$). Raised SAA has been reported in JIA, with concentrations correlating to disease activity (1160).

As IBS and food allergy are also inflammatory conditions, albeit milder, some of the pro-inflammatory cytokines were also raised. Although IL-12p70 was raised in all patient groups, it was significantly higher in IBS and food allergies only. In addition, patients with IBS also had raised IL-17, as did the IBD patients. These suggest IL-12 and IL-17 involvement in these conditions. This is consistent with previously reported increased IL-12 in children and adults with these conditions (1161-1163). As with IBD, IL-17 has also been recognised to play a role in atopy, specifically increasing asthma severity and eosinophilia (1164). Similarly, GM-CSF was reduced in IBS and food allergies as well as IBD (active and remission) thus alluding to the dysregulated homeostatic functions within the intestine.

TNF-α was not significantly increased in these patients, with it being significant only in active IBD. However, raised TNF-α has been reported in IBS and food allergies, in addition to increased IL-12, TGF-β, IL-6 and IL-1β in children and adults (1161-1163). In particular, frequent stoolsing, associated pain and anxiety were reported in those with higher TNF-α (1163).

Similarly, no differences were seen with IL-5 although there was a trend for lower concentrations in IBS. Lower concentrations were reported by Chira et al. although Vara et al. found raised IL-5 concentrations (1165, 1166).
The vascular profile in IBS and food allergies were similar to IBD. VCAM-1 concentrations were higher in IBS and food allergies, as it was in active IBD. Although VEGF concentrations were raised in HC, this may be a technical error with the HC samples being measured as a separate batch (as discussed in Section 5.6.1.2).

5.6.7 Study Limitations

As with the previous data sets, the small numbers in each cohort limited statistical analysis. From this small pool, there were samples obtained in active disease and in remission in a few of the individuals. The number of paired plasma measurements and stool SCFA were smaller still, mostly due to stool samples being more challenging to obtain. This, however, enable for the data to be thoroughly exploited thus providing a basis for future studies incorporating larger cohorts to confirm these findings.

Many of the patient samples collected were during their hospital reviews when unwell, often with active disease. When well, many of the patients were followed up at their local secondary care hospitals thus samples were not available for research. This has resulted in patient data skewed towards inflammation.

In terms of the laboratory methods, MSD assays are not equally sensitive at detecting and measuring all the cytokines in the panel. As mentioned, a number of cytokines were undetected despite likely being raised in IBD. It thus raises the question of whether the concentrations obtained were the actual circulating concentrations in the patients.

In addition, there are no published ranges of plasma cytokines and vascular mediators in healthy children using this MSD kit. Reported concentrations of these biomarkers are mostly measured in serum (Appendix 5B). In addition, these reports demonstrate age-related changes in cytokine concentrations, thus is another consideration when analysing systemic cytokines in the paediatric population.

The data on HC used for this study were obtained from adolescents recruited for another study thus not ideally matched for age. Although the HC were reported to be healthy with no GI symptoms, some of the cytokines and vascular mediators suggested that there may be at least an inter-current illness at the time of sampling. These samples were also analysed using a different batch of MSD kit; variations are known to occur between
batches. This may explain unexpected results such as the high VEGF measurements in this group.

5.6.8 Clinical Implications

Although the significant differences and trends observed will need verification with larger cohorts, it is tempting to consider some of the measurements for clinical use. This is especially as routine inflammatory markers i.e. CRP and ESR do not always reflect disease activity nor predict relapse/remission, and disease activity scores rely on honest reports of symptoms and subject to recall bias. Although stool calprotectin concentrations are helpful, stool samples are not always forthcoming, especially from adolescent patients.

Results from the cytokine analysis with treatment suggest specific cytokines that better reflect disease activity (Section 5.6.2). In CD, these cytokines were IL-7, IL-15, IL-16, IFN-γ and GM-CSF (EEN), and IL-7 (Infliximab). Similarly, IL-5 and GM-CSF may be better markers for monitoring disease activity in UC. These cytokines remained elevated in active disease despite improving ESR or CRP thus may be used in assisting with treatment decisions and disease monitoring. In addition, VCAM-1 and ICAM-1 continued to increase in early remission, perhaps being the later mediators to normalise in CD with EEN, or the first to indicate onset of relapse. This would require further clarification with a bigger cohort.

There are times when ambiguity on IBD diagnosis is encountered. These patients are thus diagnosed with IBDU. As discussed above (Section 5.6.1.1), there were clear differences in IFN-γ and ratios of IFN-γ:IL-10 and IL-17:IFN-γ, between CD and UC, thus potentially offering another diagnostic tool for differentiating between CD and UC.

The use of biomarker ratios in the clinical setting was suggested by Acarturk et al. They reported a study (66 IBD patients and 41 HC) on neutrophil-to-lymphocyte ratios as a predictor of disease severity in CD and UC. Higher values were obtained from those in active disease, with an optimum ratio threshold of 3.2 for active CD and 3.1 for UC (1167). Similarly, cytokine ratios can be utilised and may offer a more sensitive measurement thus worthy of consideration.
Results from the vascular mediator analysis highlights the crucial role of intestinal microvascular in inflammation. Increased awareness of this may initiate further anti-angiogenic agents targeting this aspect of IBD.

5.6.9 Conclusion

Measurements of cytokines and vascular mediators provide an insight into the inflammatory responses occurring in these patients. Despite the limitations of these analyses, clear trends in addition to some significant differences were demonstrated, all of which require confirmation with larger cohorts. Raised pro-inflammatory markers observed were consistent with published reports. In addition, there were also biomarkers identified that may be better indicators of disease activity, thus potentially are novel tools for disease monitoring. As we move towards personalised medicine, these biomarkers, which appear to better reflect disease processes, could be incorporated into the clinical setting.

The study analysis included associations between SCFA with immune and vascular mediators. Despite the limitations of numbers and samples available for analysis, the trends observed between cytokines and SCFA suggests links between microbial metabolites and host immune axis.
6. Chapter 6

Summary and Conclusion

6.1 Study Background

IBD encompasses diseases of different pathogenesis. Genetic and environmental factors play a crucial role in shaping the colonising intestinal microbiota in early childhood, which in turn primes the immune system for life. An event then triggers the onset of disease. Currently, IBD is classified mainly as CD or UC depending on histological findings, with two recommended guidelines for managing these overlapping and yet heterogeneous conditions. There are strong arguments for personalised management of these patients.

Research into intestinal microbiota has moved from association to causative relationships, from reporting of colonising microbes to functional consequences of dysbiosis. This study set out to investigate the microbial alterations with treatment and disease activity. Microbes influence host physiology partly through its metabolites, thus changes in SCFA were also investigated. In addition, host responses to these changes were analysed. At the start of this study, there were no published reports investigating all three parameters in the same cohort of children with IBD.

IBD patients recruited pre-treatment or soon after starting treatment were phenotyped and samples were collected prospectively. A cohort of HC were also recruited to enable comparison. Concurrently, patients with related rare conditions, offered additional perspective to the comparison between HC and IBD patients. As with most paediatric
studies, recruiting enough patients for statistical analysis was recognised as a challenge thus patients were followed up and samples collected prospectively, enabling more in-depth analysis.

6.2 Summary of the Main Findings

6.2.1 Clinical Factors in IBD

Detailed clinical history assisted interpretation of laboratory results and added another dimension to the study findings. In addition, risk factors for IBD were identified. Although the ages of first antibiotic exposure were similarly early, IBD patients had many more courses in early childhood than HC. As previously reported, this study also found that breastfeeding protects against IBD, with longer duration and greater quantities being more beneficial, even if formula milk was also offered alongside breastmilk. Weaning practices also differed between HC and IBD patients, with some IBD patients being weaned too early or too late. In addition, the quality of food may be a factor as more HC received home-cooked food rather than commercially produced food.

At the time of recruitment, nutritional assessments were also performed on HC and IBD patients. IBD patients had reduced macro- and micronutrient intake. This was associated with reduced intake of plant fibres, which could have preceded IBD development or self-imposed by the patients to reduce the symptoms of IBD. In addition, there were more IBD patients reporting food intolerances/allergies suggesting existing barrier dysfunction earlier in childhood, prior to developing IBD.

6.2.2 Intestinal Microbiota Profiles in IBD

In this study, microbial dysbiosis in IBD was defined by identifying deviations from HC and NIC. Comparison with other diseases enabled further clarification of the results. Differences between CD and UC, as well as between active disease and remission, were identified. Variations were also observed with disease severity, and with treatment. Interestingly, patients with CD who also had other GI diagnoses had microbial profiles that differed from the typical CD profile.

On immunosuppressive treatment, dysbiosis in both the stool and mucosal biopsies lessened, suggesting improved bacterial handling associated with reduction in
inflammation. EEN, however, is known to significantly reduce the microbial load, promotes the expansion of *Lactobacillus* and creates a favourable environment for recolonization with more beneficial microbes, altering the dysbiotic profile to one that promotes health.

Further analysis was also conducted on alpha and beta diversities, both of which were consistent with published reports (659, 669). This study also found positive trend of association between calprotectin and Proteobacteria, and a negative trend of association with Bacteroidetes suggesting their different effects on intestinal inflammation. Following on from this, Bacteroidetes:Proteobacteria and Firmicutes:Proteobacteria ratios were analysed and found to be lower in remission compared to HC, and lower still in active disease.

Another important finding was that stool and colonic mucosal profiles differed in IBD and in some NIC patients. A less dysbiotic stool profile may in fact be associated with significantly dysbiotic mucosal profile. These findings highlight the intestinal mucosal barrier dysfunction associated with IBD, unlike other diseases with GI symptoms such as JIA and CAPS.

### 6.2.3 SCFA Profiles in IBD

To investigate the functional consequences of dysbiosis in IBD, the stool SCFA were also analysed. As there were limited data on SCFA profiles in healthy children, a local cohort was recruited to minimise cultural variations in diet and environment, enabling genuine differences to be identified.

Although the total SCFA concentrations were similar between the groups, ratios of acetate, propionate and butyrate revealed age-dependent differences. This likely reflects the variations in the microbial profiles and perhaps differences in metabolic requirements, with infant having higher energy demands.

Utilising the SCFA concentrations and ratios of acetate:propionate:butyrate of HC, comparison was made with disease groups. Differences were also apparent between NIC, CD and UC patients. Given that NIC patients were also in an inflammatory state, albeit milder, the higher propionate and butyrate may be an appropriate response to inflammation. In active IBD, there was a trend for higher butyrate but the proportions
were not as high as in NIC. This suggests inadequate production or increased utilisation with inflammation. With inflammation in IBD, Proteobacteria increased while Bacteroidetes decreased, therefore potentially reducing the capacity for butyrate production. In remission, butyrate proportions were comparable to HC despite the higher abundance of Bacteroidetes. In addition, alterations with treatment reflected disease activity with increased butyrate in inflammation and its reduction with remission.

Proportions of propionate and butyrate appeared to differentiate CD from UC. Propionate was higher in CD (in active disease and remission) compared to UC. This was associated with dominance of Bacteroidetes in the stool of active CD patients, with both Bacteroidetes and Firmicutes dominating in remission. On the other hand, the higher proportion of butyrate in active UC, compared to UC remission and CD (in active disease and remission), was associated with dominance of Firmicutes in the stool of patients with active UC.

Correlating SCFA with calprotectin, differences were observed between CD and UC alluding to the differences in pathogenesis. In CD, the total SCFA and butyrate percentages were positively associated with calprotectin, whilst in UC, trends of associations were observed between the total SCFA and butyrate with calprotectin.

Analysis of the study results has shed light on the pathogenesis of UC. Roedinger et al. proposed UC as an energy-deficient disease, explaining the histological features and disease distribution (830). An inability to utilise the substrate was thought to be the cause. This study found lower Bacteroidetes and a trend of increased butyrate in active UC, suggesting increased production but still insufficient, especially given the state of increased energy demands with inflammation. Results by Breuer et al. however, would suggest that this energy deficient state can be reversed if sufficient SCFA is given for long enough to enable mucosal healing and repair (846).

The SCFA profiles of other IBD subtypes also correlated with the microbial profiles. IBDU patients had similar SCFA and microbial profiles to UC. In a patient with CD and Coeliac Disease, the higher total SCFA concentrations were more typical of Coeliac Disease, although the acetate:propionate:butyrate ratios were similar to other CD patients. This was associated with higher Firmicutes in the stool compared to other CD patients. Despite significant systemic inflammation, the patient with Granulomatous Polyangiitis had microbial and SCFA profiles similar to NIC while both the profiles of CAPS patients were similar to HC. The lower total SCFA concentrations in ileostomy samples was associated with reduced bacterial diversity and relative abundance. The SCFA produced
consisted mostly of acetate, and was associated with dominance of Proteobacteria in one patient, and Bacteroidetes and Firmicutes in another.

6.2.4 Cytokines and Vascular Biomarkers in IBD

It was recognised that current biochemical markers of inflammation do not always reflect intestinal inflammation, making them poor predictors of relapse or remission. In this study, raised pro-inflammatory cytokines in IBD were observed, as have been previously reported (1129, 1135, 1168). The significant role of vascular factors in IBD was highlighted.

In CD, with EEN treatment, IL-7, IL-15, IL-16, IFN-γ and GM-CSF may be more sensitive markers of disease activity thus may be more helpful in monitoring disease. Similarly, IL-17 with Infliximab in CD, and IL-5 and GM-CSF with Prednisolone in UC may be potential biomarkers for disease monitoring. In addition, IFN-γ, IFN-γ:IL-10 and IL-17: IFN-γ ratios differentiated CD from UC thus may be a useful diagnostic tool.

This study also confirmed that patients with IBS and food allergies are in a chronic inflammatory state thus are not ideal as a comparison group to IBD patients.

Correlation with SCFA was also demonstrated. TNF-beta was negatively associated with acetate but positively associated with propionate and butyrate in IBD. In addition, butyrate was found to positively correlate with IL-5. In UC, negative trends of associations were observed between propionate and IL-17 as well as IFN-γ. In individual CD patients, a positive trend of association was observed between IL-10 and butyrate.

There were also trends observed with vascular biomarkers. In IBD, negative trends were observed between VCAM-1 and butyrate, and between VEGF and acetate. VEGF was also negatively associated with acetate in UC and propionate in an individual patient with CD and Coeliac Disease. These could be trends of association or concurrent responses to inflammation.

Therefore, intestinal microbiota plays a significant role in IBD pathogenesis, by influencing host immunity, potentially via its production of SCFA. IBD treatment ameliorates inflammation via two mechanisms. The immunosuppressive medications reduce the inflammatory responses including vascular mediators VCAM-1 and ICAM-1,
unlike EEN. This allows for mucosal healing and thereby repair of the intestinal barrier to take place. Although effective, these drugs have associated side effects.

Another mechanism of treatment is by altering the intestinal luminal environment and microbial composition to increase the abundance of symbiotic microbes and reduce pathogens. This is commonly achieved with EEN in CD, experimentally with FMT, probiotics alongside conventional medications and rarely abstinence from enteral intake (supplemented with PN). In addition, some of the drugs used in IBD treatment such as Sulfasalazine also influences intestinal microbiota (1169). As our understanding and experience of these treatments gather pace, its use and application can be extended and refined.

6.2.5 Overall Summary

Intestinal microbiota and the SCFA produced influence the host in a number of ways in health and disease. This study investigated the factors affecting the intestinal microbiota and the SCFA it produces in IBD (Figure 6.1). In doing so, the luminal and mucosal microbial profiles as well as SCFA profiles for HC, NIC and IBD patients were characterised. The effects of treatment and disease severity on these profiles were subsequently analysed in CD and UC patients. This study also investigated potential associations between the microbial metabolites SCFA and systemic inflammatory and vascular profiles. Data from this study has highlighted 2 mechanisms of achieving remission with intestinal mucosal healing, by modulating the immune responses and microbial composition (Figure 6.2). Therefore, IBD management should address all aspects known to influence the disease. The management can be classified into 3 strategies, utilising immune modulators, microbial modulators and psychological therapy (Figure 6.3).

Limited by the small numbers of patients, many of the results would benefit from confirmation with larger cohorts. Regardless of this, analysis from this study has extended our understanding of UC pathogenesis and the mechanism of action of EEN. In addition, potential biomarkers have been identified for IBD diagnosis and disease monitoring.

There is now sufficient evidence for the manipulation of intestinal microbiota in managing IBD. The time is now ripe for its use in the clinical setting, as we move towards
personalised care. However, robust systems and clear guidance are needed for this to occur.

**Figure 6.1: Onset of Inflammatory Bowel Disease**

Various environmental exposures during early childhood and genotype shape the individual intestinal microbiota and programmes the immune system. An infectious or stressful episode triggers the onset of IBD. Potential preventative measures ought to be considered. *Discussed in this study*
The aim of IBD treatment is mucosal healing, which can be achieved by reducing the dysregulated immune responses (1) and favourably altering the dysbiotic luminal microbial composition (2).

IBD management ought to address the dysregulated immune system (with immune modulators), microbial dysbiosis and psychological stress. Microbial composition can be manipulated with the diet, EEN as induction therapy or regularly (in CD), probiotics and SCFA enemas (in UC). In addition, psychological wellbeing can be addressed through cognitive behavioural therapy (CBT), group or family therapy. Family dynamics may change with the diagnosis of IBD thus may be a stressful factor that could benefit from intervention and/or support.
6.3 Study Limitations

The small cohorts of this study have undoubtedly been a disadvantage, making it challenging to achieve statistical significance and to draw firm conclusions. Despite these small numbers, some of the results were significant. In addition, clear trends and associations were observed in a number of the results. In some ways, having small cohorts was a benefit as it enabled in-depth analysis of each patient/child from different perspectives and longitudinally.

Every effort was made to match the HC to IBD patients; however, it was not possible to recruit ethnicity-matched children and young adults in addition to sex- and age-matched controls. This was considered, as there may be differences in their diets.

There is a variety of methods used in microbiota research. As a result, it was not ideal to combine this data set with results published by other groups, or make direct comparison with their results. Given the small number of patients in the study, this was considered but felt to be inappropriate. These is an urgent need to standardise and align the various steps involved in microbiota analysis. This is particularly important in Paediatrics, given the challenges and restrictions in recruiting children for research studies.

For the microbiota analysis, due to restricted access to the freeze-drier, two methods of concentrating the duodenal lavages were used. In addition, the duodenal biopsies were analysed with samples of greater bacterial abundance i.e. the stool and lower GI mucosal biopsies, resulting in many of the samples being discarded due to sequence reads of <7,000.

For the SCFA analysis, two gas chromatography machines were used. This was controlled for and did not appear to affect the results, however, it would have been better avoided.

For the analysis of cytokines and vascular biomarkers, it was not possible to obtain age-matched HC due to ethical constraints. Blood samples were therefore received from adolescents and young adults, a few of whom likely had an inter-current illness that was reflected in the biomarkers measured. These HC were actually recruited for another study and the samples were analysed using a different batch of MSD kits, which may have explained some of the unexpected results. In addition, blood samples from IBD patients were received when venepuncture occurred for clinical investigations, usually when unwell thus there were fewer samples during remission.
6.4 Clinical Implications

There are a number of results from this study that has potential for clinical use but would require validation and/or confirmation with larger cohorts.

1. IBD Diagnosis and Disease Monitoring
Alterations in the microbiota and its SCFA in IBD is a dynamic process, shifting with environmental influences and perhaps reactive to the health status of the host in a symbiotic manner. The profiles observed are therefore snapshots of these processes, at that time, much like the clinical measurements of ESR and calprotectin. On the other hand, microbial and SCFA profiles offer insight into the events taking place at the mucosal surface, unlike measurements such as ESR. Therefore longitudinal profiles, as observed in this study, are much like jigsaw pieces that help us understand the molecular processes occurring. With this in mind, microbial and SCFA profiles may be utilised in disease monitoring. Also, with the differences observed between HC and IBD, and between CD and UC, it could be used as a diagnostic tool. In addition, as variations were observed with disease severity, these profiles could be used to tailor treatment management on an individual basis.

From the results of this study, there were specific cytokines and vascular biomarkers that may be more sensitive for disease monitoring. In addition, there were cytokines that could be utilised to differentiate between CD and UC, as discussed above.

2. The Clinical Use of Butyrate Enemas
This study reiterated the important role of butyrate in intestinal inflammation. Its routine use in those with defunctioned colon/rectum may prevent or ameliorate diversion colitis. This could be administered rectally or via a mucus fistula of the proximal defunctioned colon.

Butyrate is of particular importance in UC, therefore its administration rectally ought to be considered alongside concurrent conventional treatment. In addition, its administration with FMT may improve the success rates.

3. Manipulation of the Intestinal Microbiota through Alterations in the Diet
It is possible that disease severity can be lessened by manipulating the intestinal microbiota, thereby potentially reducing the need for biological therapy and/or reduce medication doses required for therapeutic effect. This could have the added benefit of minimising the exposure to the side effects and complications of these medications.
Regular dietetic input after diagnosis would be labour-intensive but the returns in terms of health and wellbeing would be invaluable. Encouraging and supporting a diet with suitable intake of plant fibres, sugars and fat would require input from a dietitian experienced in child nutrition. A dietetic change to a healthier one would not only alter the microbial profile favourably but also the SCFA production. There is little evidence for prebiotics in IBD (1170), but this may be an alternative way of increasing SCFA production.

There is, however, reasonable evidence for the use of probiotics in IBD (69, 474, 1170) but it remains a supplement to the recommended medications. Disease specific fourth generation probiotics are being developed but until such time, more regular used of butyrate-producing probiotics may benefit patients with IBD.

In addition, routine use of probiotics after a course of antibiotics may limit the period of dysbiosis. As Bacteroidetes appear to be particularly sensitive to antibiotics, this could be of particular benefit in early childhood. Although the probiotics would incur additional costs to the NHS, the longer-term benefits could far outweigh this.

4. The use of EEN to reset the Intestinal Microbiota as Treatment in other Conditions
Dysbiosis is reported in a number of diseases, and altering it to a healthy profile has been associated with improved health (1171, 1172). Convincing examples of this are EEN in IBD and FMT in refractory *C. difficile* infection. Although FMT produces dramatic results rapidly, its longer-term effects and practicalities are still issues to be addressed. EEN, on the other hand, is able to reverse severe dysbiosis and inflammation with minimal, if any adverse events. Its use in other conditions such as IBS ought to be investigated. Following such treatment, it is important that the healthy microbial profile is then maintained with a healthy diet and lifestyle.

5. Early Identification of Genetic Mutations
As it is becoming increasingly apparent that the genotype and environmental factors in early childhood are critical for the developing immune system, altering the microbiota during this period appears to modulate disease risk (159, 160, 572, 579). With this in mind, if adult IBD patients were able to test their new-born offspring for the same IBD mutation, it may influence their feeding and child-rearing practices e.g. more likely to breastfeed and for longer, more likely to offer home-cooked weaning food, more fruit and vegetable, more outdoor activities and more cautious with antibiotic administration.
6.5 Research Implications

This study has highlighted 2 points that may be worth considering in future studies on the intestinal microbiota;

1. Limitations of NIC as a Control Group
In the field of IBD research, patients with IBS are sometimes used as a control group. This study has demonstrated how these patients differ from HC and IBD patients in terms of their microbial, SCFA and immune profiles. These ought to be considered when comparisons are made during analyses.

2. Differences in Microbial Profiles of Stool and Intestinal Mucosal Biopsies
Being a disease predominantly affecting intestinal mucosa, intestinal mucosal biopsies and stool samples collected at the same time can have different microbial profiles in IBD. Therefore, stool microbial profiles cannot be assumed to represent intestinal mucosal biopsies.

3. Alterations of Microbial Profiles with Treatment
There were limited stool and mucosal samples collected from the same treatment-naïve patients who then achieved remission or improved. Pre- and post-treatment microbial profiles suggest less dysbiosis in the stool and mucosa with treatment. This raises the question of whether microbial profiles reliably reflect disease activity in IBD patients on immunosuppressive treatment.

6.6 Future Research

With existing data from this study, further analysis is planned to investigate alterations of microbial profiles with treatment and disease severity at lower taxa i.e. at family and genus levels. The Microbial Dysbiosis calculation would then be possible In addition, remaining samples could potentially be utilised to investigate other aspects of IBD.

Whilst this study has added to our understanding of the microbiota in IBD, it also raises some questions that could be further explored.
1. **The Gut-Brain Axis in IBD**

Since this study began, research into the gut-brain axis in the context of intestinal microbiota has advanced. Many of the IBD patients in this study reported emotional or psychological stress as a potential disease trigger. Stress has been reported to alter the microbiota, influencing the expression and course of diseases (352, 1173-1176). Given the profound effects it has on IBD patients, this area warrants further research.

2. **The Virome and Mycobiome in IBD**

The virome and mycobiome are part of the intestinal commensal community, exerting its influence in the functioning of its host. It is thus important that this aspect of IBD is further investigated.

3. **Valerate and Caproate in IBD**

The differences observed in valerate and caproate between HC and IBD patients may be important but little is known about these SCFA to understand the significance of this.

6.7 **Final Conclusion**

The intestinal microbiota is a dynamic entity, shifting with environmental and host factors. As part of the symbiotic relationship, the microbiota assists the host in various physiological functions in return for a suitable environment and substrates. Its active metabolites, such as SCFA, play an active role in this symbiosis.

In IBD, the microbiota and its metabolites are significantly involved in the pathogenesis, altering with disease activity and severity. Treatment appears to influence bacterial handling, resulting in differences in luminal and mucosal microbial profiles with IBD treatment.

The clinical and research implications discussed above could have wide-ranging impact if given further consideration. Each patient is unique and deserves treatment tailored to their clinical needs. The results of this study could bring children with IBD a step closer to personalised management of their care, improving their health, development and wellbeing.
Appendices
### Clinical History and Dietetic Questionnaire

**Clinical History**

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<tr>
<th>Name</th>
<th>Date</th>
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<tr>
<td>DOB</td>
<td>Ref No</td>
</tr>
<tr>
<td>Hospital No</td>
<td></td>
</tr>
</tbody>
</table>

**Diagnosis**

- Crohn’s Disease
- Upper GI
- Ileum
- Colon
- Strictures
- Penetrating
- Ulcerative Colitis
- Proctitis
- Left
- Pielitis

Date diagnosed

**Treatment**

Current:

Previous:

**PMH**

**Conception**

- Normal
- Assisted

**Antenatal**

- Normal
- Antibiotics
- Steroids

Other medications

Complications

**Perinatal**

- Antibiotics
- Medications

**Birth**

- Normal
- LSCS: Spinal
- GA

**SCBU**

- Home Day___

**Gut**

- Constipation
- Diarrhoea
- Gastroenteritis
- GOR

- Colic
- Chest
- Wheeze

**Asthma**

- Eczema
- Hayfever
- Allergies

**ENT**

- Otitis media
- Hearing problems
- Tonsillitis

**CNS**

- Headaches
- Visual concerns
- Developmental milestones

**Other**

- Joint pains
- Wound healing good/poor
- Skin changes

Menarche ___ years

Voice mature ___ years
## Medications

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<th>Antibiotics</th>
<th>After birth</th>
<th>First week</th>
<th>First month</th>
<th>First 3 months</th>
<th>First 6 months</th>
<th>First 12 months</th>
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<th>First 5-10 years</th>
<th>First 10-15 years</th>
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<td>___/month</td>
<td>___/year</td>
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<td></td>
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</tr>
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<td>___/week</td>
<td>___/month</td>
<td>___/year</td>
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<td></td>
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<td>Incomplete</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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## Family History

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<td>U/R</td>
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<tr>
<td>Smokers</td>
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<td>Pets</td>
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<td>Family history of:</td>
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<tr>
<td>GI disease</td>
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</tr>
<tr>
<td>Autoimmune disease</td>
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<td>Atopy</td>
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# Dietetic History

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<th>First week</th>
<th>First month</th>
<th>First 3 months</th>
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<tbody>
<tr>
<td>Breast fed</td>
<td>First 6 months</td>
<td>First 12 months</td>
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</tr>
<tr>
<td>Weaning</td>
<td>Puree/Baby-led</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food</td>
<td></td>
<td></td>
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<tr>
<td>Hospital No</td>
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<table>
<thead>
<tr>
<th>Samples given</th>
<th>Blood</th>
<th>Biopsy</th>
<th>Duodenal lavage</th>
<th>Stool</th>
<th>Time:</th>
<th>Urine</th>
<th>Time:</th>
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</table>

Change in medication

Change in clinical status
**Appendix 2B - Paris Classification**

**Crohn’s Disease**

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<th>Parameters</th>
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<td>Age at Diagnosis</td>
<td>A1a: 0-&lt;10y&lt;br&gt;A1b: 10-&lt;17y&lt;br&gt;A2: 17-40y&lt;br&gt;A3: &gt;40y</td>
</tr>
<tr>
<td>Location</td>
<td>L1: Distal 1/3 ileum +/- limited caecal disease&lt;br&gt;L2: Colonic&lt;br&gt;L3: Ileocolonic&lt;br&gt;L4a: Upper disease proximal to Ligament of Treitz&lt;br&gt;L4b: Upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum</td>
</tr>
<tr>
<td>Behaviour</td>
<td>B1: Nonstricturing nonpenetrating&lt;br&gt;B2: Strictureing&lt;br&gt;B3: Penetrating&lt;br&gt;B2B3: Penetrating and structuring disease, either at the same or different times&lt;br&gt;P: Perianal disease modifier</td>
</tr>
<tr>
<td>Growth</td>
<td>G0: No evidence of growth delay&lt;br&gt;G1: Growth delay *</td>
</tr>
</tbody>
</table>

- Impaired linear growth as defined by at least one of the following criteria
  1. Height z-score at diagnosis or subsequently significantly less than expected height z-score
     a. Difference between observed height z-score and predicted height z-score using the ‘Mid-parental Heights’ formula is >2.0 OR
     b. Difference between observed height z-score and the pre-illness height z-score is >1.0
  2. Current height z-score significantly less than height z-score at diagnosis
     a. Reduction in height z-score since diagnosis is >0.75

**Ulcerative Colitis**

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</thead>
<tbody>
<tr>
<td>Extent</td>
<td>E1: Ulcerative proctitis&lt;br&gt;E2: Left-sided UC (distal to splenic flexure)&lt;br&gt;E3: Extensive (hepatic flexure distally)&lt;br&gt;E4: Pancolitis (proximal to hepatic flexure)</td>
</tr>
<tr>
<td>Severity</td>
<td>S0: Never severe&lt;br&gt;S1: Ever Severe (PUCAI &gt;65)</td>
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Appendix 3A – Primers used for DNA Library Preparation

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V3V4 read 1 5' - TAT GGT AAT TGG CCT ACG GGN GGC WGC AG - 3'
V3V4 read 2 5' - AGT CAG TCA GCC GGA CTA CHV GGG TWT CTA AT - 3'
Appendix 3B - Microbial Profiles of Individual Patient

The stool bacterial profiles of 10 HC, 5 CD (Patients A14, A15, A17, A25, D4 and D6), 3 UC (Patients A8, A21 and C1) and 2 IBDU (Patients A9 and D7) patients were analysed. Percentages of bacterial phyla, Bacteroidetes:Proteobacteria ratios (B:P ratios) and alpha diversity are presented. The red stars indicated active inflammation and the blue squares indicate remission.

Healthy Controls

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Crohn’s Disease

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Patient A11

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Azathioprine

EEN

Humira

Infliximab

Prednisolone | 35 | 20 |

wPCDAI     | 10 | 5  | 10  | 10  | 40  | 40  | 20   |

CRP mg/dL | <5 | <5 | <5 | <5 | <5 | <5 | <5   |

ESR mm/hr | 95 | 66 | 67  | 70  | 60  | 55  |

Calprotectinµg/g | 2919 | | 1766 | 3083 |
Appendix 4 - Changes in SCFA with Treatment and Clinical Status for Individual Patients

4.1 Patients with Newly-diagnosed Crohn’s Disease

In 2 recruited patients with CD, exclusive elemental nutrition (EEN) and Azathioprine was commenced after diagnosis. A sample was collected before treatment in Patient A17 and 2 weeks after treatment in Patient A15 (Appendix Figure 4.1), and further samples were collected prospectively thereafter.

A week after diagnosis, Patient A15 had diarrhoea and vomiting, and was diagnosed with Norovirus gastroenteritis. The patient was admitted for intravenous fluids for 2 days, and then gradually resumed EEN. Formed stools were noted 7 days after the start of the gastroenteritis. At the same time, the patient was also found to have a urinary tract infection for which a 5-day course of Trimethoprim was given. Interestingly, the butyrate concentration decreased (from 21 to 6 µmol/g, 7% to 1%) and propionate concentration increased (from 31 to 167 µmol/g, 10% to 32%) after the antibiotics course, despite the increased total SCFA (from 297 to 528 µmol/g). The butyrate concentration then increased (by 78 to 84 µmol/g, 1% to 15%) and the propionate concentration decreased (by 90 to 77 µmol/g, 13%) 3 months post-treatment. The patient continued EEN until 6 weeks post-diagnosis, after which time normal diet was reintroduced.

The subsequent decline in butyrate (by 45 to 39 µmol/g, 15% to 13%), propionate (by 21 to 56 µmol/g, 19%) and total SCFA (from 575 to 286 µmol/g) concentrations at 9 months post-treatment coincided with increased ESR (by 55 to 67 mm/hr), PCDAI score (by 15 to 20) and raised stool calprotectin (1614 µg/g). Thus, Infliximab was commenced for poor response to first-line treatment. The total SCFA concentrations had been stable over this period but then increased 2 months later (by 866 to 1152 µmol/g). At this point, at 11 months post-treatment, this increase was associated with a rise in butyrate (by 126 to 165 µmol/g, 13% to 14%) and propionate concentration but not the percentages (by 126 to 182 µmol/g, 16%), and coincided with a reduction in ESR (5 mm/hr) but increase in stool calprotectin (2471 µg/g). The PCDAI score was stable over these 2 months (20 and 25) then reduced further (to 10) 2 months later. The acetate percentages remained stable.

In Patient A17, a month after starting EEN, a rise in total SCFA (from 332 to 592 µmol/g), propionate (from 46 to 150 µmol/g, 14% to 25%) and butyrate (from 19 to 103 µmol/g,
6% to 17%) concentrations were seen (Appendix Figure 4.2). This coincided with a reduction in ESR (by 12 to 29 mm/hr). After completing 6 weeks of EEN, this patient continued having the feed as a supplement to his diet. After 3 months, the ESR reduced further (by 7 to 22 mm/hr). The total SCFA concentration remained stable but there were decreases in butyrate (by 49 to 54 µmol/g, 17% to 10%) and propionate (by 40 to 110 µmol/g, 21%) concentrations whilst the stool consistency remained formed. Although there was only 1 calprotectin level for this patient, it is useful to note that it was low at 3 months post-treatment (237 µg/g).
Appendix Figure 4.1: Variations in Total SCFA Concentrations and Individual SCFA Percentages with Clinical Markers in Newly-diagnosed Crohn’s Disease Patients (A15 and A17)

Stool SCFA were extracted and quantified using gas chromatography. Analysis was performed with GraphPad Prism (V5) in conjunction with clinical events and parameters, which were PCDAI scores, CRP (mg/dL), ESR (mm/hr) and calprotectin (µmol/g).
4.2 Patients with Crohn’s Disease commencing Infliximab

Infliximab is an anti-TNF agent infused every 6-8 weeks, after 3 induction doses, for treatment of moderate to severe CD. Patients D2 and D4 were recruited soon after starting Infliximab. Their total SCFA, propionate and butyrate concentrations are analysed in relation to clinical events (Appendix Figure 4.2).

Patient D2 was initially diagnosed with Coeliac Disease at less than a year of age, and CD was diagnosed at the age of 9 years. The patient was commenced on Azathioprine and Budesonide but despite an increased Azathioprine dose, the patient was unwell 3 months later but responded to Prednisolone. About a year post-diagnosis, Patient D2 had a relapse thus Infliximab was commenced with an increased Azathioprine dose.

The patient initially had good responses to the Infliximab doses, but these were short-lived (PCDAI score 20, ESR 8 mm/hr). There were associated increase in total SCFA (from 1040 to 1300 µmol/g), propionate (from 236 to 279 µmol/g, 23% to 21%) and butyrate (from 146 to 235 µmol/g, 14% to 18%) concentrations 2 months after starting Infliximab. This was followed by reductions in the total SCFA (by 851 to 449 µmol/g), propionate (by 186 to 93 µmol/g, 21%) and butyrate (by 161 to 74 µmol/g, 18% to 16%) concentrations a month later followed by a plateau in total SCFA (447 µmol/g) and propionate (89 µmol/g, 20%) concentrations and increase in butyrate concentration (by 24 to 98 µmol/g, 16% to 22%). During this time, however, the patient continued to have active disease as seen by the stool calprotectin (1178 µg/g), ESR (11-16 mm/hr) and PCDAI score (15-35). Pentasa was commenced 5 months post-Infliximab (total SCFA 491 µmol/g, decreased butyrate concentration 56 µmol/g and percentage 11%, and stable propionate concentration 95 µmol/g and percentage 19%).

At 9 months post-Infliximab, Patient D2 continued to have active disease with a rise in ESR (40 mm/hr), CRP (21 mg/dL), stool calprotectin (1556 µg/g) and PCDAI score (25). This was associated with a reduction in total SCFA concentration (by 153 to 680 µmol/g) and propionate (by 43 to 132 µmol/g, 19%) but a small rise in butyrate concentration (by 10 to 87 µmol/g, 9% to 13%). Double dose Infliximab was given but again, the effects were short-lived (PCDAI increased by 20 to 45) thus Infliximab was switched to Adalimumumab, another anti-TNF agent. Four months later, clinical improvement was seen with reductions in PCDAI score (by 5 to 20), ESR (by 25 to 15 mm/hr) and CRP (to <5 mg/dL). There were also decreased total SCFA (by 428 to 252 µmol/g), propionate (by 110 to 22 µmol/g, 9%) and butyrate (by 79 to 8 µmol/g, 13% to 3%) concentrations.
Acetate percentages mildly varied with the changes in propionate and butyrate percentages.

Patient D4 had a relapse of CD on maintenance Azathioprine 18 months after diagnosis (Appendix Figure 4.2). Induction of remission was attempted with Prednisolone but due to on-going symptoms, Infliximab was commenced and Prednisolone was weaned and stopped 3 months later. Clinically, there were initial improvements as seen by the PCDAI score (25), ESR (30 mm/hr) and CRP (9 mg/dL), although the stool calprotectin remained high (2949 µg/g). The total SCFA during this time was 260 µmol/g with the propionate and butyrate concentrations being 66 (25%) and 34 (13%) µmol/g respectively.

A viral infection a month later, at 4 months post-Infliximab, triggered another relapse. An increase in the concentration of total SCFA (by 552 to 812 µmol/g), propionate (by 115 to 181 µmol/g, 22%) and butyrate (by 89 to 122 µmol/g, 13% to 15%) were seen. At clinical review a month later, a rise in stool calprotectin (3809 µg/g), PCDAI score (by 25 to 50) and ESR (by 30 to 64 mm/hr) were seen. This was associated with increased butyrate concentration (by 17 to 140 µmol/g, 15 to 19%) despite a decrease in the total SCFA (by 69 to 743 µmol/g) and propionate (by 26 to 155 µmol/g, 21%) concentrations thus an increase in butyrate percentage. This was followed by subsequent clinical improvement, with reduction in ESR (by 32 to 32 mm/hr), stool calprotectin (by 3418 to 391 µg/g)) and PCDAI score (by 15 to 35), which was associated with a stable total SCFA (762 µmol/g) and propionate concentration (159 µmol/g, 21%) and reduced butyrate concentration (by 79 to 61 µmol/g, 19% to 8%) thus a reduction in butyrate percentage.
Appendix Figure 4.2: Variations in Total SCFA Concentrations and Individual SCFA Percentages with Clinical Markers in Crohn’s Disease Patients receiving Infliximab (D2 and D4)

Stool SCFA were extracted and quantified using gas chromatography. Analysis was performed with GraphPad Prism (V5) in conjunction with clinical events and parameters, which were PCDAI scores, CRP (mg/dL), ESR (mm/hr) and calprotectin (µmol/g).
4.3. A Patient with Fistulising Crohn’s Disease

Patient A25 was recruited at diagnosis (Appendix Figure 4.3) This patient was diagnosed with CD thus was initially treated with EEN and Azathioprine Two months after diagnosis, this patient was found to have perianal fistula, and Infliximab and Ciprofloxacain were thus commenced. The patient responded to treatment and a second stool sample collected 7 months after diagnosis was associated with clinical improvement (PCDAI 15 to 10). At that time, the total SCFA concentration was less (from 1449 to 869 µmol/g), as was the propionate (from 364 to 112 µmol/g, from 25% to 13%) and butyrate (from 221 to 70 µmol/g, 15% to 8%) concentration.

Appendix Figure 4.3: Variations in Total SCFA Concentrations and Individual SCFA Percentages with Clinical Markers in a Newly-diagnosed Fistulising Crohn’s Disease Patient (A25)
Stool SCFA quantified with gas chromatography were analysis with GraphPad Prism (V5) in conjunction with clinical events and parameters, which were PCDAI scores, CRP (mg/dL), ESR (mm/hr) and calprotectin (µmol/g).

4.4 Patients with Newly-diagnosed Ulcerative Colitis

Two patients with newly-diagnosed UC were recruited to the study. Stool samples were obtained 2 months post-treatment for Patient A8 and pre-treatment for Patient A21. Patients A8 and A21 were commenced on Prednisolone and Azathioprine after being diagnosed with Ulcerative Colitis.

Patient A8 was refractory to treatment, partly due to poor compliance thus had on-going inflammation (Appendix Figure 4.4). After gradual dose reduction over 2 months, the Prednisolone was stopped and Pentasa commenced. At the same time, Azathioprine dose was increased. A month later, the reduction in ESR (by 39 to 15 mm/hr) was associated with a reduction in total SCFA concentration (from 275 to 152 µmol/g), an increase in stool calprotectin (by 475 to 1320 µg/g), and stable PUCAI score (35 to 30), propionate (from 9 to 5 µg/g, from 3% to 4%) and butyrate concentrations (16 to 11 µmol/g, 6% to 7%). Due to on-going inflammation, the patient received a second course of Prednisolone 6 months post-diagnosis. Unfortunately, the next sample was obtained at 14 months post-diagnosis. During this period, the patient remained in active disease with loose stools and raised ESR (22 mm/hr) and PUCAI score (35), however increased total SCFA (by 334 to 486 µmol/g), propionate (from 5 to 34 µmol/g, from 4% to 7%) and butyrate (by 37 to 48 µmol/g, 7% to 10%) concentrations were seen. Acetate percentages remained stable. No further samples were received but this patient was commenced on Infliximab 2 months later due to on-going active disease.

Patient A21 was very unwell at diagnosis (Appendix Figure 4.4). This patient responded well to induction of remission with Prednisolone. A month later, the reductions in ESR (by 33 to 8 mm/hr) and PUCAI score (35 to 5) were associated with decreased total SCFA (from 780 to 294 µmol/g), propionate (from 81 to 28 µmol/g, stable at 10%) and butyrate (from 184 to 26 µmol/g, 24% to 9%) concentrations seen. At 3 months post-treatment, this patient remained well on Azathioprine only. A small rise in total SCFA (by 224 to 518 µmol/g), propionate (by 24 to 52 µmol/g, stable at 10%) and butyrate (by 34 to 60 µmol/g, 9% to 12%) concentrations were then seen. It was interesting to note that this patient started taking probiotics soon after diagnosis.
Appendix Figure 4.4: Variations in Total SCFA Concentrations and Individual SCFA Percentages with Clinical Markers in Newly-diagnosed Ulcerative Colitis Patients (A8 and A21)

Stool SCFA quantified with gas chromatography were analyzed with GraphPad Prism (V5) in conjunction with clinical events and parameters, which were PUCAI scores, CRP (mg/dL), ESR (mm/hr) and calprotectin (µmol/g).
4.5 Patient with Severe Ulcerative Colitis commencing Sirolimus

Patient C1 was diagnosed with UC and commenced first line treatment but the disease progressed despite escalating treatment (Appendix Figure 4.5). Colonic resection was being considered 2 months later but in a last bid to avoid this, the patient was commenced on Sirolimus. Sirolimus is produced by a strain of Streptomyces hygroscopicus and known to have antifungal, antiproliferative and immunosuppressive properties. The first stool sample obtained for SCFA was a month after starting Sirolimus.

The first sample had low total SCFA (291 µmol/g), propionate (16 µmol/g, 5%) and butyrate (23 µmol/g, 8%) concentrations. With clinical improvement on Sirolimus, the patient no longer required surgery. Three months post-Sirolimus, the patient was well when seen in clinic however, had a relapse 3 months later at 6 months post-Sirolimus (stool calprotectin 2361 µg/g, PUCAI 15, ESR 15 mm/hr). The total SCFA concentration decreased (by 93 to 198 µmol/g) while the propionate was stable (16 to 18 µmol/g, 9%), the butyrate concentration decreased (by 13 to 10 µmol/g, 8% to 5%).

Induction of remission was achieved with Prednisolone as shown by the decrease in PUCAI score (10) and ESR (10 mm/hr). There were increased total SCFA (by 213 to 411 µmol/g) and butyrate (by 6 to 16 µmol/g, 5% to 4%) concentrations while propionate concentration decreased (by 4 to 14 µmol/g, 3%). Although there was a decrease in stool calprotectin (by 915 to 1446 µg/g), it was still high suggesting ongoing mucosal inflammation.

At 12 months post-Sirolimus, Patient C1 had a relapse again as seen by the PUCAI score (30), CRP (24 mg/dL) and ESR (15 mm/hr). This was associated with an increase in total SCFA (by 270 to 681 µmol/g), a significant rise in butyrate concentration (by 77 to 93 µmol/g, 4% to 14%) and reduction in propionate concentration (by 11 to 3 µmol/g, 0.4%). Acetate percentages remained stable. The patient went on to have mucosal endoscopic assessment and started Adalimumab, an anti-TNFα inhibitor, 2 months later.
Appendix Figure 4.5: Variations in Total SCFA Concentrations and Individual SCFA Percentages with Clinical Markers in a Patient with Severe Ulcerative Colitis Patients (C1)

Stool SCFA quantified with gas chromatography were analysis with GraphPad Prism (V5) in conjunction with clinical events and parameters, which were PUCAI scores, CRP (mg/dL), ESR (mm/hr) and calprotectin (µmol/g).
4.6 Patient with Severe Very Early Onset IBD

Patient C3 was diagnosed with VEOIBD after presenting at age 3 years with bloody diarrhoea and abdominal pain. The disease progressed rapidly despite escalation of conventional treatment. Due to the refractory disease, the patient underwent a colectomy 9 months post-diagnosis. The first stool sample obtained was prior to surgery and subsequent samples were from the ileostomy (Appendix Figure 4.6).

Although the total SCFA concentrations were on average lower than healthy children, NIC and CD patients, the propionate and butyrate concentrations were significantly lower compared to other groups, with acetate making up most of the SCFA present. The acetate percentages in this patient were significantly higher than in the other IBD patients.

Post-colectomy, there was a reduction in ESR (from 30 to 24 mm/hr) and clinical improvement, with associated stable total SCFA (from 165 to 161 µmol/g), propionate (from 7 to 8 µmol/g, from 4% to 5%) and butyrate (from 2 to 6 µmol/g, 1% to 4%) concentrations. The patient, however, became unwell post-operatively (ESR 30) with possible diversion colitis. At 3 months post-operative, a rise in total SCFA (by 406 to 567 µmol/g) and propionate (by 47 to 55 µmol/g, 10%) concentrations were seen whilst the butyrate concentration was stable (5-6 µmol/g, 1% to 3%). Various treatments were tried including Infliximab. Two months later, at 5 months post-operative, the ESR increased further (41 mm/hr), with a decrease in total SCFA (by 280 to 287 µmol/g) and propionate (by 38 to 17 µmol/g, 6%) concentration and butyrate concentrations increasing (by 3 to 8 µmol/g, 1% to 3%). This was followed by an increase in total SCFA (by 117 to 404 µmol/g) and butyrate (by 5 to 13 µmol/g, stable at 3%) concentrations and a decrease in propionate concentration (by 3 to 14 µmol/g, 3%) at 8 months post-operative. At 13 months post-operative, 5 months later, the patient was well (ESR 18 mm/hr). There were reductions in total SCFA (by 339 to 65 µmol/g), propionate (by 10 to 4 µmol/g, 6%) and butyrate (by 6 to 6 µmol/g, 3% to 9%) concentrations.
Appendix Figure 4.6: Variations in Total SCFA Concentrations and Individual SCFA Percentages with Clinical Markers in a Patient with Very-early-onset Inflammatory Bowel Disease (VEOIBD) (C3)

Stool SCFA quantified with gas chromatography were analysis with GraphPad Prism (V5) in conjunction with clinical events and ESR (mm/hr).
4.7 Patients with CAPS commencing Canakinumab

As comparison to IBD, patients with CAPS, another inflammatory condition which includes gastrointestinal symptoms, were recruited and samples analysed. Patients with CAPS are treated with 2-monthly subcutaneous Canakinumab, a human monoclonal IL-1β antibody. Two patients with CAPS were recruited to the study (Appendix Figure 4.7).

CAPS Patient 1 was recruited just before the age of 3 years, 1 month after receiving the first dose of Canakinumab. The patient was symptomatic with loose stools and abdominal pain. The symptoms settled gradually with further doses of Canakinumab although a dose increased was required after the first 2 doses. The total SCFA (from 393 to 643 µmol/g), propionate (from 50 to 104 µmol/g, from 13% to 16%) and butyrate (from 48 to 73 µmol/g, 12% to 11%) concentrations were seen to increase after the second dose at 4 months post-treatment. This was associated with reducing CRP (by 23 to 48 mg/dL) and Disease score (8 to 4). The clinical markers continued to improve (CRP 27 mg/dL, Disease score 0) with decline in concentrations of total SCFA (by 233 to 410 µmol/g), propionate (by 35 to 69 µmol/g, 17%) and butyrate (by 41 to 32 µmol/g, 11% to 8%) concentrations. Acetate percentage remained stable.

CAPS Patient 2 was relatively well at the start of treatment (CRP <5 mg/dL, Disease score 4, Amyloid A 4.6 µg/ml) with mild gastrointestinal symptoms of intermittent abdominal pain. Although the total SCFA (201-225 µmol/g) and propionate (23 µmol/g, 11%) concentrations were seen to be stable, the butyrate concentration decreased from 23 to 15 µmol/g, 11% to 6%. This decrease was associated with improvement of clinical symptoms (Disease score 2, CRP <5 mg/dL and Amyloid A 4 µg/ml).
Appendix Figure 4.7: Variations in Total SCFA Concentrations and Individual SCFA Percentages with Clinical Markers in Patients commencing Canakinumab CAPS2, CAPS2)

Stool SCFA quantified with gas chromatography were analysis with GraphPad Prism (V5) in conjunction with clinical events and parameters, which were Disease scores, CRP (mg/dL), ESR (mm/hr) and amyloid (µg/ml).

4.8 A Patient with Granulomatosis Polyangiitis

Patient A11 presented with symptoms suggestive of CD thus was managed as such. The disease course was atypical and included liver disease, hair loss and skin changes. She was referred to the Rheumatology Department and further investigations led to the diagnosis of Granulomatosis Polyangiitis, a disease of vasculitis affecting multiple systems.

The stool samples included in the study were obtained prior to the diagnosis of Granulomatosis Polyangiitis and from 2 months of diagnosis with presumed CD (Appendix Figure 4.8). During the study period there was ongoing active disease as seen by the raised stool calprotectin concentrations (1766-3083 µg/g) and ESR (55-95 mm/hr), which was reflected in the PCDAI scores (20-40) after 8 months. The disease was refractory to treatment, which included EEN, Azathioprine, Prednisolone, Infliximab, Methotrexate and Adalimumab.

Throughout this period, the total SCFA varied between 305 µmol/g and 922 µmol/g with the concentration increasing significantly after a course of Prednisolone at 3 months post-diagnosis then Methotrexate and Adalimumab at 10 months post-diagnosis. Following the course of Prednisolone, there were also increased butyrate (from 72 to 112b µmol/g, 19% to 15%) and propionate (from 138 to 150 µmol/g, 36% to 20%) concentrations.

The butyrate concentration was raised (by 12 to 124 µmol/g) over the following months despite a reduction in total SCFA (by 185 to 571 µmol/g) and propionate (by 97 to 53 µmol/g, 9%), thus raising the butyrate percentage from 15% to 22% of total SCFA. Infliximab started at 7 months post-diagnosis may have contributed to these changes. As Infliximab began to take effect, there were associated reductions in stool caprotecin (from 2919 to 1766 µg/g), ESR (from 70 to 60 mm/hr), total SCFA (by 74 to 497 µmol/g).
and butyrate (by 31 to 93 µmol/g, 22% to 19%) concentrations, but increased propionate concentration (by 42 to 95 µmol/g, 19%).

Clinical improvement i.e. PCDAI score of 40 to 20 and further decrease in ESR from 60 to 55 mm/hr were seen at 11 months post-diagnosis, however there was a sharp rise in stool calprotectin (by 1317 to 3083 µg/g), total SCFA (by 425 to 922 µmol/g), propionate (by 30 to 125 µmol/g, 14%) and butyrate (by 60 to 153 µmol/g, 19% to 17%) concentrations. This is likely due to the on-going active disease.
Appendix Figure 4.8: Variations in Total SCFA Concentrations and Individual SCFA Percentages with Clinical Markers in a Patient with Granulomatosis Polyangiitis (A11)

Stool SCFA quantified with gas chromatography were analyzed with GraphPad Prism (V5) in conjunction with clinical events and parameters, which were PCDAlI scores, CRP (mg/dL), ESR (mm/hr) and calprotectin (µmol/g).
# Appendix 5A – Immune Functions of Vitamins and Trace Elements

<table>
<thead>
<tr>
<th>Micronutrient</th>
<th>Immune Role</th>
<th>Function</th>
<th>Deficient State</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>Epithelial barrier</td>
<td>Maintains respiratory, GI mucosal/epithelial barrier</td>
<td>Increased risk of respiratory and diarrhoeal diseases, measles, malaria and tuberculosis</td>
</tr>
<tr>
<td></td>
<td>Cellular immunity</td>
<td>Reduced activity of activated macrophage, specifically phagocytic and oxidative burst activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cellular immunity</td>
<td>Reduced NK cell number and activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cellular immunity</td>
<td>Development and differentiation of Th1 and Th2 lymphocyte subsets</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antibody production</td>
<td>Defence against extracellular pathogens</td>
<td>Impaired antibody-mediated immunity, increased IL-12, TNF-α and IFN-γ production</td>
</tr>
<tr>
<td>System</td>
<td>Function</td>
<td></td>
<td></td>
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<tr>
<td>-----------------------------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cellular immunity</strong></td>
<td>Induce the expression of NOD2, HBD2, CAMP and ATG16L1. Vitamin D receptors expressed in most immune cells except B cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cellular immunity</strong></td>
<td>Inhibit DC maturation, decrease IL-12 (Th1) production and increased IL10 production</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Antibody production</strong></td>
<td>Stimulates expression of antimicrobial peptides by immune and epithelial cells of respiratory tract</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vitamin E</strong></td>
<td>Increased lymphocyte proliferation in response to mitogens, NK cell cytotoxicity, alveolar macrophage phagocytic activity and resistance to infections</td>
<td></td>
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</tr>
<tr>
<td><strong>Cellular immunity</strong></td>
<td>Increased production of IL-2 (Th1), and decreased IL-4 (Th2) production</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Epithelial barrier</strong></td>
<td>Reduced oxidative stress and intestinal epithelial leakiness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Cellular immunity</td>
<td>Stimulate leukocyte functions, especially neutrophils and monocytes, enhance neutrophil chemotaxis</td>
<td>Scurvi</td>
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<td>-----------</td>
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<td>------------------------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>Antibody production</td>
<td>Enhance T cell proliferation (in response to infection), with increased cytokine and immunoglobulin production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular immunity</td>
<td>Stimulate generation of reactive oxygen species (ROS) and bacterial killing, enhance uptake and clearance of macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>Cellular immunity</td>
<td>Essential component of nucleic acid and protein synthesis</td>
<td></td>
</tr>
<tr>
<td>Cellular immunity</td>
<td>Impaired lymphocyte maturation and growth and T cell activity</td>
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<td></td>
</tr>
<tr>
<td>Antibody production</td>
<td>Decreased antibody production (IgD)</td>
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</tr>
<tr>
<td>Cellular immunity</td>
<td>Decreased IL-1β, IL-2, IL-2 receptor, NK cell activity</td>
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<tr>
<td>Folate</td>
<td>Cellular immunity</td>
<td>Essential role in nucleic acid and protein synthesis alongside vitamins B6 and B12</td>
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<tr>
<td>Cellular immunity</td>
<td>Decreased circulating T lymphocytes and their proliferation (CD8+)</td>
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</tr>
<tr>
<td>Cellular immunity</td>
<td>Enhanced NK cell cytotoxicity</td>
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</tr>
<tr>
<td><strong>Vitamin B12</strong></td>
<td>Antibody production</td>
<td>Required for folate function</td>
<td>Secondary folate deficiency; impaired thymidine and purine synthesis thus DNA and RNA synthesis and immunoglobulin secretion</td>
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</tr>
<tr>
<td></td>
<td>Cellular immunity</td>
<td></td>
<td>Decreased lymphocyte numbers, CD8+ cells (high CD4+/CD8+ ratio) and NK cell activity</td>
</tr>
<tr>
<td><strong>Selenium</strong></td>
<td>Cellular immunity</td>
<td>Remove excess oxidative radicals, thus maintaining the redox state and protect from oxidative stress generated by macrophages in inflammation</td>
<td>Decreased immunoglobulin titres and cell-mediated immunity</td>
</tr>
<tr>
<td></td>
<td>Antibody production</td>
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<tr>
<td><strong>Zinc</strong></td>
<td>Antibody production</td>
<td>Essential for highly proliferative cells especially the immune system</td>
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<tr>
<td></td>
<td>Cellular immunity</td>
<td>Involved in cytosolic defence against oxidative stress</td>
<td></td>
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<tr>
<td></td>
<td>Cellular immunity</td>
<td>Essential cofactor for thymulin which modulates cytokine release</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cellular immunity</td>
<td>Increases cytotoxic CD8+ cells (Th1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epithelial barrier</td>
<td>Maintain skin and mucosal membrane integrity</td>
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<tr>
<td></td>
<td>Cellular immunity</td>
<td>Direct antiviral effect on Rhinovirus replication</td>
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<tr>
<td><strong>Copper</strong></td>
<td>Cellular immunity</td>
<td>With catalase and glutathione peroxidase, protects against ROS</td>
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<tr>
<td></td>
<td>Cellular immunity</td>
<td>Essential for electron transfer reactions, gene regulation, oxygen binding and transport, regulation of cell differentiation and cell growth</td>
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<tr>
<td>Iron</td>
<td>Cellular immunity</td>
<td>Essential component of peroxide and nitrous oxide generating enzymes</td>
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<tr>
<td></td>
<td>Cellular immunity</td>
<td>Involved in cytokine production and activation of protein kinase C, which regulates cell proliferation</td>
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<tr>
<td></td>
<td>Cellular immunity</td>
<td>Required for myeloperoxidase activity, which is involved in bacterial killing by neutrophils</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>In excess, may induce free radical mediated damage</td>
<td></td>
</tr>
<tr>
<td>Cytokine/Ag</td>
<td>Sample</td>
<td>Age (years) (n)</td>
<td>Median (IQR) (pg/ml)</td>
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<td>----------------------</td>
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<tr>
<td>IL-1α, β</td>
<td>Serum</td>
<td>11-17 (262)</td>
<td>10 (0-417)</td>
</tr>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>Serum</td>
<td>1-6 (7)</td>
<td>10.5 (9.4-12.2)</td>
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<td></td>
<td>Serum</td>
<td>11-17 (262)</td>
<td>26 (0-2087)</td>
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<td>IL-4</td>
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<td>11-17 (262)</td>
<td>203 (0-41099)</td>
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<tr>
<td>IL-6</td>
<td>Serum</td>
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<td>15 (4-26)</td>
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<td>Serum</td>
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<td>17 (0-495)</td>
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<td>IL-7</td>
<td>Serum</td>
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<td>12.1 (10.3-14.4)</td>
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<td></td>
<td>Serum</td>
<td>11-17 (262)</td>
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<td>IL-8</td>
<td>Serum</td>
<td>1-6 (7)</td>
<td>30.9 (23.7-32)</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>3 (10)</td>
<td>1 (1-11)</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>12-14 (58)</td>
<td>4.0-6.3</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>11-17 (262)</td>
<td>10 (0-167)</td>
</tr>
<tr>
<td>IL-10</td>
<td>Serum</td>
<td>1-6 (7)</td>
<td>11.4 (8.5-12.8)</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>11-17 (262)</td>
<td>73 (0-6476)</td>
</tr>
<tr>
<td>Protein</td>
<td>Formulation</td>
<td>Serum/Plasma</td>
<td>Detection/Interpretation</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td>--------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>IL-12A (p35), IL-12B (p40), IL-12p70 (active heterodimer)</td>
<td>Serum</td>
<td>1-6 (7)</td>
<td>IL-12p70: 44.3 (39.8-64.1)</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>11-17 (262)</td>
<td>IL-12p40: Below LOD in all subjects</td>
</tr>
<tr>
<td>IL-13</td>
<td>Serum</td>
<td>11-17 (262)</td>
<td>IL-12p70: 23% ≤ Detection</td>
</tr>
<tr>
<td>IL-15</td>
<td>Serum</td>
<td></td>
<td>Below LOD in all subjects</td>
</tr>
<tr>
<td>IL-16</td>
<td>Serum</td>
<td>1-6 (7)</td>
<td>7-17 (30)</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Serum</td>
<td></td>
<td>Below LOD in all subjects</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Serum</td>
<td>11-17 (262)</td>
<td>37% ≤ Detection</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Serum</td>
<td>3-4 (33)</td>
<td>5-11 (127)</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>11-17 (262)</td>
<td>10 (0.7-336)</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>12-14 (38)</td>
<td>0.6-0.9</td>
</tr>
<tr>
<td>TNF-β</td>
<td>Serum</td>
<td></td>
<td>Below LOD in all subjects</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Serum</td>
<td>1-6 (7)</td>
<td>78.8 (74.6-83)</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>11-17 (262)</td>
<td>95 (0-6078)</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Serum</td>
<td>1-6 (7)</td>
<td>99.6 (81.4-169.3)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Serum</td>
<td>3-5 (45)</td>
<td>1-400 (800-1900)</td>
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</tbody>
</table>
### Appendix 5C – Biomarkers in the Meso Scale Discovery (MSD) V-Plex Cytokine Panel 1, Proinflammatory Panel 1 and Vascular Injury Panel 2

<table>
<thead>
<tr>
<th>Cytokine/Ig</th>
<th>Receptor</th>
<th>Cell Source</th>
<th>Cell Target</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α, β</td>
<td>Type I IL-1R, Type II IL-1R</td>
<td>Monocytes-macrophages, B cells, fibroblasts, most epithelial cells including thymic epithelium, endothelial cells</td>
<td>All cells</td>
<td>Upregulates adhesion molecule expression, neutrophil and macrophage emigration, mimics shock, fever, upregulates hepatic acute-phase protein production, facilitates haematopoiesis</td>
</tr>
<tr>
<td>IL-2</td>
<td>IL-2IL-2Rα, β, common γ</td>
<td>T cells</td>
<td>T cells, B cells, NK cells, monocytes-macrophages</td>
<td>Promotes T cell activation and proliferation, B cell growth, NK cell proliferation and activation, enhanced monocyte-macrophage cytolytic activity</td>
</tr>
<tr>
<td>IL-4</td>
<td>IL-4Rα, common γ</td>
<td>T cells, mast cells, basophils</td>
<td>T cells, B cells, NK cells, monocytes-macrophages, neutrophils, eosinophils, endothelial cells, fibroblasts</td>
<td>Stimulates TH2 helper T cell differentiation and proliferation; stimulates B cell Ig class switch to IgG1 and IgE anti-inflammatory action on T cells, monocytes; produced by T follicular helper cells in B cell germinal centres that stimulates B cell maturation</td>
</tr>
<tr>
<td>IL-5</td>
<td>IL-5Rα, common γ</td>
<td>T cells, mast cells, eosinophils</td>
<td>Eosinophils, basophils, murine B cells</td>
<td>Regulates eosinophil migration and activation</td>
</tr>
<tr>
<td>IL-6</td>
<td>IL-6R</td>
<td>Monocytes-macrophages, B cells, fibroblasts, most epithelium including thymic epithelial cells, endothelial cells</td>
<td>T cells, B cells, epithelial cells, hepatocytes, monocytes-macrophages</td>
<td>Induces acute-phase protein production, T and B cell differentiation and growth, myeloma cell growth, and osteoclast growth and activation</td>
</tr>
<tr>
<td>IL-7</td>
<td>IL-7Rα, common γ</td>
<td>Bone marrow, thymic/intestinal epithelial cells, epithelial goblet cells, keratinocytes, dendritic cells</td>
<td>T cells, B cells, bone marrow cells</td>
<td>Differentiates B, T and NK cell precursors, activates T and NK cells; haematopoietic growth factor</td>
</tr>
<tr>
<td>-------</td>
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<td>-------------------------------------------------</td>
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<td>-------------------------------------------------</td>
</tr>
<tr>
<td>IL-8</td>
<td>CXR1, CXR2</td>
<td>Monocytes-macrophages, T cells, neutrophils, fibroblasts, endothelial cells, epithelial cells</td>
<td>Neutrophils, T cells, monocytes-macrophages, endothelial cells, basophils</td>
<td>Induces neutrophil, monocyte and T cell migration, induces neutrophil adherence to endothelial cells and histamine release from basophils, and stimulates angiogenesis; suppresses proliferation of hepatic precursors</td>
</tr>
<tr>
<td>IL-10</td>
<td>IL10Rα, β</td>
<td>Monocytes-macrophages, T cells, B cells, keratinocytes, mast cells</td>
<td>Monocytes-macrophages, T cells, B cells, NK cells, mast cells</td>
<td>Inhibits macrophage pro-inflammatory cytokine production, downregulates cytokine class II antigen and B7-1 and B&amp;-2 expression, inhibits differentiation of TH1 helper T cells, inhibits NK cell function, stimulates mast cell proliferation and function, B cell activation and differentiation</td>
</tr>
<tr>
<td>IL-12A (p35), IL-12B (p40), IL-12p70 (active heterodimer)</td>
<td>IL-12R</td>
<td>Activated macrophages, dendritic cells, neutrophils</td>
<td>T cells, NK cells</td>
<td>Induces TH1 T helper cell formation and lymphokine-activated killer cell formation; increases CD8+ CYL cytolytic activity; decreases IL-17, increases IFN-γ</td>
</tr>
<tr>
<td>IL-13</td>
<td>IL-13R/ IL-4Rα</td>
<td>Monocytes-macrophages, B cells, endothelial cells, keratinocytes</td>
<td>TH2 T cells</td>
<td>Upregulates VCAM-1 and C-C chemokine expression on endothelial cells and B cell activation and differentiation, and inhibits macrophage pro-inflammatory cytokine production</td>
</tr>
<tr>
<td>Cytokine</td>
<td>Receptor</td>
<td>Cell Type/Physiological Action</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
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<td>--------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15</td>
<td>IL-15Rα, common γ, IL-2Rβ</td>
<td>Monocytes-macrophages, epithelial cells, fibroblasts, T cells, NK cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Promotes T cell activation and proliferation, angiogenesis, and NK cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-16</td>
<td>CD4</td>
<td>Mast cells, eosinophils, CD8+ peripheral T cells, respiratory epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD4 expressing cells including monocytes, eosinophils, dendritic cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chemoattractant for CD4 expressing cells, stimulate production of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β, IL-15) by monocytes. Up-regulate IL-2Rβ, Rα on T cells. Initiates stress pathway by activating stress activated protein kinase and p38 mitogen protein kinase. Increased expression in inflamed mucosa in UC.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-17</td>
<td>IL-17R</td>
<td>CD4+ T cells, monocytes-macrophages, eosinophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD4+ T cells, monocytes-macrophages, eosinophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Promotes chemotraction of CD4+ T cells, monocytes and eosinophils; inhibits HIV replication; inhibits T cell activation through CD3/T cell receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amyloid A</td>
<td>TLR2, TLR4, formyl peptide receptor-like 1 (FPRL1), class B scavenger receptor CD36, ATP receptor P2X7</td>
<td>Hepatocytes</td>
<td>Vascular endothelial cells, intestinal epithelial cells, dendritic cells, Monocytes-macrophages, B cells, T cells, PMLs</td>
<td>Transport cholesterol to liver, recruitment of immune cell to inflammatory sites (neutrophils and mast cells), induction of enzymes that degrade extracellular matrix, induce cytokine synthesis, induces expression of pro-inflammatory and pro-thrombotic mediators including ICAM-1, VCAM-1, IL-6, IL-8, MCP-1 and tissue factor (TF) in both monocytes/macrophages and endothelial cells, and induces endothelial dysfunction—a precursor to atherosclerosis, promotes LPS clearance</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>GM-CSFR, common β</td>
<td>T cells, monocytes-macrophages, fibroblasts, endothelial cells, thymic epithelial cells</td>
<td>Monocytes-macrophages, neutrophils, eosinophils, fibroblasts, endothelial cells</td>
<td>Regulates myelopoiesis; enhances macrophages bactericidal and tumouricidal activity; mediator of dendritic cell maturation and function; upregulates NK cell function; clinical use in reversing neutropenia after cytotoxic chemotherapy</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Type I interferon receptor</td>
<td>T cells, NK cells</td>
<td>All cells</td>
<td>Regulates macrophage and NK cell activations; stimulates Ig secretion; induction of class II histocompatibility antigens; TH1 T cell differentiation</td>
</tr>
<tr>
<td>Protein</td>
<td>Receptors</td>
<td>Target Cells</td>
<td>Function</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>TNFRI, TNFRII</td>
<td>Monocytes/macrophages, mast cells, basophils, eosinophils, NK cells, B cells, T cells, keratinocytes, fibroblasts, thymic epithelial cells</td>
<td>Fever, anorexia, shock, capillary leak syndrome, enhances leukocyte cytotoxicity, enhanced NK cell function, acute phase protein synthesis, pro-inflammatory cytokine induction</td>
<td></td>
</tr>
<tr>
<td>TNF-β</td>
<td>TNFRI, TNFRII</td>
<td>T cells, B cells</td>
<td>Cell cytotoxicity, lymph node and spleen development</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>LFA-1, MAC-1, fibrinogen</td>
<td>Vascular endothelial cells, macrophages, lymphocytes</td>
<td>Stabilises cell-cell interactions; cell-cell adhesion; extravasation; facilitates leukocyte endothelial transmigration; antagonistic effects on tight junctions forming blood-testis barrier; binding site for rhinovirus entry; role in signal transduction; recruitment of inflammatory immune cells such as macrophages and granulocytes</td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
<td>VLA-4</td>
<td>Endothelial cells, smooth muscle cells, bone marrow stromal cells</td>
<td>Mediates adhesion of lymphocytes, monocytes, eosinophils and basophils to vascular endothelium; leukocyte-endothelial cell signal transduction</td>
<td></td>
</tr>
<tr>
<td>VEGF-A</td>
<td>VEGFR-1, VEGFR-2</td>
<td>Most cell types (not usually endothelial cells)</td>
<td>Stimulates endothelial cell proliferation; Increases vascular permeability</td>
<td></td>
</tr>
</tbody>
</table>
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Abstracts Presented

2015

Poster Presentation at European Society of Paediatric Gastroenterology, Hepatology and Nutrition Annual Meeting 2015, Amsterdam
Abstract Submission

**Gastroenterology**

**Inflammatory Bowel Disease**

ESPGHAN2015-1324

EXTRA-INTESTINAL MANIFESTATIONS ASSOCIATED WITH MUTATIONS IN THE IL-10 PATHWAY

Intan Yeop1,2; Mona Bajaj-Elliott3; Fevronia Kiparis3; Manoun Elawad1

1Great Ormond Street Hospital, 2UCL Institute of Child Health, London, United Kingdom

Presentation Preference: Oral

ESPGHAN Membership: No Member

Has the abstract been previously presented?: No

Objectives and Study: Loss of function in the IL-10 axis in infancy results in severe enterocolitis and extra-inestinal manifestations, the latter currently remain ill-defined.

Methods: Clinical history and results for 6 patients with IL-10 axis dysfunction, identified between 2010 to 2014, were reviewed.

Results:

<table>
<thead>
<tr>
<th>PI</th>
<th>Lg</th>
<th>Mutation</th>
<th>Origin</th>
<th>Colectomy</th>
<th>HSCT</th>
<th>Respiratory</th>
<th>Sensorineural Hearing Loss</th>
<th>Lymphome</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>18y</td>
<td>L-10</td>
<td>Northern Pakistan</td>
<td>16m</td>
<td>4y</td>
<td>Bronchiolitis wheeze, emphysematous bullae</td>
<td>moderate-3y</td>
<td>Microcephaly</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24y</td>
<td>L-10</td>
<td>Northern Pakistan</td>
<td>-</td>
<td>14m</td>
<td>Wheeze</td>
<td>Klebsiella sepsis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>327y</td>
<td>L-10R8</td>
<td>Arab</td>
<td>1y</td>
<td>-</td>
<td>Wheeze</td>
<td>Eczema</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>414y</td>
<td>L-10R8</td>
<td>Indian</td>
<td>10m</td>
<td>-</td>
<td>Severe-5y</td>
<td>Intrope-sensitive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>51y</td>
<td>L-10R8</td>
<td>Indian</td>
<td>-</td>
<td>10m</td>
<td>Respiratory failure</td>
<td>Eczema</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56y</td>
<td>L-10Ra</td>
<td>Vietnamese</td>
<td>8m</td>
<td>Due Uppertract infections</td>
<td>Recurrent Otitis Media</td>
<td>Eczema</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 1: Clinical history and systemic analyses.

**Impaired mucosal/skin defence:** A predominance of upper respiratory symptoms (4 with intermittent symptoms and 1 had HFOV+NO) in early childhood were recorded. Sensorineural hearing loss in early childhood was detected following developmental concerns. Recurrent and severe infections, including Klebsiella sepsis requiring PICU admission, indicated increased susceptibility to infections. Eczematous lesions seen were resistant to conventional topical treatment, suggesting compromised skin immunity.

**Impaired adaptive immunity:** Low T (CD3, CD4 and CD8) and B (CD19) cells counts, and PHA stimulation test were recorded. Raised IgA seen in all patients confirm mucosal immune dysfunction. IgM, IgE, IgG1 and IgG3 were also variably increased. EBV-driven lymphoma occurred later in an immunosuppressed, non-transplanted patient. Severe infections in infancy is likely to have contributed to microcephaly seen in a patient.

Conclusion: In addition to its well-established effects on gut homeostasis, defects in the IL-10 pathway leads to immune deficiency and impaired mucosal defence. Understanding this rare disease may enable prompt referral to other specialists and early treatment. It also offers to opportunity to better understand childhood IBD.

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2016

Oral Presentation British Society of Paediatric Gastroenterology, Hepatology and Nutrition Associates and Trainees Annual Meeting 2016, Liverpool

The Gut Microbiome-Immune Axis with Treatment in Paediatric Inflammatory Bowel Disease

Yeop I, Alber D, Jones H, Bajaj-Elliott M

541
Increasing evidence indicates that genetic susceptibility, dysbiotic gut microbiome and altered immunity are key determinants of Inflammatory Bowel Disease (IBD) pathogenesis. Children account for 25% of IBD patients, with the incidence continuing to rise (1). PIBD can be more aggressive and extensive, and is associated with significant morbidity affecting growth, development, education and well-being of children.

The gut microbiome of IBD patients differs from that of healthy individuals. Firmicutes and Bacteroides dominate in health but in IBD, there is dysbiosis, or microbial imbalance, with reduced bacterial diversity. Gut microbiota alters with treatment, with relapse and remission, and disease severity. It is tempting to hypothesise that treatment, when successful, does so by restoring dysbiotic microbiome to a symbiotic state leading to immune harmony.

Resident gut bacteria also influence the host by producing metabolites, i.e. microbial metabolome, from ingested food. It has been reported that high fibre and fruit intake reduces CD risk, and high vegetable intake reduces UC risk (2). In addition, short chain fatty acids (SCFA), produced from fermentation of dietary fibres, correlate with disease activity.

We hypothesise that gut microbiome composition and function (metabolome) influences disease relapse, severity and response to treatment, and these in turn affects the systemic inflammatory profile. Bacterial DNA is extracted from mucosal biopsies, duodenal lavages and stool samples. DNA extracts are then amplified using a 16s rDNA specific PCR and then sequenced using next generation sequencing. Stool SCFA content is quantified using gas chromatography and plasma cytokines concentration is analysed by Meso Scale Discovery Multiplex Assay. Samples are collected prospectively from patients with newly-diagnosed IBD, severe IBD and starting Infliximab and compared with samples from non-inflamed controls. It is hoped that the results will bring us a step closer to understanding the interaction between gut microbiota and the paediatric host immunity.

References


Investigating potential association between disease activity, short chain acid profiling with Immune and vascular status in Paediatric Inflammatory Bowel Disease

Yeop, I; Papadopoulou, C; Jones, H; d’Abbro, E; Flynn, R; Parveen, G; Alber, D; Kiparissi, F; Klein, N; Gerasimidis, K; Hunter, P; Bajaj-Elliott, M

1UCL Great Ormond Street Institute of Child Health, London; 2Great Ormond Street Hospital, London; 3University of Glasgow

Introduction: Immune dysregulation is a known factor in IBD development, with it being predominantly Th1-mediated in Crohn’s Disease (CD), Th2 in Ulcerative Colitis (UC) and Th17 in both. Intestinal vasculature is involved in IBD pathogenesis. Intestinal microbiota has a significant role in IBD, partly through its metabolites such as short chain fatty acids (SCFA).

Aims and Objectives: We aimed to (a) compare serum vascular and immune status of IBD patients with disease activity and treatment; and (b) assess any potential association with their faecal SCFA (acetate, propionate and butyrate).

Subjects and Methods: Blood samples were collected from healthy controls (HC) in the community, non-inflammatory control (NIC) and IBD patients from Great Ormond Street Hospital prior to diagnostic endoscopy or treatment then prospectively thereafter. Plasma biomarkers were quantified using the MSD kits V-PLEX Cytokine Panel 1 (GM-CSF, IL-12/IL-23p40, IL-15, IL-16, IL-17A, IL-1α, IL-5, IL-7, TNF-β, VEGF-A), V-PLEX Proinflammatory Panel 1 (IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α) and V-PLEX Vascular Injury Panel 2 (SAA, CRP, VCAM-1, ICAM-1). Stool samples collected were stored at -20°C in 1M NaOH then freeze-dried prior to extraction using diethyl ether and orthophosphoric acid (1). Gas chromatography was used to quantify acetate, propionate and butyrate in the extracted stool samples. Biomarkers and SCFA were compared with clinical parameters and disease scores pre- and post-treatment. Statistical analysis was performed using GraphPad Prism.

Results: A total of 76 blood samples from HC (n=21), NIC (n=8) and IBD (n=12) patients, and 28 stool samples from IBD (n=12) and NIC (n=6) patients were analysed. Cytokines IL-1α, IL-1β, IL-2, IL-4, IL-12p70 and IL-13 were undetectable thus excluded from analysis.

- IBD patients were found to have elevated TNF-α (p=0.007), IL-6 (p=0.0204), IL-8 (p=0.0451) and IL-15 (p=0.0012), IL-5 (p=0.001), IL-16 (p=0.004) and IL-17 (p<0.0001) compared to HC.

- Increased circulating serum amyloid A (SAA) (p=0.0353) and vascular cell adhesion protein (VCAM-1) (p<0.0001), whilst decreased concentrations of vascular endothelial growth factors (VEGF) (p=0.0299) were seen in IBD compared to HC.

- Analysing CD and UC patients separately, IL-17, IL-15, IL-5 and VCAM-1 concentrations were elevated in active CD (IL-17 p<0.0001, IL-15 p=0.002, IL-5 p=0.004, VCAM-1 p=0.0004) and
active UC (IL-17 p<0.0001, IL-15 p=0.033, IL-5 p=0.035, VCAM-1 p=0.004). In addition, SAA, IL-8 and IFN-γ were elevated in active CD (SAA p=0.005, IL-8 p=0.038, IFN-γ p=0.0009) and IL-16 was raised in UC (p=0.007).

- Following treatment, IL-12p40, IL-17, TNF-α, IL-5 and IL-6 decreased with improving ESR. Additionally, SAA in CD, and VCAM-1 and VEGF in UC reduced with decreased ESR. In contrast, IFN-γ, IL-7, IL-15 and IL-16 in CD, SAA in UC and granulocyte-macrophage colony-stimulating factor (GM-CSF) in CD and UC increased in all patients still in active disease.

- A trend for increase in IL-10 with greater butyrate concentration was observed; this reached statistical significance for IL-5 (R²=0.327, p=0.0207) and IL-8 (R²=0.2968, p=0.0291). Interestingly, TNF-β showed negative correlation with increasing acetate (R²=0.452, p=0.0043), and the reverse with propionate (R²=0.285, p=0.0043) with a trend for greater cytokine levels with increasing butyrate. Increased acetate and propionate percentages were associated with a reduction in IFN-γ, VEGF, IL-8 and IL-17 in 3 UC patients.

**Summary:** Our pilot study supports the notion that IL-7, IL-15, IL-16 and SAA may serve as potential biomarkers for disease activity in PIBD. Trends observed between cytokines and SCFA suggests intimate links between microbial metabolites and host immune axis, with a key role in UC pathogenesis.


**Poster Presentation at British Society of Paediatric Gastroenterology, Hepatology and Nutrition Annual Meeting 2019, Oxford**

*Alterations in intestinal microbial composition and metabolic profiles in response to treatment in Paediatric Inflammatory Bowel Disease*

Yeop, I1; Gates, L1; Jones, H1; Kammermeier J4; d’Abbro, E1; Flynn, R2; Parveen, G1; Kiparissi, F2; Klein, N2; Gerasimidis, K3; Alber, D1; Bajaj-Elliott, M1

1UCL Great Ormond Street Institute of Child Health, London; 2Great Ormond Street Hospital, London; 3University of Glasgow; 4Evelina London Children’s Hospital, London

**Introduction:** Intestinal microbiota and associated metabolite e.g. short chain fatty acids (SCFA), are known key players involved in paediatric IBD pathogenesis. At present, the impact of treatment on microbial biomarkers remains less studied.

**Aims and Objectives:** We aimed to characterise dynamic changes in (a) stool and mucosal microbial profiles in active IBD and the impact of treatment; and (b) the corresponding stool SCFA profiles (acetate, propionate and butyrate).
Subjects and Methods: A spectrum of IBD (IL-10Rα mutation, IL-10Rβ mutation, Infantile IBD, VEOIBD, Crohn’s Disease (CD) and Ulcerative Colitis (UC)) and non-inflammatory control (NIC) patients in addition to healthy children (HC) from the community were recruited for opportunistic, prospective collection of stool and mucosal biopsy samples.

To characterise intestinal microbiota, DNA was extracted as per manufacturer’s instructions (Stool: MPBio FastDNA SPIN for Soil; biopsies: Qiagen QIAamp Mini Kit) and the V3-V4 16s rRNA gene region amplified from each extract. Purified PCR products were pooled and sequenced on an Illumina MiSeq. Sequencing data was processed with Mothur (V1.35.1) using a published pipeline (1) to perform Operational Taxonomic Unit (OTU) picking and taxonomy assignment. Sample reads were subsampled at 2,000 to allow for comparison. Data was then imported into RStudio (V1.0.153) for further analysis.

SCFA were extracted from the same stool samples using diethyl ether and orthophosphoric acid (2) followed by gas chromatography utilising internal and external controls. SCFA concentrations were calculated based on the chromatograms. Clinical history and results were gathered for correlation with laboratory results.

Results: We present stool and mucosal microbial profiles of a patient with IL-10Rβ pre- and post-HSCT (IL-10Rβ mutation, 15y). As comparison, we present microbial profiles pre- and post-treatment with HSCT (Infantile IBD, 7y), Prednisolone (UC, 15y) and colectomy (VEOIBD, 4y) in addition to those with CD (16y), IL-10Rα (3y), NIC patients (n=2, 14y, 15y) and HC (n=2, 13y, 14y). Our findings included:

- HC stool predominantly consisted of Firmicutes (Clostridiales) and Bacteroidetes (Bacteroidales). NIC stool were similar but higher Proteobacteria abundance and with higher butyrate percentage than HC.

- Stool microbiota differed from mucosal microbiota.

- Increased Proteobacteria (particularly Enterobacteriales) was associated with more severe disease

- Bacteroidetes (Bacteroidales) was associated with disease improvement.

- Stoma samples were dominated by a single phylum, Proteobacteria (IL-10Rβ, VEOIBD) or Firmicutes (IL-10Rα), and associated with high acetate percentages.

- With treatment, the microbial profiles for the UC and Infantile IBD patients altered favourably which included reduction of Proteobacteria with associated increased Bacteroidetes. The increased Bifidobacteriales in UC was associated with increased stool propionate and butyrate percentages.

Summary: Variations in microbial profiles occur with disease severity and treatment, and is reflected in the SCFA profiles. Differences exist between luminal (stool) contents and mucosal profiles. These observations would benefit from confirmation in a bigger cohort.
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Variations in Short Chain Fatty Acids reflect the Intestinal Microbial Dysbiosis seen in Paediatric Inflammatory Bowel Disease

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Introduction: The intestinal microbiota and its metabolites e.g. short chain fatty acids (SCFA) are key determinants of host physiology. SCFA are a major source of energy for colonocytes, and participate in metabolism and maintaining mucosal and systemic immune homeostasis.

Aims and Objectives: The study aims were to (a) characterise SCFA profiles in health and IBD; and (b) investigate potential association(s) between SCFA levels and clinical/nutritional status in response to treatment.

Subjects and Methods: Healthy infants (HI) and healthy children (HC) were recruited from the community. IBD patients from Great Ormond Street Hospital were recruited prior to diagnostic endoscopy, and samples collected prospectively. Stool samples from non-IBD controls (NIC) were also included. Stool samples were stored (-20°C in 1M NaOH) then freeze-dried prior to extraction with diethyl ether and orthophosphoric acid followed by gas chromatography. SCFA concentrations were calculated utilising internal and external standards. Clinical results (calprotectin and disease scores) and dietetic assessments (24-hour recall) were gathered. Active disease was defined as PUCAI >10 and weighted PCDAI >12.5. Two-tailed Mann-Whitney U test and linear regression for assessing correlation with clinical parameters were conducted.
**Results:** Overall, the total SCFA concentrations across all groups showed a mean of 524, median 482 (65-1149) µmol/g. Our salient findings were:

- HI (n=5; age 6-16 months) had higher percentage of acetate compared to HC (n=11; age 4-7 years) (p=0.005). Significant increase in UC versus HC was also observed (active p=0.031; remission p=0.005).

- NIC (n=6; age 8-15 y) had higher propionate (p=0.009) and butyrate (p=0.017) percentages than HC.

- Lower propionate concentrations and percentages were observed in active UC (p=0.014, p=0.001) as well as UC in remission (p=0.016, p=0.005). Significant differences between UC (active and remission) and CD patients (p=0.0009, p=0.0061 respectively) were also recorded.

-Lower butyrate concentrations and percentages were observed in IBD compared to NIC (CD p=0.01, p=0.005; UC p=0.032, p=0.016). Correlation was observed with increased butyrate percentages and raised calprotectin in CD patients (R²=0.48, p=0.027).

- Valerate concentrations were lower in UC than HC (active UC p=0.002; UC in remission p=0.005).

- Ileal (n=2) total SCFA concentrations were lower than HC (p=0.019) and NIC patients (p=0.01), and predominantly consisted of acetate (>85%).

- Nutritional assessment: HC had more fruit/vegetable intake compared to IBD (p=0.016) and NIC patients (ns).

**Summary:** Infant SCFA profiles predominantly consisted of acetate, as did the ileal samples. Propionate and valerate were significantly reduced in IBD, in addition, butyrate concentrations and percentages showed a trend of decrease in UC, supporting the hypothesis that there is reduction in microbial butyrate-producers in UC. We are currently confirming our findings by 16S rDNA profiling.

**Conclusion:** There were distinct variations in SCFA profiles between healthy, NIC and PIBD. SCFA appears to play a critical role in the pathogenesis of UC. Finally, the observed differences between HC and NIC patients are likely a reflection of the well-characterised dysbiosis reported in these patients, thus caution should be exercised when comparing SCFA profiles between disease groups.

Dynamic Changes in Intestinal microbiota, associated Metabolites and Host Immunity in response to Treatment in Paediatric Inflammatory Bowel Disease

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Introduction

Intestinal microbiota and associated metabolites e.g. short chain fatty acids (SCFA), are key players implicated in paediatric IBD pathogenesis. How these entities influence host immunity and therefore clinical outcome is less clear.

Objectives

We aimed to characterise the (a) intestinal microbial alterations with treatment, (b) the associated SCFA variations and (c) potential correlation between SCFA and cytokines in the same cohort of children longitudinally.

Methods

Blood, stool, intestinal biopsies and duodenal lavages were collected opportunistically from IBD patients before and prospectively after treatment. Non-inflammatory control (NIC) patients and healthy controls (HC) from the community were also recruited. DNA was extracted from stool, biopsies and lavages and the V3-V4 16s rRNA gene region amplified. Purified PCR products were pooled, sequenced on an Illumina MiSeq and the data processed with Mothur (V1.35.1) using a published pipeline for Operational Taxonomic Unit (OTU) and taxonomy assignment. Further analysis was carried out using RStudio (V1.0.153). SCFA were extracted from the same stool samples using diethyl ether and orthophosphoric acid followed by gas chromatography utilising internal and external controls. SCFA concentrations were calculated based on the chromatograms. Plasma biomarkers were quantified using the MSD kits V-PLEX Cytokine Panel 1, V-PLEX Proinflammatory Panel 1 and V-PLEX Vascular Injury Panel 2. Statistical analysis was performed using Excel and GraphPad Prism. Clinical history, results and dietetic assessments were gathered for correlation with laboratory results.

Results

Analysis of blood, stool and biopsies from HC (n=11 stool, n=21 blood), NIC (n=6) and IBD (n=22) patients were analysed. These included patients with Infantile IBD, Very Early Onset IBD, IL-10RA and IL-10RB mutations.

Stool from HC predominantly consisted of Firmicutes (Clostridiales) and Bacteroidetes (Bacteroidales). In comparison, patients with inflammation had higher abundance of...
Proteobacteria (particularly *Enterobacteriales*) and lower abundance of Bacteroidetes (mainly Bacteroidales), with these changes being related to disease severity. Ileostomy samples, however, were dominated by a single phylum, either Proteobacteria or Firmicutes. Differences were seen between stool and mucosal microbiota, and both varied with response to treatment.

These microbial changes were reflected in the SCFA profiles. Differences in acetate, propionate, butyrate and valerate were seen between IBD patients and control groups. Propionate and butyrate proportions distinguished between CD, UC and ileostomy samples, and was associated with inflammation.

Pro-inflammatory (IL-6, IL-8, IL-16, IL-17, TNF-α) cytokine concentrations of IBD patients were higher than HC, and mirrored ESR trends. Correlations were observed between some cytokines (IL-5, IL-8, IL-17 and TNF-β) and SCFA.

**Conclusion**

The differences we report in intestinal microbiota between IBD and control patients were reflected in the SCFA profiles. These changes appear to influence the systemic cytokine milieu. Our study of associations between the 3 components in the same cohort is novel to our knowledge, and will be complete for presentation.
Gut microbiota from infant with cow’s milk allergy promotes clinical and immune features of atopy in a murine model

To the Editor,

Numerous clinical and epidemiological studies suggest an association between abnormal development of the gut microbiota in early life and development of clinical manifestations related to allergy including cow’s milk allergy (CMA). Despite advances, at present there is no consensus on a clear signature of a CMA microbiota due to variations in allergic phenotypes and methods used. Moreover, it remains unclear whether the observed microbial alterations are a cause or a consequence of allergy. Here, we report the effects of faecal microbiota transfer (FMT) of a healthy control (HC) and CMA infant in a gonadotoxic murine model of CMA. Detailed methods are provided in Appendix S1.

Infants with CMA (n = 5), under the care of Great Ormond Street Hospital, London, UK, were recruited alongside healthy controls (n = 4) from the community (Table S1 and S2) (REC 14/LO/0364). Stool samples from the infants with CMA analyzed by 16S rRNA gene sequencing showed a significant increase in bacterial diversity, decreased abundances of Bifidobacterium spp., and increased abundances of Lachnoclostridium sp., and one of its genera that is, Escherichia coli (FDR < 0.05), was compared with HC (Figure 1A–C). These specific gut microbiota signatures corroborate previous findings comparing CMA infants with breastfed HC.1,2 Significant increases in bacterial-derived short chain fatty acids (butyrate, iso-valerate, and iso-hexanoate) were observed in infants with CMA compared with HC (Figure S1). No significant differences in stool pH, acetate, glucose, lactate, VFA, and SCFA, acetate, propionate, and butyrate levels were recorded (Figure S1). All these parameters were supplemented into a principal component analysis (PCA) of microbial compositions that revealed distinct patterns between CMA and HC (Figure 1D).

We selected 2 infants, 1 with CMA and the other with HC (infants 3 and 4) matched for age (9 and 10 months old), gender (female), and delivery mode (cesarean section) for FMT into three-week-old germ-free mice (CF31/Hepa). Following 12 days of microbiota establishment, mice received, once a week for 4 weeks, whole whey protein (WW) and choline toxin (CT) (sensitized mice; S) or CT only (nonsensitized control; NC) (Figure 2A) (REC E6AAA4, JWV.03/02.12). Despite an adaptation to the murine host, the main microbiota and metabolic signatures of CMA and HC were sustained over the course of the experiment (Figure 1E, F, S2 and S3), validating our model of choice. No differences in microbiota compositions were observed between S and NC mice receiving the same fecal transfer.

Following oral allergen sensitization, CMA microbiota was associated with diarrhea-related symptoms (Figure 2A), with higher fecal scores (reflecting softer diarheic stools/ anal inflammation) that persisted at least 24 hours for the CMA-S group compared with HC-S group (P < 0.001). No significant differences in fecal scores were recorded between HC-S and HC-NS, which may indicate a protective effect of healthy microbiota upon allergen exposure as we and others observed previously.3,4 In addition, clinical scores (scratching/puffiness/loss of mobility) after oral challenge with α-lactoglobulin (α-LG) were significantly higher in CMA-S group versus CMA-NS and HC-S groups (Figure 2B). Minimal differences in mucous mast cell protease 1 (mMCP-1) and allergen-specific sensitization markers were observed between the two fecal transfers (Figure 2C–E), which contrasted with the increases observed in total IgE levels and total IgG1/IgG2a ratio in CMA-S and CMA-NS groups compared with HC-S and HC-NS groups, respectively (Figure 2F,G). The latter observations were consistent with increased gas3 mRNA expression in the colon (Figure 2H), which is a marker of Th2 lymphocytes. Interestingly, despite nonsignificant, cooccurred FCRIII mRNA expression was increased in CMA-S mice (Figure 2I, P < 0.07). FCRIII is an IgG1 binding/activating receptor, and its increased expression may imply a pathway linked to IgG1 and basophil and the potential development of anaphylaxis.5

Literature indicates that germ-free mice have increased total IgE, which can be normalized upon colonization with commensal microbiota until 8 weeks of age.6 We found that only colonization with HC-associated microbiota led to total IgE levels similar to that of nonsensitized control. This may be due to the observed enrichment of protective bacteria including bifidobacteria and Anaerococcus spp. (Figure S2), the latter of which corroborates the findings by Feeley et al7 using a similar mice model. In contrast, CMA microbiota was associated with higher total IgE, a phenomenon that is linked to poor long-term outcome in atopic dermatitis8 or an increased risk of developing other allergic manifestations.9

Our findings confirm a Th2-type immune orientation following FMT with CMA microbiota. This Th2 profile was associated with minimal differences in mMCP-1 levels or allergen-specific immunoglobulin levels between the CMA-S and HC-S groups, raising the hypothesis that non-IgE mediated immunity might be at play. Indeed, patients with non-IgE-dependent food-allergy predominantly...
FIGURE 1 Microbiota of donors and recipient mice. Richness (A) and Shannon diversity (B) of fecal samples from infants with cow's milk allergy (CMA) or without (HC). Red symbols correspond to donors selected for FMT. Cladogram (C) showing discriminant taxa identified at the genus level (dark) and family level (light). PCA (D) of donor microbiota compositions (genus level) supplemented with fecal parameters analyzed. PCA of mice microbiota compositions (genus level) of fecal pellets at D15 (E) and cecal samples at D50 (F). AA, acetic acid; BA, butyric acid; DLA, D-lactic acid; EDN, Escherichia-derived neroconol; ER/CC, Enterococcus rectale-Clostridium cocoides group; iso-BA, iso-butyric acid; iso-VA, iso-vanillic acid; LLA, L-lactic acid, PA, propionic acid; VA, valeric acid.
FIGURE 2  Mice model results: (A) Fecal scores. Pellets were collected at D15 & D16, D29 & D30, and D43 & D44. (B) Clinical score, (C) concentration of nMCP-1, (D) BLG-specific IgE, (E) BLG-specific IgG1/IgG2a ratio, (F) total IgE, and (G) total IgG1/IgG2a ratio. (H) Gene mRNA expression in colon of sensitized groups. mRNA expression is relative to HC-S mice. P-values were calculated using Mann-Whitney test: **P < 0.01; ***P < 0.001, and ****P < 0.0001.)

present gastro-intestinal tract symptoms. The diarrhea-like symptoms and signs of colonic inflammation in mice with the CMA-associated microbiota support this hypothesis.

We noted an increase in colonic foxp3 mRNA gene expression in CMA-S group compared with HC-S group. Foxp3 has been associated with the production of Th2 cytokines in several cell lines, including foxp3-“T regulatory cells, as well as with regulatory T-cells (Treg). If it is associated with Treg cells, the increased foxp3 expression may reflect a regulatory mechanism in response to the enhanced Th2 profile of the CMA-S group. Another explanation of the increased foxp3 expression could be linked to the increased Lactobacillus spp. and the associated increase in butyrate (Figure S3), which has been implicated in the induction of Foxp3 Treg cells. However, in our study, this was not associated with protection against allergic sensitization.

In summary, we demonstrate for the first time that infant microbiota with a low bifidobacteria/Lachnoclostridium ratio orients the murine immune system toward a Th2 atopic profile with enhanced clinical symptoms of allergy, showing the pivotal role of the microbiota in allergy. Our study, however, has two limitations: (a) the concomitant medication and elimination diet in infants with CMA, which are the both factors known to influence the gut microbiota, but are also inherent to the medical condition, and (b) the use of only two representative microbiota to avoid antagonistic effects that might
arise by mixing different microbiota. Despite these limitations, our model system is a valuable tool providing novel insights into the role of gut microbiota in CMA.

CONFLICTS OF INTEREST
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.