University of Alberta

The Influence of Iron Therapy on the Clinical Outcomes, the Colonic Bacteria Microbiome and the Urinary Metabolomics in Iron Deficient Subjects.

by

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Abstract

Iron deficiency is a common problem facing subjects with inflammatory bowel disease and it is of recurrent nature. It is associated with lethargy, fatigue and poorer cognition independent of anaemia. These symptoms could be reversed with iron supplementation either via oral or intravenous route. However, these two routes may have differential effects on the clinical outcomes such as quality of life and disease activity, the colonic bacterial microbiome composition and the urinary metabolomics. This thesis demonstrated that intravenous iron replacement was superior to oral iron replacement in improving the serum ferritin level and quality of life score. Moreover, compared to intravenous iron therapy, significant colonic bacterial dysbiosis was observed with oral iron therapy. The different urinary metabolite profiles between the two routes of iron therapy indicate the route of iron therapy had differential impact on the host's and gut microbial metabolisms.

Acknowledgements

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List of Abbreviations

ADE	**Adverse Drug Event**
AE	**Adverse Event**
AIEC	**Adherent Invasive E Coli**
ASA	**Aminosalicylate**
ATP	**Adenosine Triphosphates**
CAI	**Clinical Activity Index**
CRP	**C- Reactive Protein**
C difficle	**Clostridium difficile**
CI	**Confidence Interval**
CD	**Crohn's Disease**
CDAI	**Crohn's Disease Activity Index**
CT	**Computerised Tomography**
DAI	**Disease Activity Index**
DNA	**Deoxyribonucleic Acid**
DSS	**Dextran Sodium Sulfate**
E coli	**Escherichia coli**
EQ 5D VAS	**Euro Quality 5 Dimension Visual Analogue Scale**
ESA	**Erythropoiesis Stimulating agent**
F prausnitzii	**Faecalibacterium prausnitzii**
Fe	**Iron**
F	**Female**
HB	**Haemoglobin**
HBI	**Harvey Bradshaw Index**
HRQoL	**Health Related Quality of Life**
HCl	**Hydrochloric acid**
IBD	**Inflammatory Bowel Disease**

IL	**Interleukin**
IS	**Immunosuppressant**
IV	**Intravenous**
IDA	**Iron Deficiency Anaemia**
IQR	**Interquartile Range**
KOH	**Potassium hydroxide**
M	**Male**
MICA	**Microbial Community Analysis**
MW	**Molecular weight**
NIBD	**Non Inflammatory Bowel Disease**
N	**Number**
NYHA	**New York Heart Association**
NMR	**Nuclear Magnetic Resonnace**
OPLS	**Orthogonal Partial Least Squares**
OSC	**Orthogonal Signal Correction**
PLS	**Partial Least Squares**
PMS	**Partial Mayo Score**
PCR	**Polymerised Chain Recation**
PO	**Per Oral**
PCA	**Principle Component Analysis**
QoL	**Quality of Life**
RCT	**Randomised Controlled Study**
RevMan	**Review Manager**
RFLP	**Restriction Fragment Length Polymorphism**
RNA	**Ribonucleic Acid**
RDP	**Ribosomal Database project**
SCFA	**Short Chain Fatty Acid**

SF 36	**Short Form 36**
SIBDQ	**Short form Inflammatory Bowel Disease Questionnaire**
SI	**Similarity Index**
SD	**Standard Deviation**
STfR	**Soluble Transferrin Receptor**
SWI	**Shannon Weiner Index**
TE	**Thromboembolism**
TIBC	**Total Iron Binding Capacity**
TNF	**Tumour Necrosis Factor**
TRFLP	**Terminal Restriction Fragment Length Polymorphism**
UC	**Ulcerative Colitis**

1. INTRODUCTION

GENERAL

1.1. Definition of Iron Deficiency and Anaemia

Inflammatory Bowel Disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract as a result of the aberrant host immune response to the gut microbiota. It consists of Crohn's disease (CD) which is characterized by transmural inflammation with normal intervening intestines, and ulcerative colitis (UC), which is characterized by continuous colonic mucosal inflammation with a variable extend from rectum (1). Anaemia is defined as haemoglobin (HB) less than 130g/L in males and less than 110g/L in females. Components of iron studies such as ferritin and iron saturation are used in conjunction with serum C-Reactive Protein (CRP) to interpret one's iron store in patients with IBD. The definition of iron deficiency is less clear-cut in the setting of inflammation because ferritin is an acute phase reactant and it is frequently elevated in this setting despite deficient iron store. Ferritin ≤30µg/L is considered iron deficient; however, in the setting of inflammation, as indicated by an elevated CRP, serum ferritin is frequently elevated. It is generally accepted that ferritin <100 µg/L in the setting of elevated CRP indicates iron deficient (2-4). Transferrin saturation is calculated by dividing the serum iron by the total iron binding capacity (TIBC) and due to significant diurnal fluctuation in serum iron and TIBC, the interpretation of transferrin saturation can be difficult (5). One way to overcome this was to have blood draws around the same time of the day in order to minimize this fluctuation. Unlike ferritin, transferrin saturation is not influenced by inflammation and is a more reliable guide to monitor iron deficiency state in patients with IBD. Bone marrow biopsy with iron staining is the traditional gold standard in the assessment of body's iron reserve; however, the systematic review by Guyatt on the laboratory diagnosis of IDA concluded that serum ferritin immunoassay is as accurate and more practical in assessing iron store (6).

In the setting of active coexisting systemic inflammation, independent from serum ferritin, interpretation of 'functional' iron deficiency is made when the iron saturation is less than 16%

(7), although many other studies used 20% as the cut off (8,9). This approach is reflected in clinical practice and also in published clinical trials' definition of iron deficiency (10,11).

1.2. Prevalence and significance of iron deficiency anaemia in inflammatory bowel disease

Iron deficiency anaemia (IDA) is one of the most common management issues in IBD and it is of recurrent nature. During the course of IBD diagnosis, the prevalence of IDA had been reported to be as high as 75% in the older literature (12), up to 20% in the more recent studies and 30% had isolated iron deficiency. Moreover, Scandinavian data suggested IDA recurs about 10 months after treatment and iron deficiency without anaemia recurs about 19 months after treatment (13). Currently, there is no clear clinical predictor for who may need recurrent or ongoing iron replacement therapy (14,15). IDA significantly impairs one's quality of life with common symptoms such as fatigue; lethargy and poor concentration span (16-18). Therefore, the management of IDA or more importantly the management of iron deficiency before anaemia occurs is critical in the maintenance of a good quality of life. However, awareness of this fundamental issue is suboptimal as indicated by a recent survey of 236 gastroenterologists from 5 European countries. Only 82% (56-88%) of patient with anaemia had ferritin and 25% (19-33%) had iron saturation checked as part of the investigation (19).

The occurrence of IDA in patients with IBD is frequently multifactorial. Contributing factors include active blood loss from intestinal mucosal ulcerations, dietary aversion of iron containing food and inflammation related iron sequestration in the recticuloendothelial system – termed functional iron deficiency.

BASIC SCIENCE

1.3. The role of non-haeme iron

In addition to iron's role in oxygen transportation, it plays an important role in the non-haeme component of cellular function. Iron participates in the electron transfer during mitochondrial

respiratory chain reaction in energy (ATP) production, is the key component of non-enzymatic protein myoglobin in storing oxygen, and is a cofactor in Krebs cycle and oxidative phosphorylation. Therefore it is not surprising that iron replacement therapy in non-anaemic iron deficient subject improves many aspects of one's life. Examples include, 1) neurons are the most metabolically active cells in the body, neuron transmitters and their packaging are all iron dependent. Consequently, it is not surprising that iron deficiency has a negative impact on the neuromuscular junctions, as well as on cognition, independent of anaemia. (20-23), 2) Iron supplementation in non-anaemic iron deficient athletes and military personnel reported significantly improved exercise tolerance (24, 25). 3) Iron supplementation reduces the severity of restless leg syndrome (26, 27). 4) Correction of iron deficiency improves the symptom of fatigue (16, 28) and the quality of life in both anaemic and non-anaemic subjects (8, 29). Therefore, treating iron deficiency only when anaemia occurs is inappropriate.

Moreover, a recent clinical trial demonstrated the benefit of iron replacement therapy in chronic cardiac failure patients with iron deficiency +/- anaemia. This large RCT compared intravenous iron therapy versus placebo in 459 ambulant patients and revealed a significant improvement in the self-reported patient global assessment, NYHA (New York Heart Association) functional class, 6 minute-walk test and Euro Quality-5 Dimensions Visual Analogue scale at 24 weeks, in both anaemic and non anemic iron deficient subjects (8).

1.4. Iron transportation and its regulation

1.4.1. Iron absorption and transportation

Dietary inorganic iron is found in ferric (Fe^{3+}) form and it is converted to ferrous (Fe^{2+}) state at the duodenal enterocytes brush border prior to its absorption (fig 1, step 1) (7). Haeme-iron of animal origin is in ferrous form and easily absorbed. Some of the absorbed irons are stored as ferritin within the enterocytes and most are reduced to ferric form and bind to transferrin prior to reaching the systematic circulation (fig 1 step 3). Ferric state is more soluble and non reactive therefore safer for transportation. Transferrin then bound to transferrin receptor on the surface of erytheoid precursors and is subsequently endocytosed into the cytoplasm and endoplasmic reticulum. During this process, ferric iron is reduced to ferrous form and released into the cytoplasm for utilization. Uptake of iron by non-haematopoietic cells does not require transferrin receptor. Iron regulation occurs at the absorption level because it is not actively excreted. The

main ways in which iron is 'lost' include the shedding of enterocytes, dermal epithelia or blood loss.

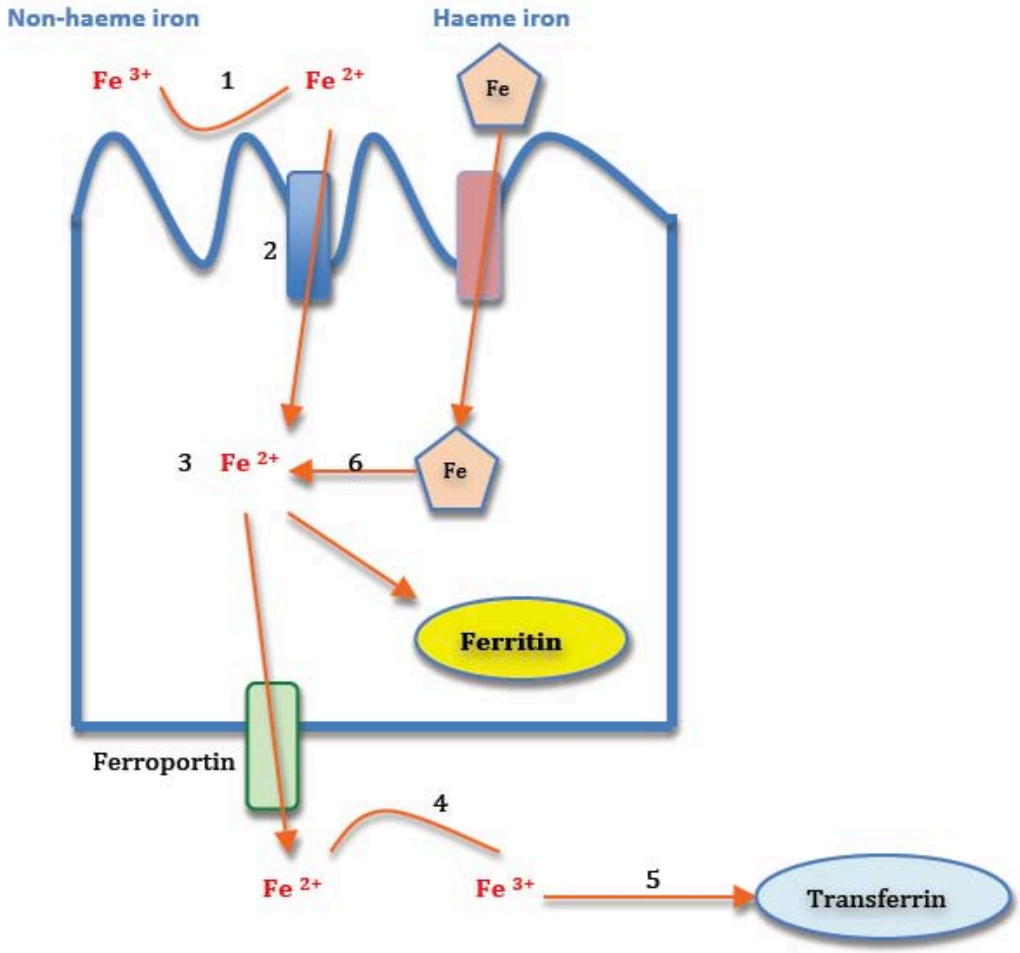


Figure 1-1 Mechanisms of duodenal absorption of haeme and non-haeme iron

Legend: **1.** Non-haeme iron is reduced from Fe^{3+} to Fe^{2+} by either gastric acid or duodenal cytochrome B. **2.** Fe^{2+} is taken up into mucosal cells via the divalent cation transporter 1(DMT1). DMT1 is upregulated during iron deficiency and down regulated during iron excess. **3.** Fe^{2+} either binds to ferritin or directly released into the circulation via ferroportin. 4. Hephaestin oxidizes Fe^{2+} to Fe^{3+}. **5.** Fe^{3+} binds to transferrin and released into the systemic circulation. **6.** Haeme iron is liberated from its porphyrin framework by haeme oxygenase and enters the common pathway as non-haeme iron.

1.4.2. The role of soluble transferrin receptor (sTfR)

Anaemia in IBD may be the result of iron deficiency and/or chronic inflammation. One way to differentiate between these two entities is by measuring soluble serum transferrin receptors (sTfR). TfR mediates the erythroid cellular uptake of ferritin and its expression is up regulated in the setting of diminished intracellular iron availability. TfRs are eventually shed into the circulation and the measured serum level is an accurate reflection of the total body TfR concentration and therefore erythropoietic activity. As iron deficiency begins to impair haemopoiesis, there is a corresponding increase TfR expression on the cell surface, therefore higher measurable serum transferrin receptor level. IDA is associated with raised sTfR concentration, which is in contrast with normal sTfR level in patients with anaemia of chronic disease. It is important to note that iron deficiency without impaired marrow haemopoiesis is not associated with raised serum TfR (30). Therefore the increase in sTfR is a reflection of increased haemopoiesis and not specific to iron deficiency (4, 31). Diagnoses associated with increased haemopoiesis such as hemolytic anaemia, thalassemias, myelodysplastic syndrome (32) , chronic lymphocytic leukemia (33) and the use of erythropoietin are associated with increased sTfR level. Measuring sTfR is helpful in identifying iron deficient anaemic patients and it is still a research tool only. The target study population for this thesis is patients with iron deficiency and anaemia is not required, however, we did not exclude patients with IDA.

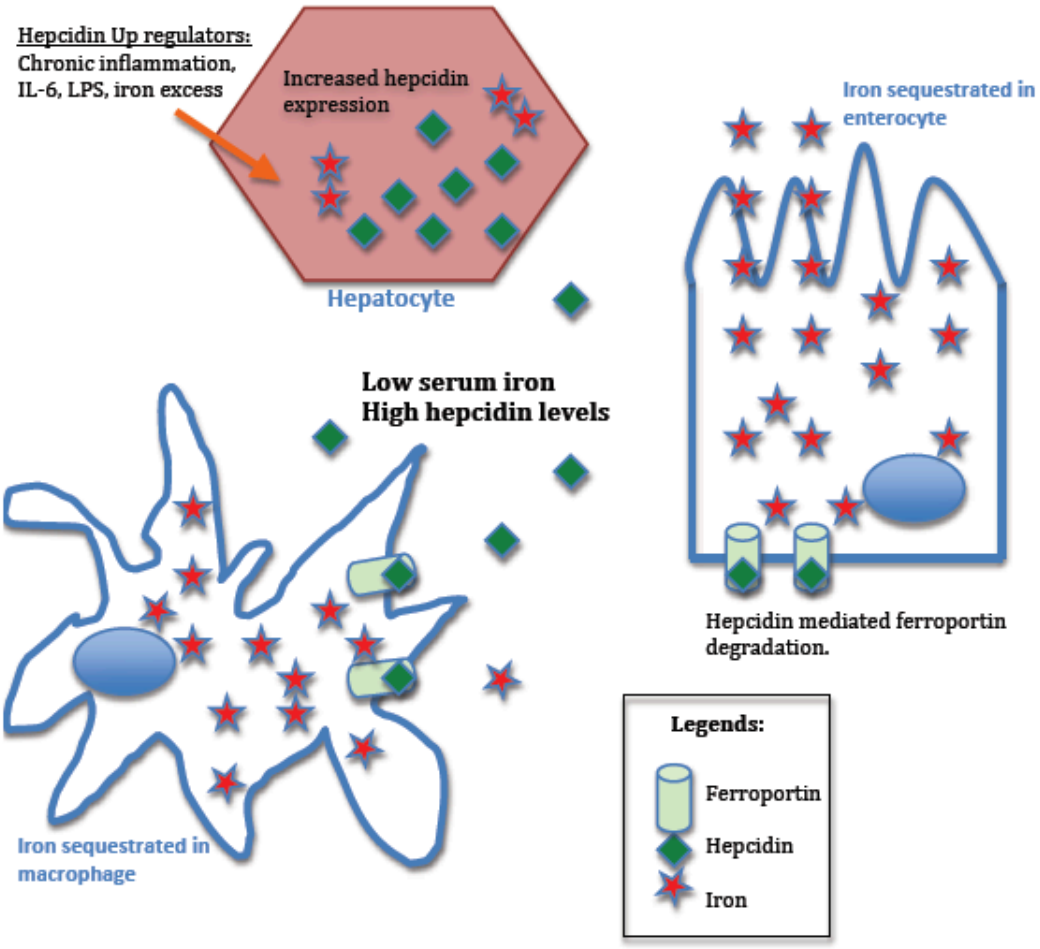
1.4.3. The role of hepcidin

Hepcidin is a 25 amino acids peptide synthesized by the liver and has the dual actions of being a regulator of iron homeostasis and a bactericidal protein (34). Its discovery was part of Krause and colleagues' screening of human blood ultra filtrate for antimicrobial peptides (35). The word hepcidin was coined from the composite of ***hep***atic synthesized bacteri***cid***al prote***in***. Increased hepcidin is the proposed link between inflammation and iron sequestration in the reticuloendothelial system leading to anaemia of chronic disease (36, 37). In the context of elevated hepatic iron store, elevated interleukin 6 and lipopolysaccharide stimulation, the hepatic synthesis of hepcidin is increased.

Mechanistically, hepcidin binds to ferroportin in the macrophages and the enterocytes thereby preventing the release of iron into the systemic circulation and sequestrate iron intracellularly. The binding of ferroportin by hepcidin leads to the internalization of ferroportin and its

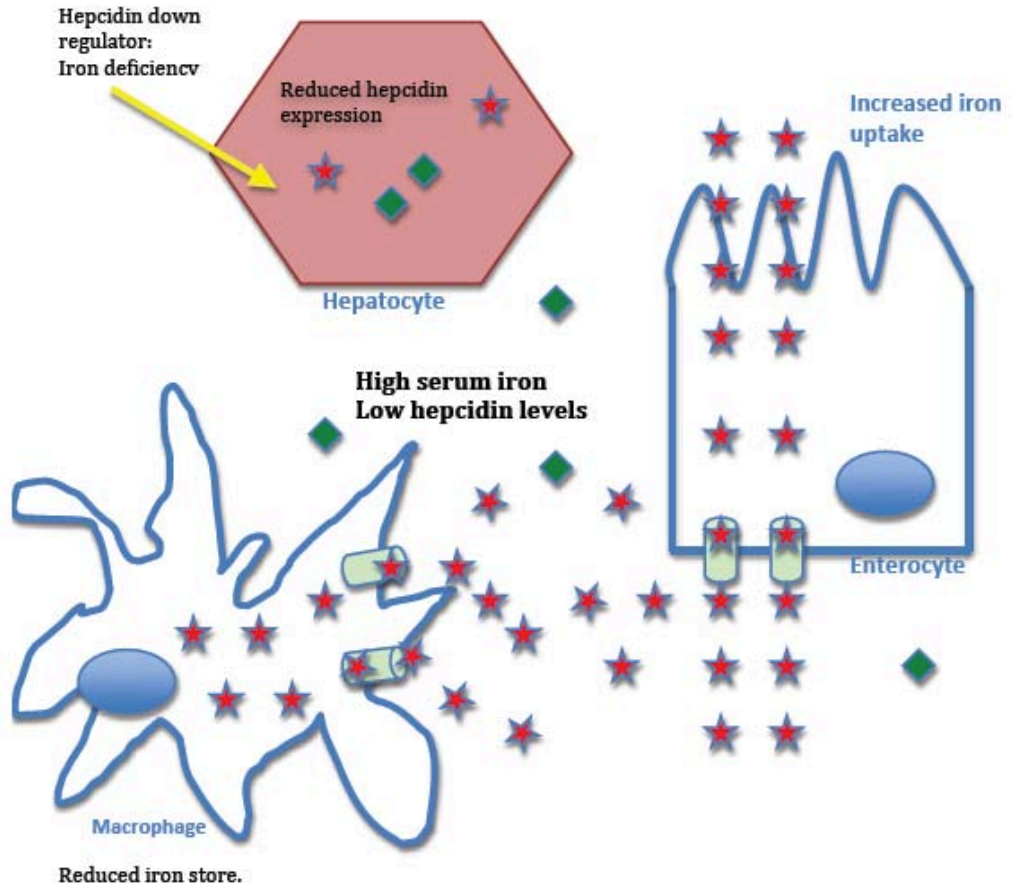
subsequent lysosomal degradation. (Fig 1.2) Interestingly, patients with juvenile hereditary haemochromatosis have virtually undetectable serum hepcidin and behave as the most severe form of hereditary haemochromatosis, i.e., as if they were iron deficient. Figure 1.3 illustrates the interaction among iron, hepcidin, ferroportin in the setting of iron deficiency. With reduced hepatic hepcidin production, more intestinal absorbed iron and more macrophage iron store is mobilized and released into the systemic circulation.

Figure 1-2 Hepcidin in the setting of chronic inflammation



This figure illustrates the mechanism of iron sequestration in the setting of chronic inflammation. Hepcidin binds to ferroportion and triggers ferrorportin's internalization and degradation thereby preventing the release of iron into the systemic circulation.

Figure 1-3 Hepcidin in the setting of iron deficiency



Reduced hepcidin production enables mobilization of intestinal absorbed iron to enter the systemic circulation via ferroportin and the mobilization of iron store from macrophage.

1.5. Gut microbiota and Inflammatory Bowel Disease

The gastrointestinal tract is home to many micro-organisms including bacteria, Archaea, fungi, viruses and parasitic nematode such as hookworm. Majority of the literature to date has focused on bacteria and this thesis elected to examine the effect of bacteria in particular on disease state.

1.5.1. Iron and Bacteria

Bacteria have a variety of iron acquisition systems because iron is a scarce and critical nutrient for their growth and survival. Siderophores belong to a family of small molecules produced by

bacteria that could effectively compete with mammalian iron binding proteins for free iron (38). Often, the virulence of a bacterium relates to its iron acquisition capability. This is beautifully demonstrated by *Vibrio cholerae*, causative bacteria for cholera, which has multiple mechanisms for extracellular iron scavenging, multiple dedicated iron transportation systems for both haeme and non-haeme iron as well as using transport systems from other microorganisms (39). Similarly, uropathogenic *E. coli* preferentially express yersiniabactin and salmochelin, both are siderophores, capable of scavenging iron to support their growth and survival in a virtual iron free environment of the urinary tract (40).

1.5.2. The role of nutrients in the shaping of gut microbiota

The intestinal microbiota is constantly shaped by our dietary intake of various food groups. For example, higher levels of *E. coli* and *C. difficile* were found in the stools of formula-fed infants compared to breast milk-fed infants and this has been linked to increased risk of eczema development (41). Similarly, non-digestible fermentable fibers known as prebiotic have been used to manipulate colonic microbiota towards a more favourable composition. (42) Iron is an important nutrient for bacterial growth and survival and it is a concern that oral iron therapy may deliver excess free luminal iron and selectively promotes the growth of pathogenic species resulted in further dysbiosis. Moreover, it is possible that this dysbiosis may further exacerbate IBD and becomes a vicious cycle.

1.5.3. Microbial dysbiosis

Microbial dysbiosis refer to the imbalance between the potentially harmful and the helpful microbes in the host and significant dysbiosis had been described in patients with IBD (43). Thus far, no single bacterium has been confirmed as the causative agent for IBD despite the suggestion that some had been implicated – *Mycobacterium paratuberculosis* (44) and *Adherent-invasive E. coli* (45). As most gut bacteria are difficult to culture and/or unknown, one approach is to examine the bacterial community as whole – microbiota, and examine the community genomic makeup or microbiome. For example, compared to a healthy population, Crohn's Disease (CD) is associated with a significant reduction in Firmicutes diversity, especially in the butyrate producing *Clostridium leptum*, *Clostridium coccoides* (46) and *F. prausnitzii*. (47)

Conversely, increases in Proteobacteria and Bacteroidetes have been reported (48). Interestingly no significant difference between healthy individuals' and ulcerative colitic subjects' tissue associated microbiota was found (48). Biopsy associated microbiota in untreated UC and CD patients are different with greater bacteria abundance in the ulcerative colitic patients (49).

1.5.4. Gut microbial diversity

Oral iron therapy is associated with reduced colonic microbial diversity by reducing the number of 'good' bacteria whilst increase the number of 'harmful' bacteria (50). This change in the microbiota composition and further dysbiosis has the potential to alter the clinical course of IBD as the mucosal adherent bacteria can modulate mucosal immune activity. Therapeutic manipulation of the colonic microbiota with prebiotic, probiotic or antibiotic had been shown to be somewhat efficacious in managing mild ulcerative colitis and in the prevention of postoperative recurrence of anastomotic ileal Crohn's disease (51, 52). Animal models have demonstrated that a paternal route of iron replacement therapy had less impact on the native colonic microbiota compared to an oral route of iron replacement but no study has been done in human IBD patients with iron deficiency.

1.6. Iron and oxidative stress

1.6.1. Mucosal oxidative stress

Animal models demonstrated that 'topical' iron supplement was associated with increased production of pro-inflammatory cytokines and worsening of IBD activity (53, 54) and depletion of luminal iron through dietary restriction could prevent chronic Crohn's ileitis. Possible mechanisms include a marked reduction in the cellular endoplasmic reticulum stress response and pro-apoptotic mechanisms (55). Moreover, high dose oral iron replacement in colitis animal model was associated with worse histological score for inflammation (56). A study involved healthy human volunteers, where additional oral iron sulfate supplement was given confirmed a higher quantity of faecal iron during iron supplementation and increased stools markers of oxidative stress (57). Conversely, use of chelating agents (desferrioxamine chelates Fe3+ and

1,10-phenanthroline chelates Fe2+, in an in vitro study on human colonic mucosal biopsies, has demonstrated a reduction in the amount of mucosal reactive oxygen radicals (58).

1.6.2. Serum oxidative stress

The only human IBD study involving patients with IDA and supplementation with either oral or intravenous iron revealed a higher plasma malondialdehyde (MDA) level, a marker of lipid peroxidation, in the iron sulfate group (Oral) and not in the iron polymaltose group (IV). However, neither group reported worsening of IBD activity. (59) In contrast, a non- IBD human study had suggested intravenous iron use and NOT oral iron use was associated with elevated plasma markers of oxidative stress and tissue damage (60). With these conflicting findings and no definite link to worsening of IBD activity, the significance of oxidative stress induce by iron therapy needs further clarification.

CLINICAL SCIENCE

1.7. Other factors to consider

Other factors to consider in the management of anaemic IBD patients include other nutritional deficiency such as folate (5% prevalence) and/or vitamin B12 (5% prevalence) (13), overt blood loss secondary to active disease, drug related bone marrow suppression (thiopurines, methotrexate) and anaemia of chronic disease. Moreover, the tendency of IBD patient to have deliberate lower dietary iron intake compared to normal population has been reported and this dietary avoidance may be due to subjective food intolerance (61, 62). The prevalence of drug induced anaemia is not known but it is suspected to be low as suggested by our clinical experience. Indeed, the aetiology of iron deficiency in patients with IBD is multifactorial and the approach should be wholistic. The first step is to take control of the underlining inflammatory process with close monitoring for treatment related side effects. Although the prevalence of coeliac disease in IBD is low when compared to the general population (63, 64), its exclusion is important because intravenous iron replacement therapy should be used instead.

1.8. Therapeutic options

Currently, iron may be replaced orally with iron pills, intravenous infusion or intramuscular injection, which is gradually phased out as iron absorption is erratic, it is painful, and iron may stain the skin as if one had a tattoo. The total iron deficit is calculated based on the Ganzoni's equation and it determines the amount of intravenous iron replacement needed.

Iron deficit = body weight (kg)*(target HB – actual HB g/L)*0.24 + 500mg (iron depot)

According to the Ganzoni equation, non anaemic patient would receive one 500mg of iron depot. However, it has been our clinical practice to give 3*300mg iron infusions in this population. For those with IDA, 1200mg of iron sucrose was given.

1.8.1. Role of erythropoiesis-stimulating agents (ESAs)

The use of ESA in iron deficient anaemic IBD patients not responding to intravenous iron therapy has been discussed in the literature. Theoretical arguments for its use included studies showing inappropriately low serum erythropoietin level for the degree of anaemia in some IBD patients (65, 66) and a higher erythropoietin level needed to overcome the antagonising effect of pro-inflammatory cytokines such as tumour necrosis alpha on the haemopoiesis. (67) Thus far, ESA is considered as an adjunct therapy in IBD patients not responding to adequate IV iron replacement (15, 65, 68, 69). Moreover, it does not work if the patient is iron deficient. Arguments against ESA use include concerns regarding the speed in which anaemia is corrected with resultant increased cardiac mortality in haemodialysis patients (70). Therefore, erythropoietin supplementation in iron deficient anaemic IBD patient is not the current standard of care.

1.8.2. Oral Iron replacement

Oral iron formulations include ferrous sulfate (300mg tablet contains 60mg of elemental iron), ferrous gluconate (300mg tablet contains 35mg of elemental iron) and, ferrous fumarate (300mg tablet contains 100mg of elemental iron). The advantages of using oral iron include being less expensive and less interference with one's life. But, it is associated with up to a 20% intolerance

rate and frequently reported side effects include nausea, abdominal bloating and pain as well as altered bowel habits (71, 72). Moreover, active inflammatory state is associated with impaired oral iron absorption compared to inactive disease state (73) and iron absorption appears to be saturable with no benefit in using a higher oral dose (74). Enteric-coated iron pills are less effective as the bulk of iron absorption occurs in the duodenum and proximal jejunum and its reduction to Fe^{2+} by gastric hydrochloric acid helps with iron absorption. Other arguments against oral iron replacement include poor patient adherence to therapy, slow improvement in iron store with the need for a prolonged course of therapy and its potential in worsening underlying IBD activity. Our study elected to use ferrous sulfate because it is the most commonly available formulation, being used in similar IBD clinical trials and the decision to use bid frequency was in anticipation of poor adherence rate with tid dosing.

1.8.3. Intravenous Iron replacement

The safety profiles of intravenous iron formulation have improved significant from the highly anaphylactic high molecular weight iron dextran to the newer formulation – iron sucrose. Table 1 summarizes the three intravenous iron formulations available in Canada as well as their safety profile. Thus far, there have been three randomized control trials comparing the clinical efficacy of intravenous and oral iron therapy in the management of iron deficient anaemia in patients with IBD. From these studies, the incidence of intravenous iron related side effects ranged from 1-3%. These include nausea and vomiting, localized and generalized rash, injection site reaction, thrombophlebitis, myalgia, arthralgia. Infusion related arthralgia could be easily prevented with methylprednisone premedication (75). More serious side effects included a case of thrombocytopenia possibly related to iron sucrose (11) and a case of cardiac death with iron carboxymaltose, although no causal link was established (10). Iron sucrose was chosen for this clinical trial because it has a better safety profile than low molecular weight iron dextran as well as greater familiarity of iron sucrose in the medical and nursing staff.

Table 1.1 Intravenous iron formulations available in Canada

	Low MW Iron Dextran	Iron Sucrose	Iron Gluconate
Trade names	Infufer®,DexIron®	Venofer	Ferrlecit
Availability in Canada	yes	yes	yes
Plasma half-life (h)	30	6	1
Dosing			
Iron concentration (mg/mL)	50	20	12.5
One dose iron repletion	yes	no	no
Test dose required	yes	no	no
Max. injectable single dose	20mg/kg	500mg	125mg
Premedication	yes	no	no
Safety Profile			
Life-threatening ADE (per 10^6 doses)	3.3	0.6	0.9
Death rate (per 10^6 doses)	0.78	0.11	0.25
Pregnancy category	C	B	B
Cost CAD$/per 100mg vial	14.70	37.50	23.44

1.9. The clinical efficacy comparison between oral and intravenous iron therapy

The question: '*is oral iron replacement as clinically efficacious as intravenous iron replacement in iron deficiency anaemic patients with IBD?*' has been addressed in a systematic review of all published randomized control trials comparing the efficacy of oral and intravenous iron replacement therapy in adult IBD patients with IDA (Chapter two). Primary endpoint was to compare the mean haemoglobin at the end of study between oral and intravenous therapy. Secondary endpoints included the mean change in ferritin, quality of life score, IBD activity status and the adverse reaction rate.

1.10. Medication Adherence

Adherence is defined as the extent to which a patient's behaviour matches the agreed recommendations from their health professionals' (76). It describes a therapeutic relationship between the patient and the healthcare professional and sees treatment as a partnership rather

than a one-way street where the patient complies with doctor's order passively (77). Methods of measuring adherence include i) direct methods – such as directly observed therapy or measure the blood or urine level of a medication or its metabolite or ii) indirect methods – such as pill counts or review of the prescription refills rates. The prevalence of non-adherence in inflammatory bowel disease ranged from 35% to 72% (78).

Non-adherence to medication is associated with disease relapse and increased hospitalization rate. (79) It was estimated to cost over $100 billion in the United States in 2007 and over £100 million in the United Kingdoms in 2009. In IBD, younger age (<40 years old) and short disease duration (<5 years) were associated with non-adherence, with odds ratio for non-adherence were 1.5 CI: 1.01-2.13 and 2.1 CI: 1.3 – 3.39, respectively (80). Other significant factors included psychological distress, patient's belief about medication and the doctor-patient discordance (76). Given at least 20% of patients reported intolerant to oral iron replacement, there would be concerns regarding patients' adherence to it and efficacy in improving the body iron store.

1.11. The cost of iron therapy

Cost of the intravenous iron replacement therapy is considerable compare to oral iron therapy. Intravenous iron itself is more costly than iron pills. Moreover, the need for a medically supervised environment, a registered nurse and all the accessories going with an infusion making it by far more costly than taking iron pills. It is estimated that the cost of a patient receiving three 300mg of iron sucrose infusions through the clinical investigation unit at the University of Alberta Hospital is about $CAD 1000. This is in comparison to $CAD 30 for three months supply of ferrous sulfate 300mg pills and likely one year's worth of oral iron is needed to accomplish as much as IV iron. Moreover, these costing calculations do not factor in the time loss and/or loss of income as patients would need to take at least 4-5 hours off work per infusion.

However, the cost of iron therapy in IBD should to be compared with the cost of other medications used in IBD such as infliximab, which average CAD$25 000 per year.

OBJECTIVES

1.12.1. Primary Objective

1. To compare the mean iron saturation level in patients treated with oral compared to those treated with intravenous iron replacement therapy after 2 month of iron replacement therapy. (discussed in chapter 4- clinical results)

1.12.2. Secondary Objectives

1. To compare the mean and the mean change in ferritin and hemoglobin, at 3-month from baseline, in those treated with oral compared to those treated with intravenous iron replacement therapy. (discussed in chapter 4- clinical results)

2. To compare the number of iron-therapy associated adverse events in those treated with oral to those treated with intravenous iron replacement therapy. (discussed in chapter 4- clinical results)

3. To correlate the above biochemical and haematological changes with patients' quality of life scores and IBD activity index in those treated with oral compared to those treated with intravenous iron replacement therapy. (discussed in chapter 4- clinical results)

4. To compare the colonic microbiome biodiversity and composition in those treated with oral compared to those treated with intravenous iron replacement therapy. (discussed in chapter 5- colonic microbiome)

5. To explore the urinary metabolomics profile of iron deficient versus iron sufficient states as well as the effect of oral and intravenous iron replacement. (discussed in chapter 6- Urinary metabolomics)

2. IRON REPLACEMENT THERAPY IN INFLAMMATORY BOWEL DISEASE PATIENTS WITH IRON DEFICIENCY ANAEMIA: A SYSTEMATIC REVIEW AND META-ANALYSIS.

Title Page

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Dr Thomas Lee, Dr Michael Kolber and Dr Sander van Zanten have nothing to declare. Dr Fedorak served as a speaker, consultant and an advisory board member for Merck Canada Inc and has received research funding from Merck Inc.

2.1. Abstract

Goals: To compare the clinical efficacy of intravenous versus oral iron replacement in adult inflammatory bowel disease patients with iron deficiency anemia.

Background: Iron deficiency anemia (IDA) is a common problem in patients with Inflammatory Bowel Disease (IBD) and can have a significant negative impact on quality of life.

Study: A systematic search for randomized controlled trials comparing the efficacy of intravenous iron infusion versus oral iron replacement therapy in the treatment of IDA in adult IBD patients. The primary outcome was the mean change in the hemoglobin at the end of study and secondary outcomes include mean change in ferritin, clinical disease activity index, quality of life score and the adverse reaction rate.

Results: The search strategy identified 757 articles while only three articles met the inclusion criteria for systematic review and meta-analysis. The total sample size of the included studies was 333 patients, 203 patients received IV therapy. Intravenous compared to oral iron was associated with a 6.8 g/L higher mean hemoglobin increment and 109.7ug/L higher mean ferritin increment. There was no difference in IBD activity index and Quality of Life scores between the two treatment groups. More adverse events were reported in the oral treatment group with the odds for discontinuation being 6.2 (CI 2.2, 17.1).

Conclusions: Intravenous iron treatment was better tolerated and more effective than oral iron treatment in improving hemoglobin and ferritin in adult IDA patients with IBD. However, the sample size of the combined studies was small and further clinical trials are required.

Key words: iron deficiency; anemia; inflammatory bowel disease; Crohn's disease; ulcerative colitis; iron sucrose; iron sulfate

2.2. Introduction

The prevalence of anemia during the course of inflammatory bowel disease (IBD) diagnosis has been reported to be as high as 75% with iron deficiency being the most common (3,12). Recent Scandinavian data indicated the prevalence of iron deficiency anemia (IDA) at 20%, and 30% had isolated iron deficiency (without anemia). After treatment is stopped, IDA normally recurs at about 10 months and iron deficiency recurs about 19 months after treatment (13,81). IDA is associated with reduced quality of life with complaints including fatigue, concentration difficulties and slower cognitive response (16,21). IDA has traditionally been treated with oral iron supplements, however, concerns regarding its variable absorption in patients with IBD (73) and the possibility that oral (PO) iron could exacerbate IBD has led to an increase in the use of intravenous (IV) iron. Animal models have suggested that PO iron therapy might exacerbate IBD through oxidative radicals mediated mucosal injury (55,56,82)or by alterations in the luminal microbiota (50,55). Moreover, animal models have demonstrated that oxidative radicals may promote or up regulate carcinogenic pathways as evident by a significantly higher dysplasia rate in the colonic mucosa of mice given PO iron replacement (83-85). It is unclear whether these animal data have any consequences in the management of human IBD patients with IDA. Thus far, one human study demonstrated PO iron replacement therapy in patients with Crohn's disease (CD) reduces plasma antioxidants levels (such as cysteine and glutathione) and increases CD activity (86). Another disadvantage of PO iron supplementation is the potential for side effects such as nausea, abdominal cramping or pain and altered bowel habits. Intolerance to oral iron therapy leading to discontinuation has been reported to be as high as 20% (87,88).

The advantage of using IV iron is that it bypasses the need for gastrointestinal absorption, which is known to be variable in IBD patients. Moreover, adherence with daily medication is less of an issue with IV iron as total iron replacement can be achieved with 1-4 doses of iron infusions depending on the iron formulation. However, there had been concerns with the safety profile of IV iron infusion, especially with the high molecular weight Dextran formulation with an increased risk of anaphylaxis. More recent formulations such as iron sucrose and iron carboxymaltose appear to have a better safety profile (8, 89, 90). Despite the improved safety profile, IV iron infusions add additional costs to the health care system which limits its widespread use.

Despite the fact that a few reviews have been published on this topic (3,4,91,92)including two qualitative systematic reviews (81,93), controversy still exists as to what is the best way to treat IDA in patients with IBD. The general awareness of the management options for IDA in patients with IBD is suboptimal among some gastroenterologists (19). Some authors propose that the route of iron replacement therapy should depend on the degree of anemia. For example, it has been suggested that IV replacement should be considered in patients with hemoglobin (HB) less than 100g/L and those with HB >100g/L receive PO iron replacement (94).

A problem with the existing reviews is the inclusion of studies that used concurrent Erythropoiesis-Stimulating Agents (ESA) in the treatment of IDA in IBD (15, 65). This is relevant as recent studies in hemodialysis patients using ESA in the treatment of anemia have raised concerns regarding the association between the rapidity of HB improvement and adverse cardiac outcomes. (70)Therefore the addition of erythropoietin to the treatment of IBD patients with IDA cannot be considered part of current accepted standard of care.

As more recent clinical trials have been published, the objective of this systematic review and meta-analysis is to compare the efficacy of IV versus PO iron replacement in the treatment of IDA in patients with IBD. The primary outcome measure was the mean change in HB and the secondary outcome measures included the mean change in ferritin, clinical disease activity indices, quality of life scores and adverse reaction rate.

2.3. Material and Methods

2.3.1. Search methods

A systematic search of the following databases was performed in January 2010. MEDLINE (1950 to February 2010, Ovid interface), EMBASE (1980 to 2010 Week 04, Ovid interface), Web of Science (2000- January 2010), Cochrane Database of Systematic Reviews, Cochrane Central Register of Controlled Trials (1991-January 2010), ClinicalTrials.gov (2000-January 2010) and Database Abstracts of Reviews of Effectiveness (1991-Januray 2010) was performed. MeSH subject headings and text-words used include inflammatory bowel disease, Crohn's disease, Crohn's colitis, ulcerative colitis, anemia, iron deficiency, ferric or ferrous compounds and administration & dosage, adverse effects, deficiency, therapeutic use. Abstracts from the American Gastroenterology meeting - Digestive Disease Week (2004-2009) and the European

Gastroenterology meeting - United European Gastroenterology Week (2004-2009) were hand searched for additional publication. This search strategy was updated in January 2011.

2.3.2. Selection criteria

Randomized controlled clinical trials comparing the efficacy of IV versus PO iron replacement therapy in adult IBD patients with IDA were included. Anemia was defined as HB <105 g/L for females and HB< 115g/L for males. Iron deficiency was defined as a serum ferritin <100ug/L or saturation <20% if C Reactive Protein (CRP) was raised. Studies with concurrent use of erythropoietin and those published in non-English or that employed a cross over study design were excluded. Cross-over study design was excluded because the assumptions made in crossover study design are that symptoms would return to baseline during the washout period and the disease state is constant overtime (95). The measured HB and ferritin improvement at the end of the second period of intervention is likely influenced by the treatment given during the first period. Therefore the crossover design is not appropriate in IDA patients undergoing treatment for IDA.

One author (TL) searched the database and screened the retrieved citations by examining the abstracts. Publications that met the broad inclusion criteria were selected for further review. By following a pre-determined inclusion and exclusion form, two reviewers (TL and MRK) independently graded the abstracts as relevant (meeting all of the pre-determined inclusion criteria), possibly relevant (meeting some, but not all of the inclusion criteria), unclear or rejected (failure to meet any of the inclusion criteria). Finally, both reviewers independently reviewed the full text of all relevant and possibly relevant articles. The final decision regarding eligibility was reached by consensus and any disagreement was resolved through discussion.

2.3.3. Outcome Measures

The primary outcome measure was the mean difference in HB at the end of study. Secondary outcomes include the mean difference in ferritin, the quality of life (QoL) score using Short Form-36 questionnaire, inflammatory bowel disease activity indices such as Colitis Activity Index (CAI) for ulcerative colitis and Crohn's Disease Activity Index (CDAI) for CD and the adverse event rate. Study authors were contacted to help with data clarification where needed.

2.3.4. Data extraction

Both reviewers independently extracted the data using a pre determined data extraction form. In studies where the outcome measures were reported as medians, they were accepted as means for the purpose of analysis. The inter-quartile Range (IQR) was converted to an estimated standard deviation (SD) using the formula 'IQR/1.35' and the range was converted to an estimated standard deviation using the formula 'range/4' (96). The study by Kulnigg et al expressed their results as median and range; we successfully contacted the author and obtained the results expressed as mean and standard deviation. The mean end of study HB and ferritin in the study by Lindgren et al. were presented graphically; therefore these values were directly taken from the graph. After attempts to contact the authors of the study were unsuccessful to provide standard deviations pertaining to the end of study HB and ferritin, it was decided to use the standard deviation from Kulnigg's study instead, as the two studies had similar methodology and study population (97). TL and MRK assessed the methodological quality of the studies independently based on the Cochrane Collaboration's tool for assessing risk of bias form (98). A final decision regarding the overall risk of bias was reached through discussion.

2.3.5. Statistical analysis

Data was analyzed using Review Manager (RevMan) Version 5.1. [Copenhagen: The Nordic Cochrane Centre, the Cochrane Collaboration, 2011]. The methodology and outcome measures of the included studies were similar and this allowed for pooling of results. All data were analyzed on an intention-to-treat basis. A random effect model was used as it better reflects clinical practice and provides a more conservative estimate of effect size. Heterogeneity of the studies was assessed by I^2. Publication bias was not assessed because the small number of included studies.

2.4. Results

The literature search identified 757 potential articles, 25 of which were duplications. After initial review of the titles and abstracts of 732 articles, 719 articles were further excluded. Two

reviewers independently examined the full text of the 13 remaining articles resulting in exclusion of further 10 retrieved articles (figure 2.1). Table 2.1 summarizes the characteristics of the included studies and table 2.2 summarizes the reasons for excluding the 10 retrieved articles. The three included studies have the total sample size of 333 patients, 203 patients received IV iron replacement and 130 patients received PO iron replacement. An updated literature search was performed in January 2011 and it did not yield any new relevant clinical trial for inclusion in this review.

For the three studies, treatment allocation was done by an external clinical trial company (Kulnigg study), by computer generated random number table (99) and by an Internet based method.(100) The allocation was not concealed in the Schroder et al study as a computer generated random number table was used to assign treatment group. There was no blinding of participants or study staff members in any study; however, study personnel were blinded to treatment allocation in the Lindgren study.

Table 2.1 Characteristics of the included studies

	Route	N	Baseline HB (g/L)	Baseline ferritin (µg/L)	EOT HB (g/L)	EOT Ferritin (µg/L)	mean total Fe given (g)	Rx time (wk)
Schroder 2005 (median, IQR) F: Hb≤105g/L M: Hb≤115g/L TSAT ≤20% or Ferritin≤20ug/L	PO	24	96(93-101)	8(5-39)	117 (111-129)	24 (11-49)	Fe Sulfate 4.2g (4.2-8.4)	6
	IV	22	98(88-104)	12(5-37)	123 (109-126)	240 (186-427)	Fe sucrose 1.4g (1.4-1.5)	6
Kulnigg 2008 (median, range) Hb<100g/L TSAT <20% or Ferritin <100ug/L	PO	60	91(53-111)	6.5(1-383)	121 (65-174)	28.5(**2-255**)	Fe Sulfate 16.8g	12
	IV	136	87**(50-115)**	5 (1-399)	123 (60-159)	43.5(**2-586**)	Fe carboxymaltose 1g	12
Lindgren 2009 (mean ± SD) Hb<115g/L Ferritin<300ug/L Low TSAT	PO	46	103.8**±11.4**	12.4**±14.5**	114 (**using SD from Kulnigg**)	70 (**using SD from Kulnigg**)	Fe Sulfate 38.4g± 20	20
	IV	45	104.9**±9.0**	14.0**±17.6**	129 (**using SD from Kulnigg**)	140 (**using SD from Kulnigg**)	Fe sucrose 1.7g± 0.3	20

Legends: IQR: Interquartile Range; SD: Standard Deviation; HB: Hemoglobin; EOT: End of Treatment; Fe: Iron; F: Female; M: Male

Figure 2-1 Flow chart of literature search outcomes

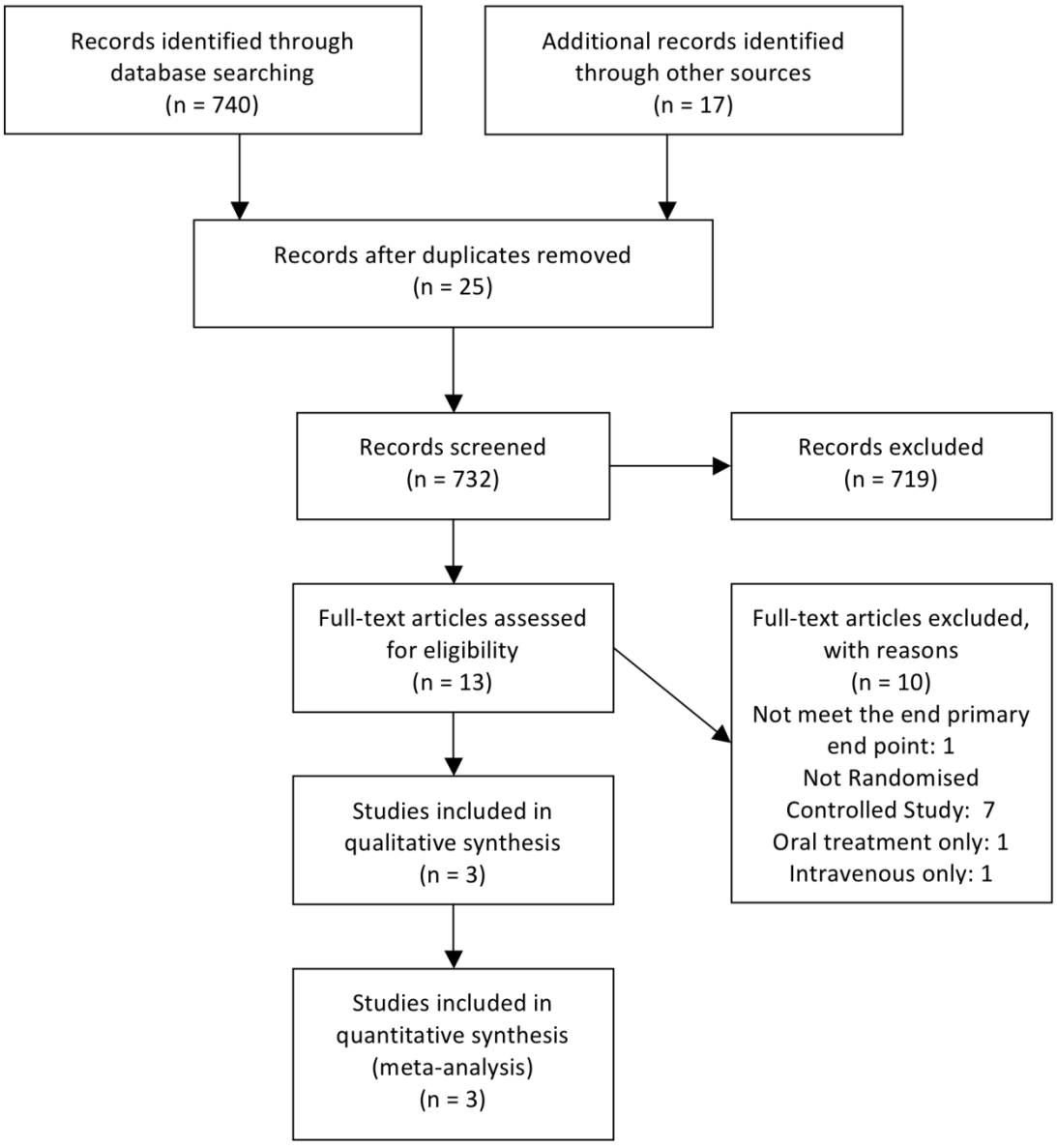


Table 2.2 Characteristics of excluded studies

Authors	Year	Journal	Reason for exclusion
Krafft A et al	2000	Acta Obstet Gynecol Scand	Case reports on intravenous iron therapy in pregnancy
De Silva A et al	2003	Inflamm Bowel Dis	Retrospective review, oral iron therapy
Rosado B et al	2003	Gastroenterology DDW Supplement	Retrospective review of intravenous iron replacement therapy
Bodemar G, et al	2004	Scand J Gastroenterol	Retrospective review of iron sucrose infusions
De Silva A et al	2005	Aliment Pharmacol Ther	Prospective study with oral iron therapy in IBD and non-IBD patients
Erichsen K et al	2005	Scand J Gastroenterol	Prospective crossover design; sub-therapeutic dose of iron used how defined Patients were treated with either iron fumarate 120mg/d for 2 weeks or 600mg IV iron sucrose. The treatment duration was too short to enable measurable improvement in Iron study or Hb.
Maslovsky I	2005	Am J Hematol	Prospective study giving oral iron intolerant patients iron infusions
Gasche C et al	2006	Semin Hematol	Review article
Katsanos K et al	2007	J Crohn's and Colitis	Prospective iron infusion study
Gisbert J Pet al	2009	Inflamm Bowel Dis	Prospective non-randomized study

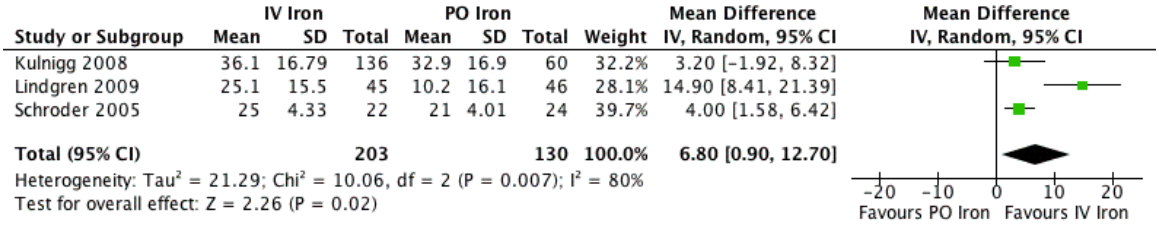
There was incomplete outcome data reporting by Lindgren. The end of treatment HB and ferritin were presented graphically and no standard deviation was reported. The other two studies presented all relevant outcome data, including the reasons for screen failures. An external clinical trial company on behalf of the sponsor in the Schroder and Kulnigg study performed the data analysis. It is unclear from the manuscript if the authors had full access to the collected data. It is also unclear why Kulnigg performed an interim analysis that resulted in the early termination of the study. Based on the results from the interim analysis the authors felt that sufficient statistical power was achieved with a lower recruitment number. Therefore 52 fewer subjects were recruited. However, it is known that by performing an interim analysis power may be overestimated. This could potentially bias the results in favour of the intravenous cohort. Finally, all three studies received financial sponsorship from the makers of IV iron and this could influence the study outcomes and the decision regarding its publication. The risk of bias from the study design and reporting point of view is low for study by Schroder et al. Risk of bias was high in the study by Kulnigg et al because the reason for conducting the interim analysis which led to early termination of the study was unclear. The Risk of bias was also high in study by Lindgren et al because of incomplete data reporting. Moreover, having pharmaceutical support and the possibility of limited access to the study data were the basis for the overall high risk of bias assessment.

2.4.1. Primary Endpoint

Mean change in hemoglobin

The range of the mean baseline HB was 89.7-103.8g/L for PO and 85.4-104.9g/L for IV route and the range of the mean end of study HB was 114-122.6g/L for PO and 121.5-129g/L for IV route. Figure 2.2 shows the forest plot comparing the mean difference in the amount of HB improvement between IV and PO iron replacement from baseline to end of study. The IV route was associated with a greater improvement in the HB level than the PO route, weighted mean difference in HB of 6.8g/L (CI 0.9, 12.7). This was statistically significant, p = 0.02. There was significant heterogeneity in the data with I^2=80%. Heterogeneity may be explained by the difference in the magnitude of HB improvement not the direction of treatment effect and the duration of iron therapy and follow up, which ranged from 6 weeks to 20 weeks.

Figure 2-2 The mean hemoglobin improvement between IV and PO routes of iron replacement therapy. CI: confidence interval.

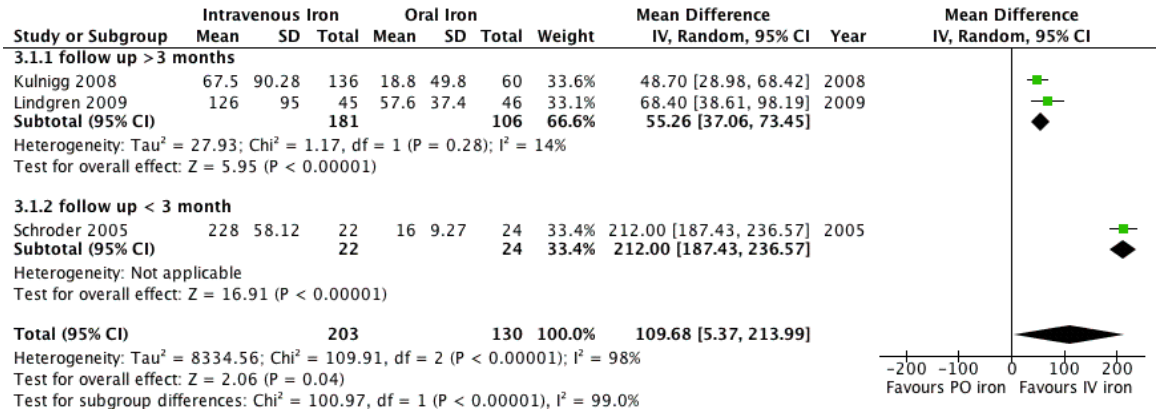


2.4.2. Secondary Endpoints

2.4.2.1. Mean change in the end of treatment ferritin

The baseline ferritin ranged from 5-383ug/L for PO and 5-399ug/L for the IV route. Figure 2.3 demonstrates that IV iron replacement was superior in improving serum ferritin level over PO iron replacement therapy: the mean difference was 109.7ug/L (CI 5.37, 214), p=0.04. There was significant heterogeneity in the data with I^2= 99%. The high I^2 value observed reflects the difference in the magnitude but not the direction of ferritin improvement and this is likely explained by differences in the duration of iron therapy, which ranged from 6 weeks to 20 weeks.

Figure 2-3 The mean difference in ferritin improvement between IV and PO routes of iron replacement therapy. CI: confidence interval.



2.4.2.2. Adverse Events/Discontinuation

Table 2.3 describes the number and the nature of adverse event that led to the discontinuation of iron replacement therapy. Five out of 203 patients who received IV iron discontinued because of infusion related reactions (2), small bowel hemorrhage (1), thrombophlebitis (1) and thrombocytopenia (1). In comparison, 21 out of 130 patients in the PO iron replacement cohort discontinued iron replacement because of gastrointestinal related side effects such as nausea, abdominal pain and diarrhoea. The odds ratio for discontinuing PO iron treatment due to side effects compared to IV iron replacement was 6.2 (CI 22.1, 17.1) (figure 4). One patient with a history of cardiac disease died of cardiac arrest one day after receiving iron carboxymaltose. The study authors reported that 'the event was considered unrelated to study medication but related to the underlying cardiac disease' (10).

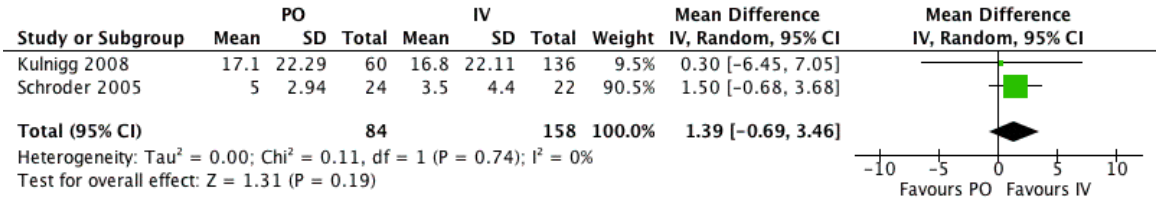
Table 2.3 Number of patients discontinued iron replacement therapy due to adverse event as reported by the study authors

	Studies	Oral	Intravenous
Number of patient discontinued	Schroder	5/24: Gastrointestinal side effects	2/22: rash, nausea, edema; thrombophlebitis
	Kulnigg	5/60: flare of ulcerative colits; diarrhea; asthma; vomiting; Stomach pain	2/136: (+1 death) erythematous rash; small bowel hemorrhage
	Lindgren	11/46: Gastrointestinal symptoms	1/45: (thrombocytopenia)

2.4.2.3. Quality of life score

The studies by Schroder et al and Kulnigg et al reported an increase in SF-36 score at the end of iron replacement therapy irrespective of the route of replacement: 7-17 points increment with IV iron and 8-17 points increment with PO iron therapy. Both routes had a comparable improvement in SF 36 scores, the pooled mean difference between PO and IV was 1.4 (CI -0.7, 3.5), which was not statistically significant (Figure 2.5). Lindgren et al did not report on the effect of iron therapy on quality of life.

Figure 2-4 The mean change in SF-36 score between IV and PO routes of iron replacement therapy. CI: confidence interval.



2.4.2.4Effects of treatment on disease activity

Schroder et al and Kulnigg et al did not demonstrate worsening of IBD activity in their study participants. The median Crohn's Disease Activity Index (CDAI) and the median Colitis Activity Index (CAI) were lower at the end of the study compared to baseline in both IV and PO iron replacement cohorts suggesting an improvement in the clinical disease status. The mean serum C-reactive protein levels at the end of study were normal with both routes of iron therapy.

Both Schroder et al and Kulnigg et al listed the use of concurrent medications including 5 amino-salicylates, immunomodulator such as azathioprine and corticosteroids. During the 6 weeks of iron replacement therapy in the study by Schroder et al, 25% of study participants had ongoing active IBD as indicated by a persistently elevated disease activity index. Successful prednisone tapering occurred in 75% of the study participants suggesting that clinically these patients were improving. The study by Kulnigg et al did not report on concurrent medication usage.

2.5. Discussion

The comprehensive literature search for randomized controlled trials comparing IV and PO iron therapy in iron deficient anemic IBD patients identified three studies, which combined, included a total of 333 patients. There was a small (6.8 g/L) but statistically significant difference in favour of IV iron therapy in improving HB levels. Whether this small difference is also clinically important is a matter of debate as there is no agreement in the literature and none of the studies a priori defined what amount of improvement would be considered clinically important. For the outcomes of serum ferritin levels and rate of adverse events IV iron therapy clearly favoured over PO therapy. There were no differences in quality of life or IBD activity but the number of patients available for these outcome measures is small.

This review has several methodological limitations. Most importantly the total sample size was small. In addition there were problems with data reporting, especially in the study by Lindgren et al (100). That study did not report the standard deviations for the end of study HB and ferritin levels. For that reason these values were derived from the study by Kulnigg et al, as they used a similar study design and patients.

Another limitation is that the duration of post iron replacement therapy follow up, varying from 8 to 20 weeks, was relatively short which makes it difficult to interpret if the higher ferritin level is of clinical significance in terms of a more durable HB improvement. Further clinical trials with a longer duration of post infusion follow up are needed to investigate this aspect. Some may also argue that the PO dose of iron replacement therapy was low which would bias the results in favour of IV therapy. In that regard the severity of side effects of oral therapy is important as the withdrawal rate was higher in patients receiving PO iron therapy. Higher doses of PO iron may therefore affect patient compliance further.

All three studies were analyzed on an intention to treat basis. Using Cochrane Collaboration's risk of bias tool, the overall risk of bias of the included studies was determined to be high on multiple levels, including incomplete data reporting of the primary end point by Lindgren et al, an interim analysis which led to early termination of study by Kulnigg et al and industry sponsorship in all three included studies.

The cost of using IV iron replacement therapy is also important. Direct costing components include the intravenous iron itself. In Canada the cost of one vial of iron sucrose containing 100mg iron is $37.50 which is more expensive than oral iron pills ($30/100 tablets of 300mg iron sulfate). The need for a medically supervised environment to give the iron infusion ($238/infusion) and nursing time (4 h infusion time + 1 h preparation/observation time, @$52/h) also need to be considered as well as the indirect costs related to travel costs and possible time lost from work. The estimated total cost for 900mg iron sucrose infusion is CAD $1831.50 ($337.50 for iron sucrose + $714 for infusion facility fee + $780 for nursing). In contrast, 100 tablets of 300mg iron sulfate costs CAD $30. A cost effective study comparing different intravenous iron formulations (iron carboxymaltose versus iron sucrose) has been done in anemic IBD patients (101)but none comparing PO versus IV iron therapy for the treatment of IDA in adult IBD patients. Compared to iron sucrose, treatment with iron carboxymaltose would save CAD $475 (US $460) per patient because total iron replacement can be achieved with fewer iron carboxymaltose infusions.

In conclusion, IV iron replacement therapy is superior in improving HB and ferritin levels in IBD patients with iron deficiency anemia but the overall sample size of included studies was small. The difference in the mean HB increment was small. IV iron was associated with fewer adverse events in patients with IBD. Further studies are needed to examine this important area to help establish the optimal management of iron deficiency in these patients and to determine whether IV iron therapy is cost effective.

2.6 Acknowledgments

Marlene Dorgan: assistance in comprehensive literature search.

Donna Dryden: systematic review methodology advice

Ben Vandermeer: statistical advice

3. METHODS

PART A CLINICAL EXPERIMENTATION

3.1. Clinical Subject and Study Process (Results in Chapter four)

3.1.1 Subject selection

Eligible subjects were identified through the University of Alberta hospital and Royal Alexandra Hospital gastroenterology and inflammatory bowel disease outpatient clinics and inpatient services.

3.1.1.1. Inclusion criteria

1. Iron deficiency as defined by ferritin<30ug/L or iron saturation <16% measured within 2 weeks of enrolment.
2. No oral iron therapy within 2 weeks of enrolment.
3. No intravenous iron therapy within 3 months of enrolment.
4. 18 and over year of age and able to give written consent.

3.1.1.2. Exclusion criteria

1. Coeliac disease
2. Known hypersensitivity to iron sucrose
3. History of intolerance to oral iron sulfate
4. Severe or multiple medical co-morbidities
5. Active IBD that may require surgery within 12 weeks of enrolment.
6. Ferritin >200ug/L
7. Pregnant

3.1.2. Study design

Open label randomized control study.

3.1.3. Treatment allocation

Written consent was obtained prior to Internet based randomization for treatment group allocation. The randomization ratio was 1:1 for oral (PO): intravenous (IV) therapy. In clinical

practice, the standard dose of oral iron replacement therapy in the setting of IDA is ferrous sulfate 300mg PO BID for a minimum of three months is used. For simplicity, all subjects in the PO group received 300mg iron sulfate bid for 3 months regardless of anaemic status. In the IV group, *non anaemic iron deficient* patients were given 900mg of iron sucrose and those with *iron deficiency anaemia* were given 1200mg of iron sucrose. Iron sucrose infusions were given at 300mg per infusion over 4 hours, one infusion per week, i.e. over 3-4 weeks. A copy of the iron sucrose infusion protocol is attached as appendix 3.1.

The study duration was determined to be three months in order to accurately measure the effect of iron therapy on haemoglobin in anaemic subjects as the life span of erythrocyte is about 120 days.

The total iron deficit was calculated based on the Ganzoni's equation and used as a guide to determine the amount of intravenous iron replacement needed. According to the formula, non anaemic iron deficient subjects would receive 500mg of iron sucrose in total; however, we elected to give 900mg iron sucrose in this setting in order to avoid under-replacement. Moreover, the study was design to mirror current institutional clinical practice.

Iron deficit = body weight (kg)*(target HB – actual HB g/L)*0.24 + 500mg (iron depot)

3.1.4. Sample size calculation

The primary end point was iron saturation at 2 month. Iron saturation was chosen as it is not affected by the inflammatory state unlike ferritin. The two months end point was chosen because published clinical trial data suggested maximum difference in iron saturation level between the IV and PO group was at 2 months. (10)Based on a two sample 2-sided t-test, a sample size of 85 will provide 80% power to detect a minimally important clinical difference of 5% between oral iron and IV iron groups, with a standard deviation of 13 in each group. Assuming a drop out or loss to follow up rate of 15%, the sample size was increased to 100. Based on these assumptions, 50 patients per arm were needed, i.e. a total of 100 patients with IBD and 100 patients without IBD are needed. Although the primary endpoint was at 2 months, all the biochemical and clinical outcome measurements were collected at 3 months as well in order to provide the entire clinical picture.

3.1.5. Blinding

This was an open label study where the patient and investigators were not blinded. However, the final results were coded prior to analysis, therefore the final analysis was performed in a blinded fashion.

The study was not double blinded because of the unavailability of placebo iron sucrose and placebo ferrous sulfate pills. The costs involved in using 'coloured' iron infusion giving sets would be high and do not ensure blinding as iron sucrose was mixed in the Clinical Investigation Unit and double checked by another registered nurse. The costs involved in the making of placebo iron pills which is able to discolour the stools is beyond the budgetary allocation for the study, Moreover, we were unable to allocate the existence of such pills.

3.1.6. Statistical Methods

The analysis was performed as per protocol. One and two sample t tests were used to compare the biochemical outcomes such as ferritin, iron saturation, haemoglobin and quality of life score. Non-parametric analysis: Wilcoxon Signed Rank test and Mann-Whitney test were used in analyzing disease activity index and Microbiome diversity index. Intention to treat analysis was not performed as the reasons for drop out or adverse event may not be related to iron replacement therapy.

3.1.7. Efficacy and Safety Monitoring

Any iron replacement therapy related adverse event (AE) was recorded in a descriptive fashion and all significant AEs were discussed with the principle investigator. Appropriate standard of care action was taken as needed for each AE.

3.1.8. Study activity schedule (Table 3.1)

As part of the baseline screening blood draw, anti-tissue transglutmate antibody titre and serum IgA level were checked to exclude coeliac disease. Coeliac patients have duodenal inflammation which impairs oral iron absorption therefore should receive IV iron and not eligible for the study. Nutritional markers Vitamin B12 and Folate were checked and replaced as needed prior to iron therapy.

Reticulocyte count was performed as a surrogate measure of adequate bone marrow response to anaemia.

C reactive protein was measure as a surrogate marker for systemic inflammation. It was performed at baseline, 2 and 3 months to assist in the interpretation of serum ferritin level.

Serum ferritin, iron saturation, haemoglobin were performed at baseline, 2 and 3 months as a measure of response to intervention.

Stool specimens were collected at baseline and 3-month to examine the microbiome composition. (see Chapter five)

Urine specimens were collected at baseline and 3-month to examine the urinary metabolomics. (see Chapter six)

Optional sigmoid mucosa biopsies were collected at baseline and 3-months for the examination of mucosal microbiome and mucosal markers for oxidative stress (Collaboration with Prof Dirk Haller, Munich Germany).

Euro-Quality 5-Dimension Visual Analogue (EQ5D VAS) were performed at baseline and 3-months and Short form Inflammatory Bowel Disease Index (SIBDQ) were collected at baseline, 2 and 3-months to monitor the change in the quality of life during and after iron therapy.

Disease activity indices; Harvey Bradshaw Index (HBI) for Crohn's disease and partial Mayo score were collected at baseline, 2 and 3-months to monitor the change in the disease activity status during and after iron therapy.

The use of concurrent antibiotics/ probiotics/ prebiotics during the study was noted. Where there is the use of antibiotics, stools specimens were collected 2 weeks after completion of antibiotics.

Table 3.1 Study Activity schedule

	Baseline (wk 0)	Week 8	Week 12
Haemoglobin	X	X	X
Reticulocyte	X		
Ferritin	X	X	X
Iron saturation	X	X	X
Anti-tTG /IgA	X		
Vit B12	X		
Folate	X		
CRP	X	X	X
Stool specimen	X		X
Urine specimen	X		X
Flex Sig (optional)	X		X
EQ 5D	X		X
SIBDQ	X	X	X
HBI/partial Mayo	X	X	X
Adverse event		X	X
Adherence			X

3.1.9. Overview of specimen handling and processing

3. 1.9.1. Blood draws for haematology and biochemistry were performed at the local pathology laboratories as part of standard patient care.

3.1.9.2. Urine specimens were collected into a sterile sodium azide coated urine cups and stored in a -80 °C freezer within 4 hour of collection. There was no dietary restriction or a specified time of day for the urine collection.

3.1.9.3. Stools specimens were collected on the day of randomization where possible or before the initiation of iron therapy. They were fresh frozen until ready for processing in batches.

3.1.9.4. Four sigmoid mucosal biopsies were collected, snap frozen in liquid nitrogen and stored at -80°C, Two were used to examine the gut microbiome composition and two were sent to Prof Dirk Haller's lab in Germany.

3.1.10. Quality of Life Questionnaires

3.1.10.1. EuroQoL 5 Dimension (EQ 5D) and Visual analogue scale – Appendix 2.2

EQ 5D is a simple, generic, self-administering questionnaire used to describe health related quality of life (HRQoL) (102). It consists of five dimensions: mobility, self-care, usual activities, pain ⁄discomfort and anxiety ⁄depression and a visual analogue scale (VAS) for general well being scored that ranged from 0 – worse possible imagined health state and 100 – best possible imagined health state. Each dimension has three levels of severity: no problem (1), some problem (2) and extreme problem (3). EQ 5D has been validated against Short Form 36 (SF-36), a representative questionnaire for general QoL and against Inflammatory Bowel Disease Questionnaire 32 (IBDQ 32) for validity (does the measure correspond to what is predicted by well developed theory), reliability (internal consistency of results) and responsiveness (ability of the measure to detect important changes in HRQOL) (103). EQ 5D was selected for this study because it is a generic QoL assessment and user friendly - short and easy to complete, making it an excellent complementary questionnaire to a disease specific QoL questionnaire.

3.1.10.2. Short form of the IBDQ (SIBDQ) – Appendix 2.3

Inflammatory bowel disease questionnaire (IBDQ) is a validated IBD specific questionnaire used in clinical trials to assess patient's response to intervention in terms of changes in HRQoL (104). However, due to its lengthy nature, two short forms of the IBDQ (SIBDQ) [10 items, 4 dimensions: bowel symptoms (3 items), systemic symptoms (2 items), emotional function (3 items) and social function (2 items)] were developed and validated. The 10 items in SIBDQ were selected by logistic regression methods. The first version was validated from a cohort of Crohn's disease and ulcerative colitis patients in 1996 (105) and the second was developed and validated within ulcerative colitis patients in 2000 (106). Despite only have 3 out of 10 questions in common between these two versions, latter version was not shown to be superior to the first version in terms of validity and responsiveness. The SIBDQ by Irvine et al was chosen for this

study as it was validated in both CD and UC cohorts.

3.1.11. Disease Activity Questionnaires

3.1.11.1. Crohn's Disease: Harvey Bradshaw Index (HBI) – Appendix 2.4

Crohn's disease activity index (CDAI) is an eight items disease activity questionnaire mostly used in the setting of clinical trial (107). Due to its lengthy nature, a simplified version with 5-item questionnaire - Harvey Bradshaw Index (HBI) was developed. It has 93% correlation with CDAI (108). Therefore HBI was chosen to clinically assess CD activity during iron therapy. By convention, an increase of HBI by 3 points or more indicates worsening of disease and HBI≤ 5 indicates clinical remission.

3.1.11.2. Ulcerative colitis: Partial mayo score (PMS) - Appendix 2.5

Multiple disease activity indices had been developed since 1955 for ulcerative colitis. Some were a composite of clinical and endoscopy score – mayo score and disease activity index (DAI) and others were a combination of biochemical and clinical score – Truelove and Witts severity index and clinical activity index (CAI) (109), all of which had been used in clinical trials to assess response to treatment. The Mayo score has four components – bowel frequency, per rectal bleeding, physician global assessment and endoscopy score. It was used in the pivotal infliximab clinical trial for the management of ulcerative colitis. It has good correlation with SIBDQ and SF 36 (110). Partial Mayo Score (PMS) is based on Mayo Score without the endoscopy sub-score. PMS performed as well as mayo score in identifying patients with clinical response, it has good sensitivity and specificity in identifying patients in clinical remission or with clinical improvement (111). By convention, 2 points reduction in PMS indicates clinical improvement and PMS ≤2 indicates clinical remission. PMS was chosen in this study because it is a widely accepted non invasive tool in monitoring UC activity and is user friendly.

The study was approved by the Human Research Ethics board.

PART B BASIC SCIENCE EXPERIMENTATION

3.2. Colonic Microbiome Assessment (Results in Chapter five)

3.2.1. Specimen Handling

3.2.1.1. Faecal Material Collection

Stools were collected from patient on the day of their baseline and 3-month study visit. Stools were then aliquoted into two 2mL tubes and kept frozen at -80°C. Where bowel preparation was required for a colonoscopy for flexible sigmoidoscopy, stool was collected prior to the administration of bowel prep.

3.2.1.2. Sigmoid Mucosal Biopsy Collection

No enema preparation was used routinely unless the procedure was performed for a primarily clinical care reason. With a flexible sigmoidoscope, four biopsies were obtained at 20cm from the anal verge. The biopsies were snap frozen in liquid nitrogen and stored at -80 °C.

3.2.2. Terminal Restriction Fragment Length Polymorphism (TRFLP) Methods

FastDNA® SPIN Kit for FECES was used (MP Biomedicals, LLC, Solon, OH).

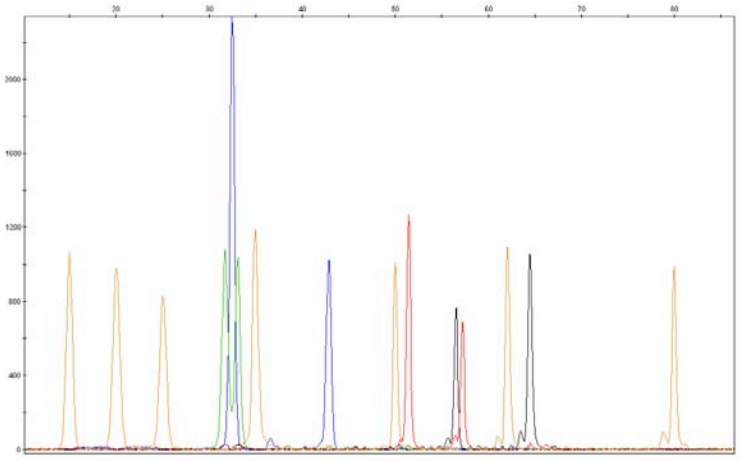
3.2.2.1. The specimens were agitated in order to 'release' the DNA from the bacterial cell walls.

3.2.2.2 Ethanol precipitation method was used to clean and concentrate extracted DNA.

3.2.2.3 Hpall restriction enzyme was then used to digest the PCR products over night for 16 -18 hours at 37 °C.

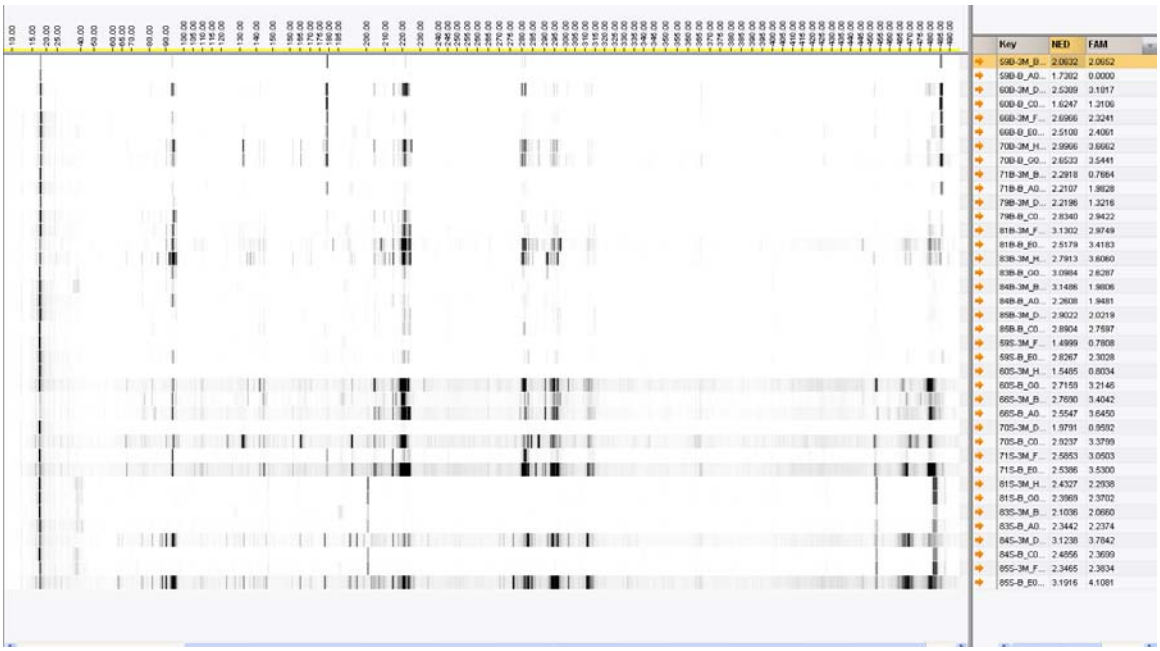
3.2.2.4 Restriction fragments were separated by a DNA sequencer to determine the fragment lengths and electropherograms were generated. (Figure 2-1)

Figure 3-1 An example of electropherogram



3.2.2.5 Terminal restriction fragments profile was then generated by BioNumerics (Applied Maths, Belgium) based on the electropherograms. A copy of the terminal restriction fragment profile is shown below and the fragment traces are in paired for ease of comparison. (Figure 2-2)

Figure 3-2 An example of terminal restriction fragment profile as seen on BioNumerics



The horizontal numbering across the top of the figure (increasing from left to right) indicates the size of the restriction fragments as determined by the number of base pairs.

3.2.2.3. The DNA samples were diluted to the same concentration with 50ng/µl EB Buffer.

3.2.2.4. PCR was performed using labeled primers 1) RFLP_B16sFAM8f and 2) RFLP_B16sNED926R.

3.2.2.5. PCR products were run on QIAxcel to check if the PCR had successfully amplified the 16S DNA.

3.2.2.6. PCR products were then cleaned using Qiagen MinElute PCR purification kit (Qiagen sciences, Maryland). This removes primers and other residual protein products.

3.2.2.7. The terminal restriction fragment length profile was then uploaded and run through the Microbial Community Analysis (MICA) website to obtain accession numbers (http://mica.ibest.uidaho.edu). An accession number for a bacterium is a unique serial number that identifies it universally.

3.2.2.8. The saved accession numbers were then run through the Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu) for RNA sequence match and subsequent classification into plausible bacteria phylum, class, family, and order with 95% confidence.

3.2.3 Terminal Restriction Fragment Length Polymorphism (TRFLP) Interpretations

3.2.3.1 *Similarity Index* (Percentage)

This is determined mathematically by BioNumerics software. Each individual's terminal restriction fragment traces was aligned with another as shown in figure 2.1. It assumes similarly length TRFLP fragments represent similar organisms. A higher similarity index indicates the greater similarity in the distribution of the terminal restriction fragments between the two aligned traces. With this, we were able to compare the trace pattern for the baseline and the 3 months within each individual. This index does not take into account the actual bacteria composition or abundance. The similarity index is thus a gross measure of how similar the terminal restriction fragment trace patterns are and infer similarity in the microbiome.

3.2.3.2 *Shannon Weiner index of diversity* (SWI-absolute number)

BioNumerics determines the diversity index based on the number of peaks on the electropherogram (simplified as the number of unique species) and the peak area as the relative species abundance. The diversity index is a measure of both species richness and relative species abundance of the community.

3.3. Urine Metabolomics Assessment (Results in Chapter six)

3.3.1. Urine specimen handling and processing

3.3.1.1. Sterile urine containers were coated with 6 drops of sodium azide (27.3mg/mL)

3.3.1.2. A fresh urine specimen was collected prior to commencement of iron replacement and a further fresh urine specimen was collected at 3-month. There was no predetermined condition/diet/ time for which the urine was collected. Subjects were encouraged to maintain their usual diet and medications.

3.3.1.3. Collected specimens were refrigerated at 4°C as soon as it was collected and transferred to -80°C freezer within 4 hours.

3.3.1.4. The frozen urine specimens were thawed and aliquoted into four 1mL samples into eppendorf tubes along with 50µL of sodium azide (27.3mg/mL) and freeze at -80°C.

3.3.1.5. Specimens were analysed in batches for practical reason – personnel availability and cost. Moreover, the specimen is stable for up to 1 year when frozen. (112)

3.3.2. Sample preparation

3.3.2.1 **Day prior to NMR**

The samples were thawed and each was diluted with 75 µL of Chenomx internal standard (1:10) to achieve a total volume of 750 µL. Then stored at 4 °C overnight

3.3.2.2 **Day of NMR**

3.3.2.2.1. The pH of each sample was checked and either HCl or NaOH was added to achieve pH 6.7 – 6.8.

3.3.2.2.2. 700 µL of the urine samples were aliquot into 5mm NMR tubes and capped.

3.3.3. NMR acquisition (Chapter six)

3.3.3.1. All samples were analysed on Oxford 600Hz NMR spectrometer with a Varian VNMRS two-channel console. Automatic sample-handling routines developed in-house were coupled with VNMRJ software version on a RHEL 4 host computer

3.3.3.2. The NMR tubes were cleaned with Kimwipes®

3.3.3.3. Samples were then inserted into NMR magnet

3.3.4. Post NMR Acquisition

3.3.4.1. Sample pH were rechecked and recorded for quality assurance

3.3.4.2. NMR tubes were cleaned with bleach, soapy water, alcoholic KOH (120g/L) and concentrated HCl (360g/L). The tube was rinsed with double distilled water five times between the two chemical washes.

3.3.5. Metabolite Analysis

Once the spectra were acquired, quantification of metabolites was carried out using the targeted profiling technique Chenomx NMRSuite v7.0 (Chenomx, Inc. Edmonton, Canada). It compares the integral of a known reference signal (Dextran Sodium Sulfate- DSS) with signals derived from a library of known compounds to determine metabolite concentration relative to the reference signal. The quantification process was done by one individual and verified by a second individual to optimize accuracy. The spectral analyses were also spot checked by a third individual. Metabolites of non human metabolism such as medications –ibuprofen, salicylurate and the internal standard DSS were excluded from analysis. Over 240 metabolites were considered and 71 were found to be significant, that is, the spectral peaks of 71 metabolites in the compound library were identified in the spectra of the study samples.

Normalization of the metabolites may be required as the concentration of the metabolites varies

by hydration status. Creatinine normalization is traditionally used to adjust for this but by doing so, creatinine is eliminated from further analysis (113).

Moreover, logarithmic transformation could be done to account for the non-normal distributive nature of the concentrations in the SIMCA-P+ v12.0.1 (Umetrics, Umea, Sweden) program. Therefore in situation where a statistical model cannot be generated from the original dataset, creatinine normalization and/or logarithm transformation may help. Neither approach was helpful in the generation of a better orthogonal partial least squares (OPLS) model.

3.3.6. Metabolite Statistical Analysis

SIMCA-P+ v12.0.1 (Umetrics, Umea, Sweden) were used to perform the projection-based methods analysis such as principal component analysis (PCA) and orthogonal partial least squares (OPLS). These methods compress the multi-dimensional data down to a more manageable 2 or 3 main components based on variance. Projection based models are able to handle many variables and correlate predictor variables in a simple and straightforward way.

3.3.6.1. Principal Component Analysis (PCA)

A PCA model is unsupervised analysis (the model is blinded to the two groups) and provides an overview of all observations or samples demonstrating groupings, trends and outliers. PCA makes it possible to extract and display systematic variation in the data. Each PCA model is generated based on the direction in the data demonstrating the highest variation, i.e. gender, age, diet, concurrent medication, genes, disease or other unknown factors, which might be distinctly different from the direction separating the classes (114).

3.3.6.2. Partial Least Squares (PLS)

Conventional PLS is supervised analysis (the model is informed of two distinct groups and the aim is to find the metabolites that cause maximum separation between the two groups) describing a quantitative relationship between two data variables X & Y; it uses X (various metabolites) to construct a model of Y(PO or IV route). The objective is to predict Y from the X for new samples

in the prediction set (114).

3.6.3. Orthogonal Partial Least Squares (OPLS)

OPLS is an extension to the supervised PLS regression method with an integrated Orthogonal signal correction (OSC) filter, which removes the uncorrelated signals resulting in information of the within-class variation. The OPLS method is designed to handle variation in X that is orthogonal to Y. OPLS separates the systematic variation in X into two parts, one that is linearly related (and therefore predictive) to Y and one that is orthogonal to Y. The predictive variation of Y in X is modeled by the predictive components. The variation in X which is orthogonal to Y is modeled by the orthogonal components. OPLS enable a clearer and more straightforward interpretation.

3.3.7 Metabolite Model Characteristics

The quality of a model is represented by R^2 and Q^2. The range for these parameters is 0 to 1, where 1 indicates a perfect fit. A large R^2Y (close to 1) is a necessary condition for a good model and a large Q^2Y ($Q^2Y > 0.5$) indicates good predictivity. R^2 is the percent of variation of the training set – X: metabolites and Y: IV or PO – explained by the model. It is a measure of fit, i.e. how well the model fits the data. Q^2 is the percent of variation of the training set – X with PCA and Y with PLS – predicted by the model according to cross validation. It indicates how well the model predicts new data in 7-fold cross validation.

4. RESULTS

CLINICAL EXPERIMENTATION

4.1. Study Subject flow (Figure 4-1)

Subject recruitment started April 2010 and completed March 2011. Written consent was obtained from 110 subjects who met the inclusion criteria. These study subjects were randomized to receive either intravenous (IV) iron or oral (PO) iron therapy for 3 months. Eighteen subjects were withdrawn within 1 to 2 weeks after randomization as the route of iron replacement didn't meet subjects' expectation or work schedule. ***Figure 4-1*** describes the overall study subject flow.

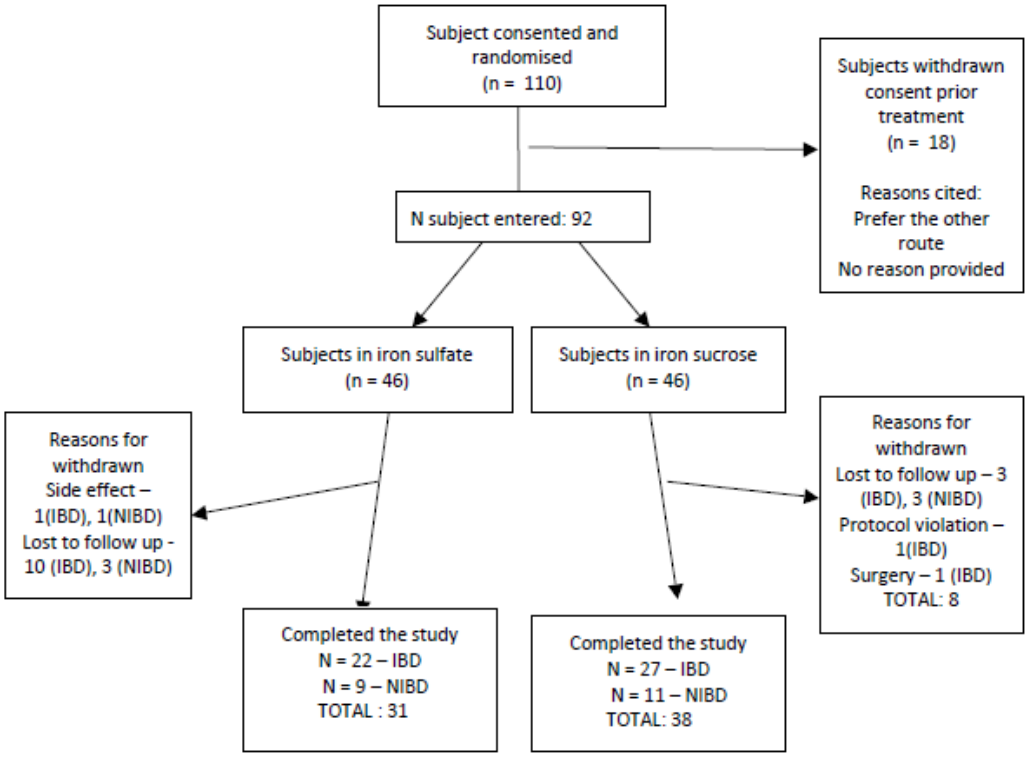
4.1.1. Intravenous iron treatment group.

Forty six subjects were thus randomized to receive IV *iron sucrose infusions.* Of these 46 subjects 38 completed the 3-month study interval. Two subjects were not included in the final analysis because - one was given intramuscular iron injection during the study period by the family physician and the other developed fulminate colitis required total colectomy during the study period. Six subjects were not contactable or did not return for follow up visit despite numerous reminders.

4.1.2. Oral iron treatment group.

Forty six subjects were thus randomized to receive *PO iron sulfate.* Of these 46 subjects 31 completed the 3-month study follow up. Two subjects were withdrawn from the study due to intolerance to the study drug. Thirteen subjects were lost to follow up or did not return for follow up visits despite numerous reminders.

Figure 4-1 Study subjects flow chart.



Legend: N: number; IBD: inflammatory bowel disease; NIBD: Non inflammatory bowel disease

4.2. Baseline characteristics (Table 4.1)

The baseline characteristics between the IV and the PO group were similar in terms of age, sex, haemoglobin, CRP in patients with IBD, disease activity index and quality of life scores, short form inflammatory bowel disease questionnaire (SIBDQ) and EuroQaulity 5 Dimension Visual Analogue Scale (EQ5D VAS). However, within the IBD subgroup there were more subjects in the IV group (15/26) with a history of iron deficiency compared to the PO group (6/23), (p=0.03) and this is mostly contributed by the CD subgroup – 11/18 subjects in the IV group have a history of iron deficiency compared to 5/12 in the PO group, (p=0.3).

Within the Crohn's disease (CD) group, higher mean baseline ferritin was reported in the oral group (63.8ug/L) compared to the IV group (29.2ug/L) (p=0.04) and higher mean baseline iron

saturation was reported in the oral IBD group (10.9%) compared to the IV IBD group (8.5%) (p=0.03).

In terms of concurrent medications, the most striking differences were 1) more subjects in the IV group took prebiotic/probiotic/antibiotic (7/20) compared to the PO group (0/23) p=0.002 and 2) more subjects in the IV group (6/20) took either or a combination of an immunosuppressant (IS) and a biological drug (infliximab, adalimumab or vedolizumab) compared to oral group (3/23), p=0.17.

4.3. Mean dose of iron taken

Ganzoni equation was used to calculate iron deficit and determine the amount of iron replacement required. (Chapter 1 section7, page 17) Subjects with iron deficiency and not anemic, a standard dose of 900mg iron sucrose was given. Subjects with iron deficiency anemia, a standard dose of 1200mg iron sucrose was given.

4.4. Medication adherence

The iron pill count was performed at 3 months in the PO iron group to determine the total amount of iron pills taken during the three months period. The mean percentage of pills taken was 80% (range 70% to 100%). The total mean ferrous sulfate intake for the study was 33.6g (range 16.8g to 33.6g) or 6.6g (range 3.3g to 6.6g) of elemental iron. The total mean IV iron intake for the study duration was 965mg (range 900mg to 1200mg). We a priory defined non adherence to PO iron as having ingested less than 90% of the iron pills – ie with 18 or more pills left at 3 months and non adherence to IV therapy as missing at least one iron infusion.

4.4.1 Non inflammatory Bowel Disease

The number of subjects who took less than 90% of the prescribed iron pills were eight out of nine. In contrast, all 11 subjects in the IV group completed their prescribed course of iron infusions indicating 100% adherence. PO route was associated with a higher risk of medication non adherence compared to IV route with a risk ratio of 12 (CI 1.8-78.4).

4.4.2 Inflammatory Bowel Disease

The number of subjects took less than 90% of the prescribed iron pills were 14 out of 23. In contrast, all 26 subjects in the IV group completed their prescribed course of iron infusions indicating 100% adherence. PO route was associated with a higher risk of medication non adherence compared to IV route with a risk ratio of 3.9 (CI 2.2-6.8).

4.4.2.1 Crohn's disease

The number of subjects took less than 90% of the prescribed iron pills were eight out of 12. In contrast, all 18 subjects in the IV group completed their prescribed course of iron infusions indicating 100% adherence. PO route was associated with a higher risk of medication non adherence compared to IV route with a risk ratio of 5.5 (CI 2.3-13.4).

4.4.2.2 Ulcerative colitis

The number of subjects took less than 90% of the prescribed iron pills were six out of 11. In contrast, all eight subjects in the IV group completed their prescribed course of iron infusions indicating 100% adherence. PO route was associated with a higher risk of medication non adherence compared to IV route with a risk ratio of 2.6 (CI 1.3-5.2).

	CD		UC		IBD = CD+UC		NIBD	
	IV	PO	IV	PO	IV	PO	IV	PO
Number	18	12	8	11	26	23	11	9
Age (years) mean (SD)	41(18)	40(19)	39(18)	39(19)	42(18.7)	40(19)	41(19)	41(19)
Sex male (n)	7	7	6	4	13	11	3	3
History of bowel resection (n)	1	4	1	2	2	6	0	0
History of Iron Deficiency (n)	11	5	4	1	15	6	4	3
Haemoglobin mean (SD) g/L	125.7(16.3)	130.9(8.6)	123.7 (17.1)	129.9(19.4)	126.5(16.9)	130.4(14.5)	121.2(17.7)	111.1(15.6)
Ferritin mean (SD) ug/L	29.2(39.3)*	63.8(47.4)*	33.9 (43.5)	28.2(29.3)	32.1(43.9)	33.9(42.2)	21(23.3)	12.1(12.2)
Iron saturation mean (SD) %	8.6(3.1)	10.6(3.1)	8.25 (4.2)	11.3(5.6)	8.5(3.3)#	10.9(4.3)#	8.8(4.6)	9.9(5.4)
QoL								
SIBDQ mean (SD) max 70	42.9(6.2)	47.3(11.8)	46.5(18.2)	41.8(12)	44(11)	44.7(12)	49.4(11.8)	47.8(14.4)
EQ5D VAS mean (SD) %	59.9(21.7)	65.6(16.9)	65.8(21)	57.3(12.7)	64.2(18)	61.2(15.4)	69.9(18.3)	63.4(17.2)
Disease activity								
CRP mean (SD) mg/L	17.4(38.2)	12.3(10.5)	12.6(13.8)	5.7(8)	15.9(32.4)	9.2(9.8)	4.3(3.6)	4.5(6.6)
Partial Mayo score median (IQR)	na	na	1.5(5)	3(4)				
HBI median (IQR)	6(3)	6.5 (6.5)	na	na				
DRUGS								
Prebiotic (n)	3	0	1	0				
Probiotic (n)	2	0	0	0				
Antibiotic (n)	0	0	1	0				
5ASA (n)	3	1	2	7				
Immunosuppressant (IS) (n)	2	3	1	1				
Biological (n)	3	2	1	1				
IS+biological (n)	4	2	2	1				

Table 4.1 Baseline characteristics of the study population

***p=0.04, # p=0.03,**
Legend: CD: Crohn's disease; UC: ulcerative colitis; IBD: Inflammatory Bowel Disease; NIBD: Non inflammatory bowel disease; IV: intravenous; PO: Oral

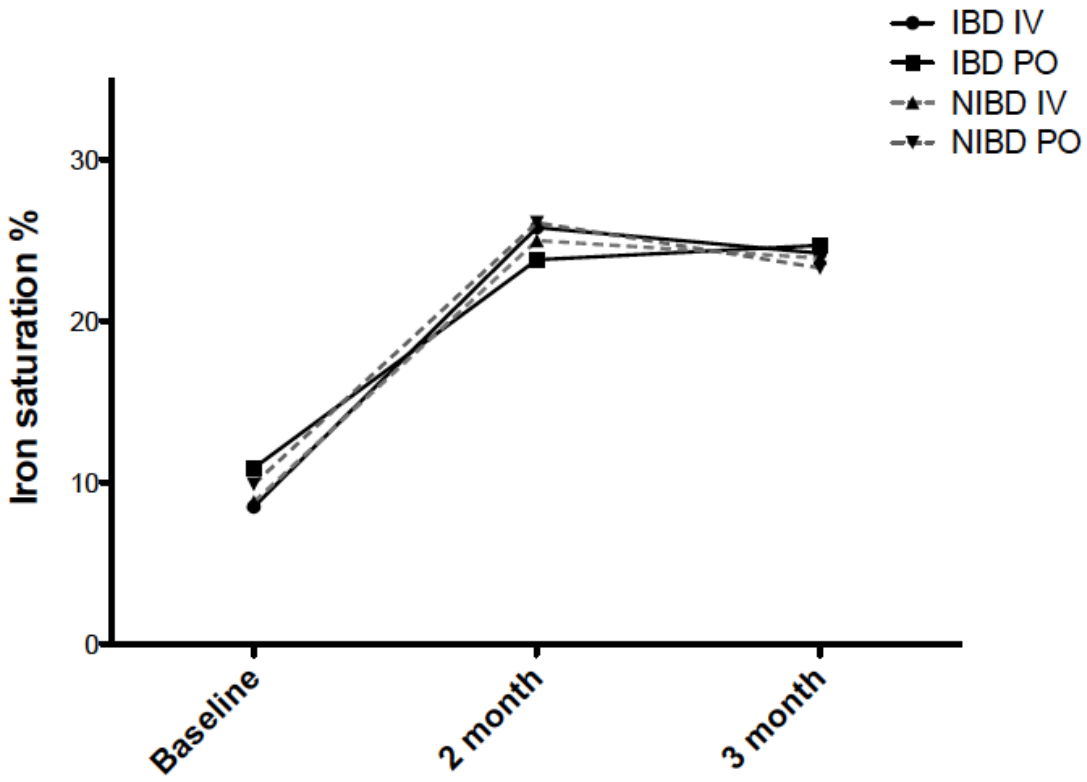
4.5. Primary endpoint

4.5.1. Iron saturation at 2 months (table 4-2)

4.5.1.1. Non Inflammatory Bowel Disease Group (Table 4-2, Figure 4-2)

The mean iron saturation at 2 months were 25% (SD 9.5) in the IV group and 26.1% (SD 26.8) in the PO group, which was not statistically different, p=0.9. Numerically more patients in the IV treatment group (72%) normalized their iron saturation than those in the PO treatment group (66%), however, this did not reach statistical significant (p=0.44).

Figure 4-2 Percentage iron saturation at 2 and 3 months



Legend: IBD: Inflammatory Bowel Disease; NIBD: Non-IBD, IV: Intravenous, PO: Oral

There was no statistical difference in the iron saturation at 2 and 3 months between the PO and IV routes.

Table 4.2 Clinical outcomes at 2 months

	CD		UC		IBD=CD+UC		NIBD	
	IV	**PO**	**IV**	**PO**	**IV**	**PO**	**IV**	**PO**
Hb mean (SD) g/L	136.1(8.4)	135.3(11.3)	130.6(15.4)	138.5(9.9)	134.4(11)	136.8(10.5)	127.5(10.6)	136.3(14.4)
Ferritin mean (SD) ug/L	136.9(116.1)#	62.3(57.7)#	179.1(156.5)*	41.7(33.5)*	149.9(128.1)^	52.4(47.8)^	143.4(126.3)$	29(9.8)$
Iron sat mean (SD) %	25.7(13.9)**a**	18.6(5.1)**a**	26.1(15.2)b	29.5(13.2)b	25.8(14)c	23.8(11)c	25(9.5)d	26.1(26.8)d
QoL								
SIBDQ mean (SD) – out of 70	52.6(8.3)	53.8(11.7)	52.3(14.5)	44(12.7)	52.5 (10.2)	48.5(12.8)	54.7(9.4)	51(8.2)
Disease activity								
CRP mean (SD) mg/L	6.9(8.1)	8.1(8.5)	10.6(10.6)e**	2.9(4.1)e	8.1(8.9)	5.6(7.1)	2.8(2)	7.3(7.9)
Pmayo median (IQR)	na	na	1.5(5)	2(2)				
HBI median (IQR)	5(3)	5.5(3)	na	na				

#p=0.049, *p=0.01, ^p=0.001, $p=0.015, a p=0.1, b p=0.6, c p=0.58, d p=0.9, e p=0.04.

Legend: CD: Crohn's disease; UC: ulcerative colitis; IBD: Inflammatory Bowel Disease; NIBD: Non inflammatory bowel disease; IV: intravenous; PO: Oral

4.5.1.2 *Inflammatory Bowel Disease Group* (Table 4.2, Figure 4.2)

The mean iron saturation at 2 months were 25.8% (SD 14) in the IV group and 23.8% (SD 11) in the PO group, which was not statistically different, p=0.74. Numerically more patients in the IV treatment group (76.3%) normalized their iron saturation than those in the PO treatment group (67.7%), however, this did not reach statistical significant (p=0.24).

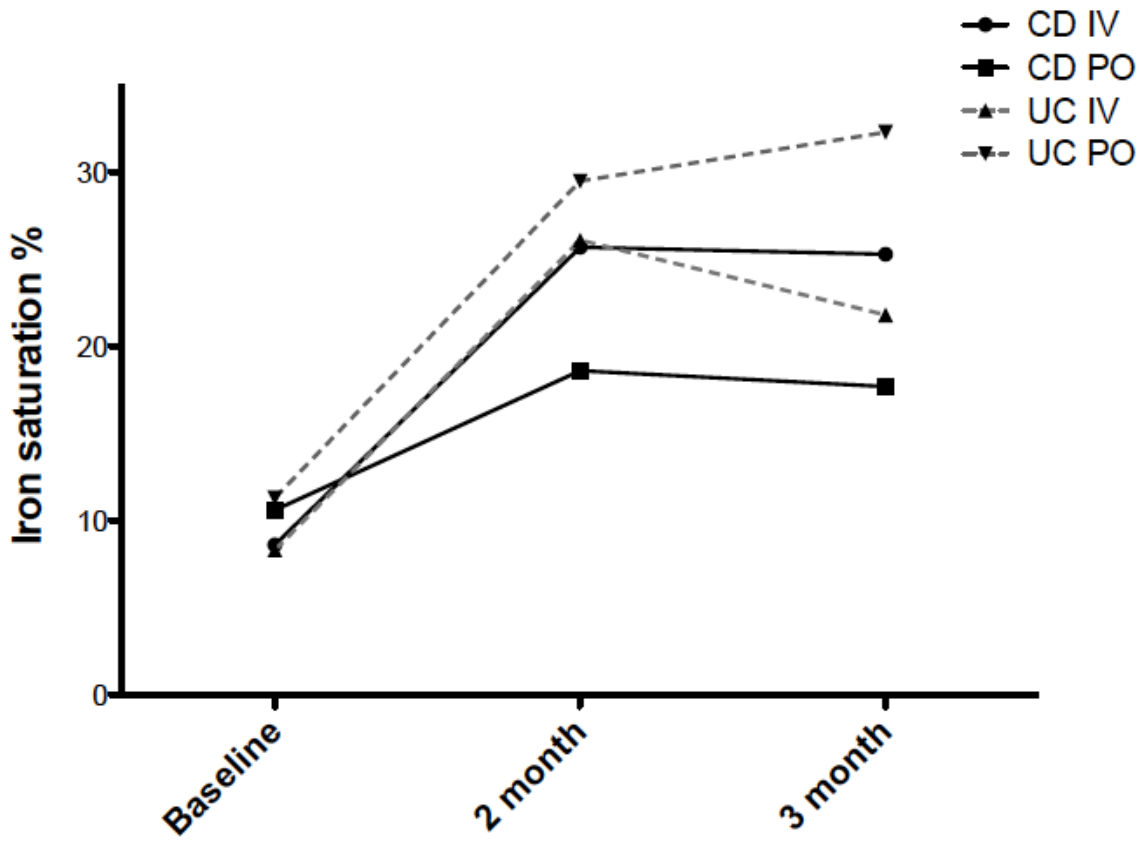
4.5.1.2.1 Crohn's Disease Group *(Table 4.2, Figure 4.3)*

The mean iron saturation at 2 months were 25.7% (SD 13.9) in the IV group and 18.6% (SD 5.1) in the PO group, which was not statistically different, p=0.1. Numerically more patients in the IV treatment group (77%) normalized their iron saturation than those in the PO treatment group (44%), however, this did not reach statistical significant (p=0.12). This is likely due to the small sample size.

4.5.1.2.2 Ulcerative colitis Group *(Table 4.2, Figure 4.3)*

The mean iron saturation at 2 months were 26.1% (SD 15.2) in the IV group and 29.5% (SD 13.2) in the PO group, which was not statistically different, p=0.6. Numerically more patients in the PO treatment group (78%) normalized their iron saturation than those in the IV treatment group (71%), however, this did not reach statistical significant (p=0.7). This is likely due to the small sample size.

Figure 4-3 Percentage iron saturation at 2 and 3 months for CD and UC.



Legend: CD: Crohn's disease, UC: Ulcerative colitis; IV: Intravenous, PO: Oral

There was no statistical difference in the iron saturation at 2 and 3 months between the PO and IV routes.

4.6. Results at two months of treatment (Table 4.2.)

4.6.1. Serum ferritin

4.6.1.1. Non Inflammatory Bowel Disease Group (Table 4.2, Figure 4.4.)

The mean serum ferritin after 2 months of PO iron therapy was 29 µg/L (SD 9.8) and the mean serum ferritin after 2 months of IV iron therapy was 143.4 µg/L (SD 126.3), which is statistically

superior than PO therapy, p=0.015. The mean ferritin increment for the PO group was 122.3µg/L (SD 110), p= 0.004 and the mean ferritin increment for the IV group was 16.9µg/L (SD 14.3), p=0.008. IV iron group was associated with a statistical significant higher serum ferritin gain of 105.4µg/L (SD 37) over PO iron group, p=0.01.

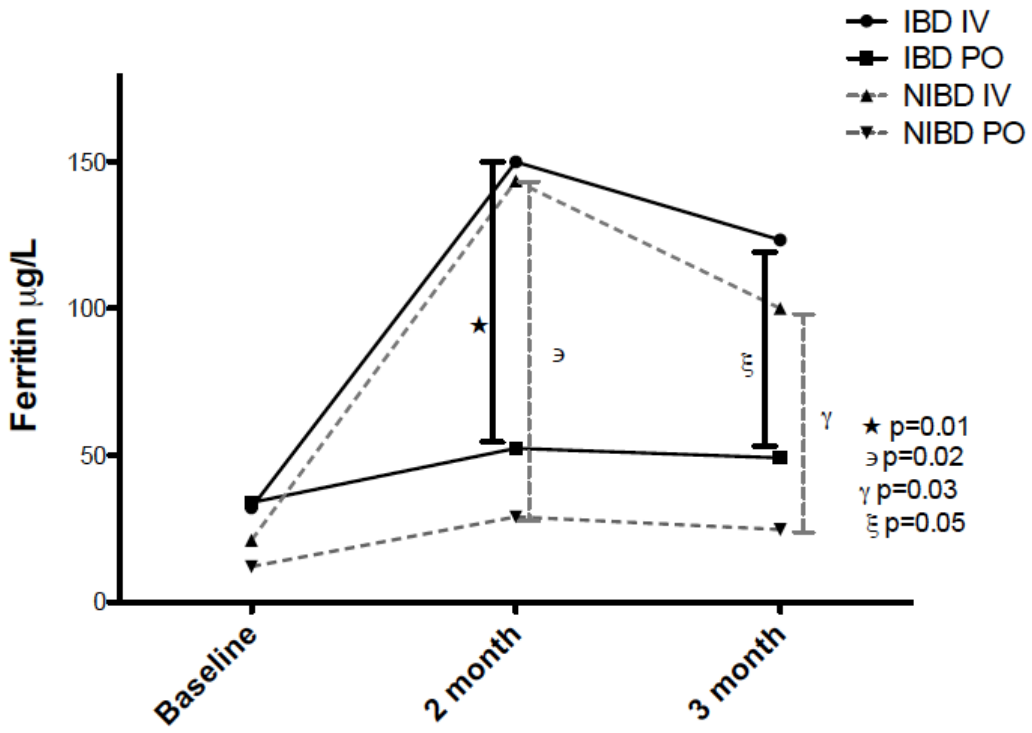
4.6.1.2. Inflammatory Bowel Disease Group (Table 4.2, Figure 4.4)

The mean serum ferritin after 2 months of PO iron therapy was 52.3 µg/L (SD 47.8) and the mean serum ferritin after 2 months of IV iron therapy was 149.9 µg/L (SD 128.1), which is statistically superior than PO therapy, p=0.001. . The mean ferritin increment for the PO group was 5.7 µg/L (SD 41), p= 0.5 and the mean ferritin increment for the IV group was 141µg/L (SD 193), p=0.001. IV iron group was associated with a statistical significant higher serum ferritin gain of 135.7µg/L (SD 41) over PO iron group, p=0.002.

4.6.1.2.1 Crohn's Disease Group *(Table4.2, Figure 4.5)*

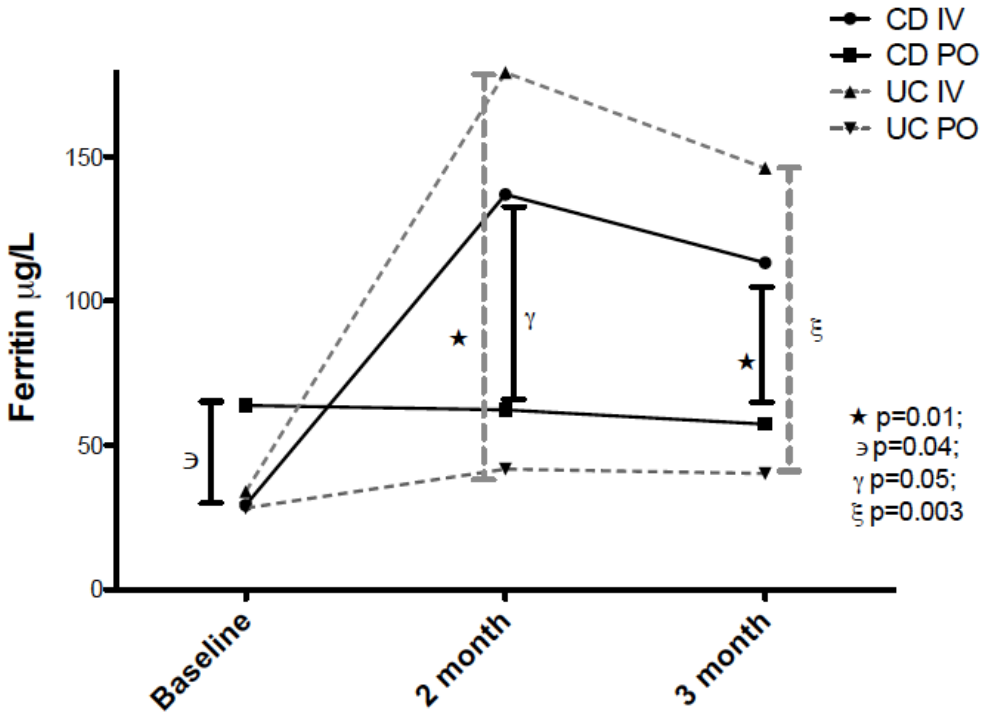
The mean serum ferritin after 2 months of PO iron therapy was 62.3 µg/L (SD 57.7) and the mean serum ferritin after 2 months of IV iron therapy was 136.9 µg/L (SD 116.1), which is statistically superior than PO therapy, p=0.049. . The mean ferritin increment for the PO group was -1.5µg/L (SD 56), p= 0.9and the mean ferritin increment for the IV group was 107.7µg/L (SD 98.7), p=0.0002. IV iron group was associated with a statistical significant higher serum ferritin gain of 109.2µg/L (SD 31.5) over PO iron group, p=0.002.

Figure 4-4 Serum ferritin at 2 and 3 months.



Intravenous iron therapy was superior to oral iron therapy in achieving a greater statistical significant gain in the serum ferritin at 2 and 3 months.

Figure 4-5 Serum ferritin at 2 and 3 months for UC and CD.



Intravenous iron therapy was superior to oral iron therapy in achieving a greater statistical significant gain in the serum ferritin at 2 and 3 months.

4.6.1.2.2 Ulcerative colitis Group *(Table 4.2, Figure 4.5)*

The mean serum ferritin after 2 months of PO iron therapy was 41.7µg/L (SD 33.5) and the mean serum ferritin after 2 months of IV iron therapy was 179.1 µg/L (SD 156.5), which is statistically superior than PO therapy, p=0.01 The mean ferritin increment for the PO group was 13.5µg/L (SD 12.8), p= 0.0057 and the mean ferritin increment for the IV group was 217µg/L (SD 318), p=0.095. IV iron group was associated with a statistical significant higher serum ferritin gain of 203.6µg/L (SD 95) over PO iron group, p=0.047.

4.6.2. Haemoglobin at 2 month

4.6.2.1. Non Inflammatory Bowel Disease Group (Table 4.2, Figure 4.6)

The mean haemoglobin after 2 months of PO iron therapy was 136.3 g/L (SD 14.4) and the mean haemoglobin after 2 months of IV iron therapy was 127.5 g/L (SD 10.6). There was no statistical difference. It is important to note that the majority of patients entering this study, while iron deficient, had normal hemoglobin levels. Thus we would not anticipate significant changes in the hemoglobin levels.

4.6.2.2. Inflammatory Bowel Disease Group (Table 4.2, Figure 4.6)

The mean haemoglobin after 2 months of PO iron therapy was 136.8 g/L (SD 10.5) and the mean haemoglobin after 2 months of IV iron therapy was 134.4 g/L (SD11), with no statistical difference.

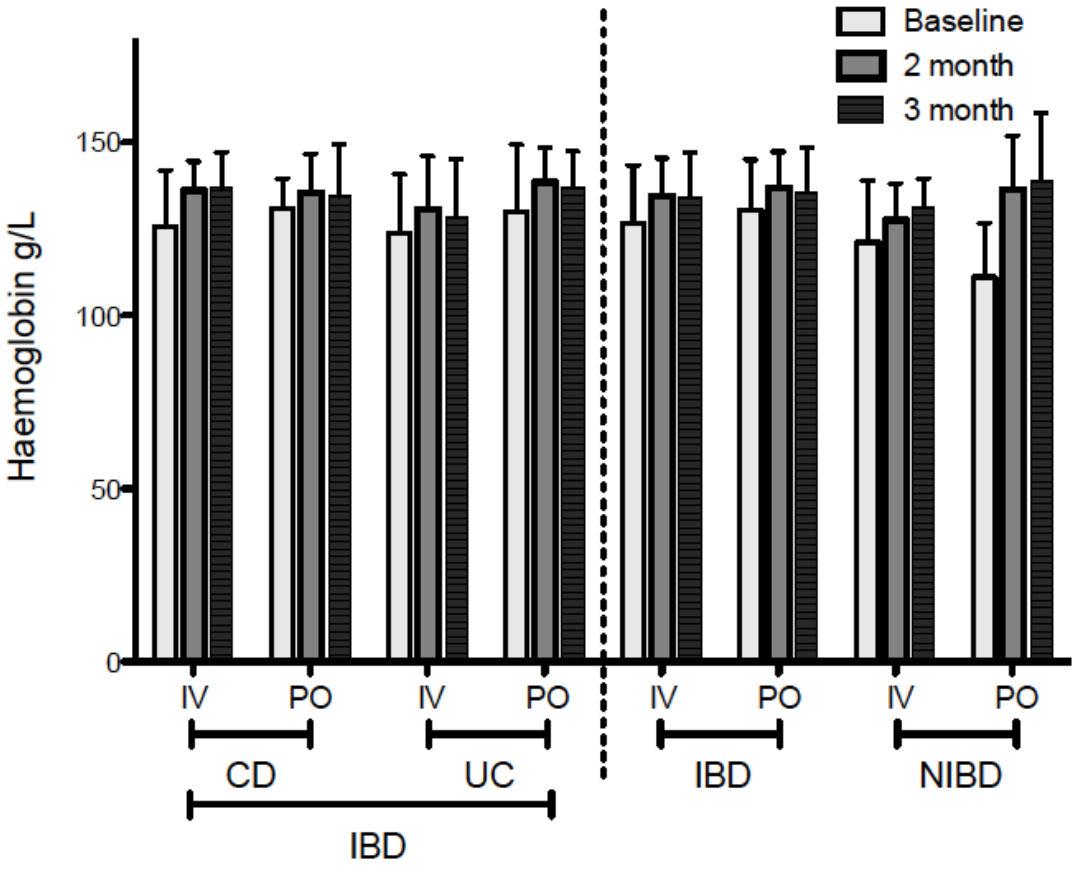
4.6.2.2.1 Crohn's Disease Group *(Table 4.2, Figure 4.6)*

The mean haemoglobin after 2 months of PO iron therapy was 135.3 g/L (SD 11.3) and the mean haemoglobin after 2 months of IV iron therapy was 136.1 g/L (SD 8.4), which was not statistically significant.

4.6.2.2.2 Ulcerative colitis Group *(Table 4.2, Figure 4.6)*

The mean haemoglobin after 2 months of PO iron therapy was 138.5 g/L (SD 9.9) and the mean haemoglobin after 2 months of IV iron therapy was 130.6 g/L (SD 15.4), which was not statistically significant.

Figure 4-6 Haemoglobin at baseline, 2 and 3 months for each subgroup.



Legend: CD: Crohn's disease, UC: Ulcerative colitis; IBD: Inflammatory Bowel Disease; NIBD: Non-IBD IV: Intravenous, PO: Oral

It is not surprising that the mean haemoglobin at baseline, 2 and 3 months were comparable between IV and PO iron groups as more than 75% of the subjects were not anaemic.

4.6.3. Quality of life score: Short form Inflammatory Bowel Disease Questionnaire (SIBDQ)

4.6.3.1. Non Inflammatory Bowel Disease Group (Table 4.3)

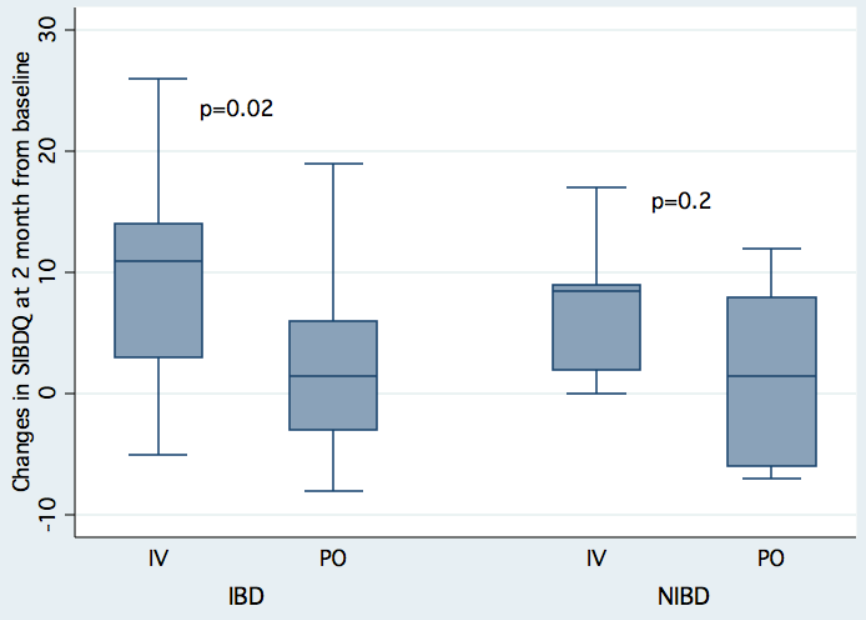
Table 4.3 Quality of Life – SIBDQ at 2 month in Non- inflammatory bowel disease subjects

Route	SIBDQ (Maximum score 70) -NIBD			
	Baseline	2 month	Improvement in SIBDQ	P value
IV Mean (SD)	49.4 (11.8)	54.7(9.4)	7.5(6)◊	0.03
PO Mean (SD)	47.8 (14)	51(8.2)	1.6 (7.6)†	0.6
IV versus PO comparison for SIBDQ improvement				0.2*

Legend: IV: Intravenous, PO: Oral; SIBDQ: Short form Inflammatory Bowel Disease Questionnaire.

The mean SIBDQ score after 2 months of PO iron therapy was 51 (SD 8.2) which is a median gain of 1.6 (IQR 7.6)†. The mean SIBDQ score after 2 months of IV iron therapy was 54.7 (SD 9.4), which was associated with statistical significant median gain of 7.5 (IQR 6)◊, p=0.03. There was no statistical difference in the SIBDQ at 2 month. Although IV group was associated with a higher SIBDQ score gained than PO group, it was not statistically significant, p=0.2*. (Figure 4-7)

Figure 4-7 Change in SIBDQ score at 2 month.



4.6.3.2. Inflammatory Bowel Disease Group (Table 4.4)

The mean SIBDQ score after 2 months of PO iron therapy was 48.5 (SD 12.8) which is a median gain of 2 .5 (IQR 7.7)†. The mean SIBDQ score after 2 months of IV iron therapy was 52.5 (SD 10.2), which was associated with statistically significant median gain of 9.9 (IQR 8.2)◊, p=0.0001. IV group was associated with a higher SIBDQ score gained than PO group and it was statistically significant, p=0.02. IV iron therapy improved SIBDQ by 9.9 points (p=0.0001) compared to oral iron 2.5 points (p=0.3) at 2 month. IV iron appears to be a superior therapy in patients with IBD. p=0.02*. (Figure 4-7)

Table 4.4 Quality of Life – SIBDQ at 2 month in inflammatory bowel disease subjects

Route	SIBDQ (maximum score 70) -IBD			
	Base line	2 month	Improvement in SIBDQ	P value
IV Mean (SD)	44 (11)	52.5(10.2)	9.9(8.2)◊	0.0001
PO Mean (SD)	44.7(12)	48.5(12.8)	2.5(7.7)†	0.3
IV versus PO comparison for SIBDQ improvement				0.02*

Legend: IV: Intravenous, PO: Oral; SIBDQ: Short form Inflammatory Bowel Disease Questionnaire

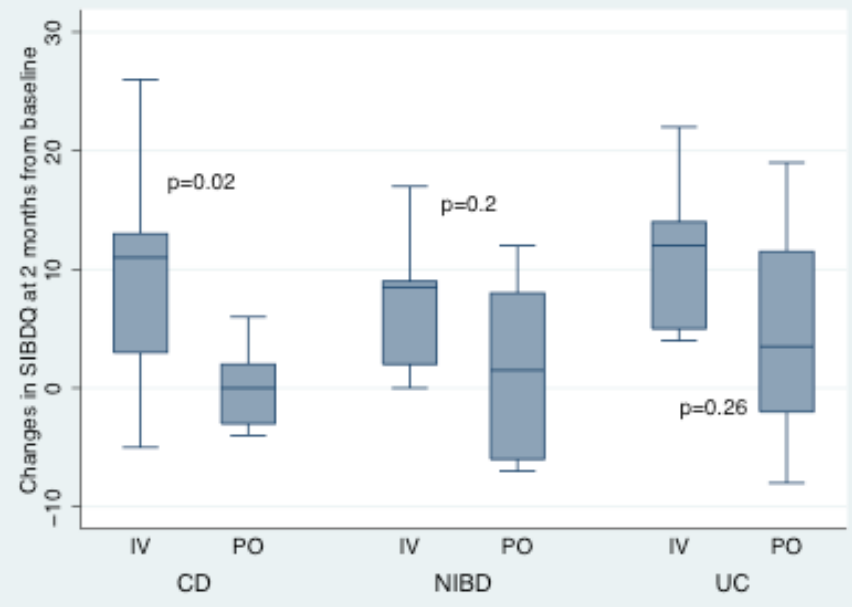
4.6.3.2.1. Crohn's Disease Group *(Table 4.5, Figure 4.8)*

The mean SIBDQ score after 2 months of PO iron therapy was 53.8
(SD 11.7) representing a median gain of 0.2 (IQR 3.7)†. The mean SIBDQ score after 2 months of IV iron therapy was 52.6 (SD 8.3), which was associated with statistical significant median gain of 9.7 (IQR 9)◊, p=0.03. There was no statistical difference in the SIBDQ score at 2 month. IV group was associated with a higher SIBDQ score gained than PO group and it was statistical significant, p=0.02*.

Table *4.5* Quality of Life – SIBDQ at 2 month in Crohn's disease subjects

Route	SIBDQ (maximum score 70) -CD			
	Base line	2 month	Improvement in SIBDQ	P value
IV Mean (SD)	43 (6.2)	52.6(8.3)	9.7(9)◊	0.03
PO Mean (SD)	47.3 (11)	53.8(11.7)	0.2 (3.7)†	0.6
IV versus PO comparison for SIBDQ improvement				0.02*

Figure 4-8 Change in SIBDQ score at 2 month.



4.6.3.2.2 Ulcerative colitis Group *(Table 4.6, Figure 4.8)*

The mean SIBDQ score after 2 months of PO iron therapy was 44 (SD 12.7) representing a median gain of 4.6 (IQR 9.9)†. The mean SIBDQ score after 2 months of IV iron therapy was 52.3 (SD 14.5), which was associated with statistical significant median gain of 10.3 (IQR 7)◊, p=0.02. IV group was associated with a higher SIBDQ score gained than PO group, however, it was not statistical significant, p=0.26*.

Table 4.6 Quality of Life – SIBDQ at 2 month in ulcerative colitis subjects

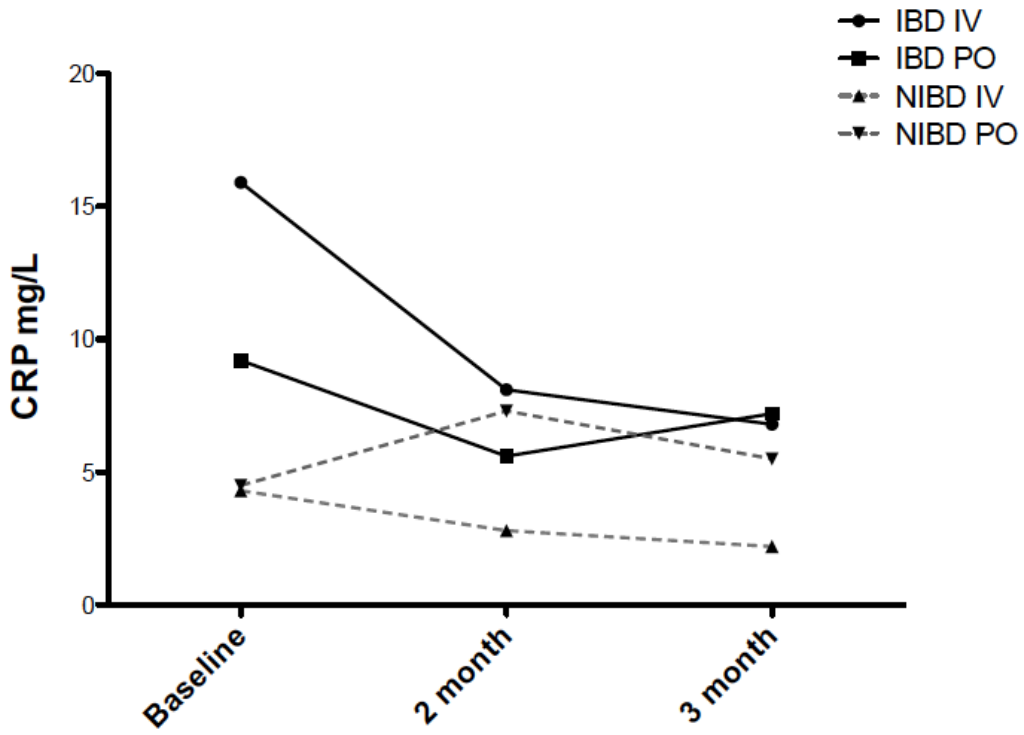
Route	SIBDQ (Maximum 70)			
	Baseline	2 month	Improvement in SIBDQ	P value
IV Mean (SD)	46.5 (18.2)	52.3(14.5)	10.3(7)◊	0.02
PO Mean (SD)	41.8 (12)	44(12.7)	4.6 (9.9)†	0.3
IV versus PO comparison for SIBDQ improvement				0.26*

4.6.4 Disease activity by CRP

4.6.4.1 Non Inflammatory Bowel Disease Group (Table 4.2, Figure 4.9)

The mean CRP after 2 months of PO iron therapy was 7.3 (SD 7.9) and the mean CRP after 2 months of IV iron therapy was 2.8 (SD 2). There was no statistical difference.

Figure 4-9 Serum CRP at baseline, 2 and 3 months for IBD and NIBD patients.



4.6.4.2 Inflammatory Bowel Disease Group (Table 3.2, Figure 3.9)

The mean CRP after 2 months of PO iron therapy was 5.6 (SD 7.1) and the mean CRP after 2 months of IV iron therapy was 8.1 (SD 8.9). There was no statistical difference.

4.6.4.2.1 Crohn's Disease Group *(Table 4.2, Figure 4.10)*

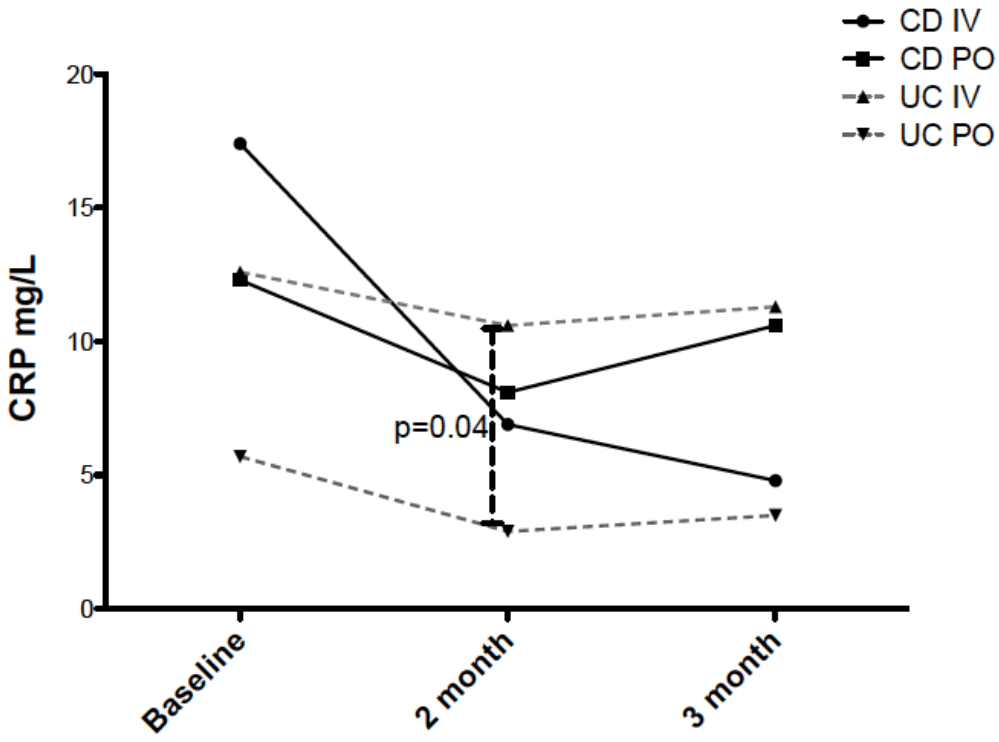
The mean CRP after 2 months of PO iron therapy was 8.1 (SD 8.5) and the mean CRP after 2 months of IV iron therapy was 6.9 (SD 8.1). There was no statistical difference.

4.6.4.2.2 Ulcerative colitis Group *(Table 4.2, Figure 4.10)*

The mean CRP after 2 months of PO iron therapy was 2.9 (SD 4.1) and the mean CRP after 2 months of IV iron therapy was 10.6 (SD 10.6). The difference in CRP was statistically significant, p=0.04. There were more patients in the IV group with active disease (4/8) required

corticosteroid +/- a biological agent compared to 1/9 in the PO group with active disease on oral prednisone.

Figure 4-10 Serum CRP at baseline, 2 and 3 months for UC and CD patients.



4.6.5 Disease activity by clinical disease activity index

4.6.5.1 Crohn's Disease Group (Table 4.7, Figure 4.11)

<< HBI <5 indicates clinical remission **>>**

The median Harvey Bradshaw Index (HBI) after 2 months of PO iron therapy was 5.5 (IQR 3) and the median HBI after 2 months of IV iron therapy was 5 (IQR 3). There was no statistical difference.

4.6.5.2 Ulcerative colitis Group (Table 4.7, Figure 4.11)

<< PMS <2 indicates clinical remission **>>**

The median partial Mayo clinic score (PMS) after 2 months of PO iron therapy was 2 (IQR 2) and the median PMS after 2 months of IV iron therapy was 1.5 (IQR 5). There was no statistical difference.

Figure 4-11 Change in clinical disease activity index at 2 month.

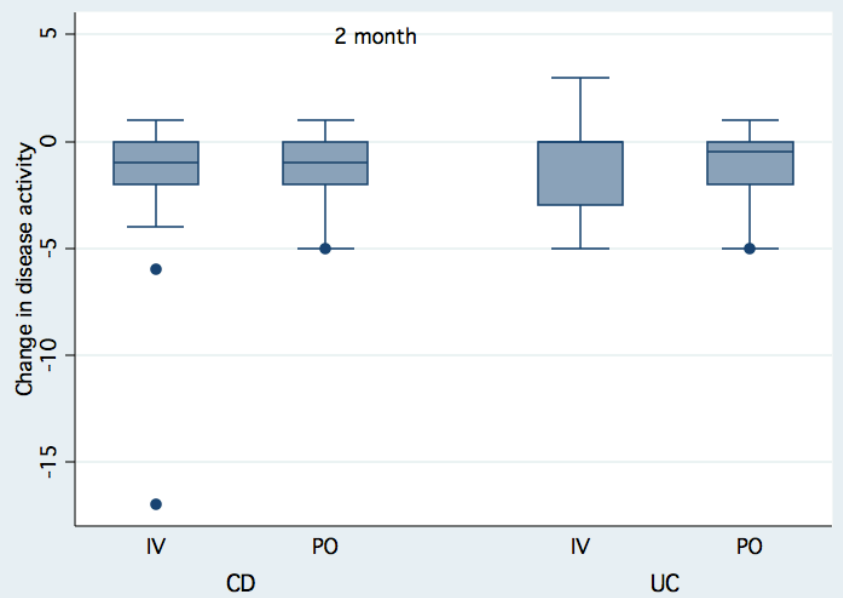


Table 4.7 Influence of iron replacement on disease activity index at 2 months

	Crohn's Disease				Ulcerative Colitis			
	HBI Wk 0	HBI Wk 12	ΔHBI	P value*	PMS Wk 0	PMS Wk 12	ΔPMS	P value*
IV Median (IQR)	6 (3)	5.5 (2.8)	-2.4 (4.6)†	0.0002	1.5 (5)	2.8 (3.2)	0(1) ‡	0.94
PO Median (IQR)	6.5 (6.5)	5 (3.7)	-1.4 (2.3)†	0.056	3 (4)	1.9 (1.2)	0(3) ‡	0.11
PO versus IV in change of disease activity index				0.81†				‡0.66

***Wilcoxon Sign rank test**
†Mann-Whitney test compared the changes in the HBI between PO and IV route
‡Mann-Whitney test compared the changes in the PMS between PO and IV route

4.7. Results at <u>three months</u> of treatment

4.7.1 Iron saturation at 3 months

4.7.1.1 Non Inflammatory Bowel Disease Group (Table 4.8, Figure 4.2)

The mean iron saturations at 3 months were 23.9% (SD 9.9) in the IV group and 23.3% (SD 6.3) in the PO group, which was not statistically different. Comparable proportion of patients in the IV treatment group (91%) and in the PO treatment group (100%) normalized their iron saturation.

4.7.1.2 Inflammatory Bowel Disease Group (Table 4.8, Figure 4.2)

The mean iron saturation at 3 months was 24.2% (SD 13.4) in the IV group and 24.7% (SD 15.8) in the PO group, which was not statistically different. Numerically more patients in the IV treatment group (82%) normalized their iron saturation than those in the PO treatment group (76%), however, this was not statistical significant (p=0.5).

4.7.1.2.1 Crohn's Disease Group (Table4.8, Figure 4.3)

The mean iron saturation at 3 months was 25.3% (SD13) in the IV group and 17.7% (SD 10.5) in the PO group, which was not statistically different. Numerically more patients in the IV treatment group (77%) normalized their iron saturation than those in the PO treatment group (50%), however, this did not reach statistical significant (p=0.3). This is likely due to the small sample size.

4.7.1.2.2 Ulcerative colitis Group (Table 4.8, Figure 4.3)

The mean iron saturations at 3 months were 21.8% (SD 14.7) in the IV group and 32.3% (SD 17.4) in the PO group, which was not statistically different. Numerically more patients in the PO treatment group (64%) normalized their iron saturation than those in the IV treatment group (50%), however, this did not reach statistical significant (p=0.4).

Table 4.8 Clinical outcomes at 3 months

	CD		UC		IBD=UC+CD		NIBD	
	IV	PO	IV	PO	IV	PO	IV	PO
Hb mean (SD) g/L	136.8(10.4)	134.5(14.9)	128.6(16.4)	137.2(10.2)	134.3(12.8)	135.8(12.7)	131.4(8.1)	139.1(19.4)
Ferritin mean (SD) ug/L	113.2(106)#	57.3(34.3)#	146(143.8)b	40.2(21.4)b	123.3(116.9)a	49.1(29.5)a	100(74.4)^	24.7(9.1)^
Iron saturation mean (SD) %	25.3(13)	17.7(10.5)	21.8(14.7)	32.3(17.4)	24.2(13.4)	24.7(15.8)	23.9(9.9)	23.3(6.3)
QoL								
BEST SIBDQ mean (SD)	54(6.8)	53(9.6)	54(12.8)	47.5(13)	54(8.8)	50(11.6)	59(7)	59(7.3)
EQ5D VAS mean (SD) %	79.8(10)*	67.3(18.5)*	71(22.3)	60.9(20.9)	77(14.9)#	64.2(19.5)#	83.3(9.9)	74.7(14.8)
Disease activity								
CRP mean (SD) mg/L	4.8(4.8)	10.6(13.8)	11.3(12.6)	3.5(4.3)	6.8(8.4)	7.2(10.8)	2.2(1.8)	5.5(7.3)
Pmayo median (IQR)	-	-	2(4)	2(3)				
HBI median (IQR)	3.5(4)	5(3.5)	-	-				

***p=0.02, # p=0.01, ^ p=0.007, a p=0.005, b p=0.003**

4.7.2 Serum ferritin

4.7.2.1 Non Inflammatory Bowel Disease Group (Table 4.8, Figure 4.4)

The mean serum ferritin after 3 months of PO iron therapy was 24.7 µg/L (SD 15.8) and the mean serum ferritin after 3 months of IV iron therapy was 100 µg/L (SD 74.4), which was statistically superior than PO therapy, p=0.0007.

4.7.2.2 Inflammatory Bowel Disease Group (Table 4.8, Figure 4.4)

The mean serum ferritin after 3 months of PO iron therapy was 49.1 µg/L (SD 29.5) and the mean serum ferritin after 3 months of IV iron therapy was 123.3 µg/L (SD 116.9), which is statistically superior than PO therapy, p=0.005.

4.7.2.2.1 Crohn's Disease Group *(Table 4.8, Figure 4.5)*

The mean serum ferritin after 3 months of PO iron therapy was 57.3 µg/L (SD 34.3) and the mean serum ferritin after 3 months of IV iron therapy was 113.2 µg/L (SD 106), which is statistically superior than PO therapy, p=0.01.

4.7.2.2.2 Ulcerative colitis Group *(Table 4.8, Figure 4.5)*

The mean serum ferritin after 3 months of PO iron therapy was 40.2 µg/L (SD 21.4) and the mean serum ferritin after 3 months of IV iron therapy was 146 µg/L (SD 143.8), which is statistically superior than PO therapy, p=0.003.

4.7.3 Haemoglobin at 3 months

4.7.3.1. Non Inflammatory Bowel Disease Group (Table 4.8, Figure 4.6)

The mean haemoglobin after 3 months of PO iron therapy was 139.1 g/L (SD 19.4) and the mean haemoglobin after 3 months of IV iron therapy was 131.4 g/L (SD 8.1). There was no statistical difference.

4.7.3.2 Inflammatory Bowel Disease Group (Table 4.8, Figure 4.6)

The mean haemoglobin after 3 months of PO iron therapy was 135.8 g/L (SD 12.7) and the mean haemoglobin after 3 months of IV iron therapy was 134.3 g/L (SD 12.8), with no statistical difference.

4.7.3.2.1 Crohn's Disease Group *(Table 4.8, Figure 4.6)*

The mean haemoglobin after 3 months of PO iron therapy was 134.5 g/L (SD 14.9) and the mean haemoglobin after 3 months of IV iron therapy was 136.8 g/L (SD 10.4), which was not statistically significant.

4.7.3.2.2 Ulcerative colitis Group *(Table4.8, Figure 4.6)*

The mean haemoglobin after 3 months of PO iron therapy was 137.2 g/L (SD 10.2) and the mean haemoglobin after 3 months of IV iron therapy was 128.6 g/L (SD 16.4), which was not statistically significant.

It is not surprising that the mean haemoglobin at baseline, 2 and 3 months were comparable between IV and PO iron groups as more than 75% of the subjects were not anaemic.

4. 7.4 The best quality of life score achieved during the study period: Short form Inflammatory Bowel Disease Questionnaire (SIBDQ)

Definition: The best SIBDQ score achieved refers to the best score obtained by the patient at either 2 or 3 months. Different patients may have achieved their best quality of life score at different time point in time during the study.

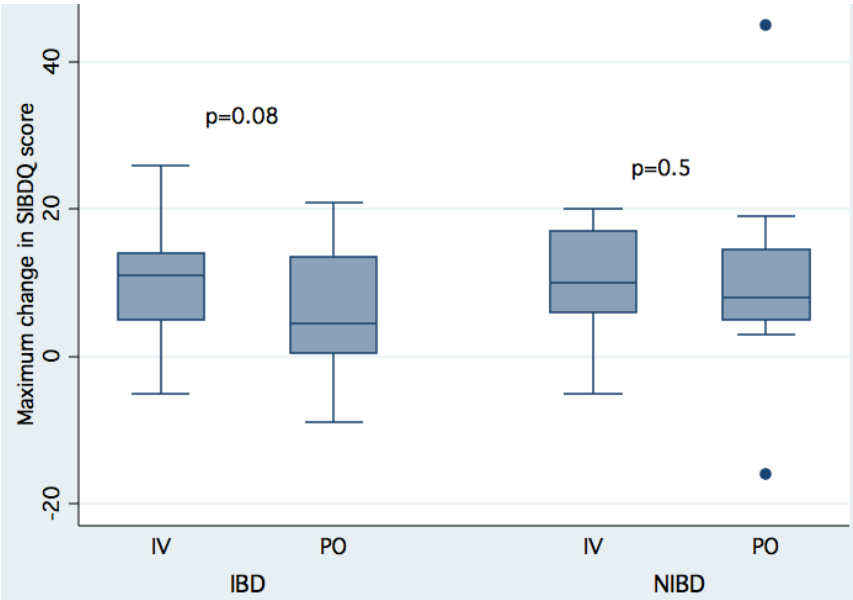
4.7.4.1 Non Inflammatory Bowel Disease Group (Table 4.9, Figure 4.12)

The best mean SIBDQ score during IV iron therapy was 59 (SD: 7), with a statistically significant median gain of 10 (IQR: 7.5), p=0.001. The best mean SIBDQ score achieved during PO iron therapy was 59 (SD: 7.3), with a median gain of 10.5 (IQR: 17), p=0.1. There was no statistical difference in the best mean SIBDQ achieved during the study between the two groups. There was no statistical difference in the magnitude of SIBDQ score gain during the study between the two groups. P=0.9.

Table 4.9 Best quality of life score achieved– Maximum SIBDQ achieved in Non-inflammatory bowel disease patients

Route	SIBDQ (total score is 70) -NIBD			
	Base line	Maximum gained	Improvement in SIBDQ	P value*
IV Mean (SD)	49.4 (11.8)	59(7)	10(7.5)†	0.001
PO Mean (SD)	47.8 (14)	59(7.3)	10.5 (17)†	0.1
IV versus PO comparison for SIBDQ improvement				0.9†

Figure 4-12 Maximum changes in SIBDQ score during the study period.



4.7.4.2 Inflammatory Bowel Disease Group (Table 4.10, Figure 4.12)

The best mean SIBDQ score during PO iron therapy was 50 (SD 11.6), with a median gain of 5.7 (SD 8.2), p=0.003. The best mean SIBDQ score achieved during IV iron therapy was 54 (SD 8.8), with a median gain of 10 (SD 8.4), p<0.0001. There was no statistical difference in the best mean SIBDQ achieved during the study between the two groups. There is a trend towards statistical significance that the higher SIBDQ score improvement in the IV group was significant compared to the PO group, p=0.07.

Table 4.10 **Best quality of life score achieved– Maximum SIBDQ achieved in inflammatory bowel disease patients**

Route	SIBDQ (maximum score is 70) -CD			
	Base line	Maximum gained	Improvement in SIBDQ	P value*
IV Mean (SD)	43 (6.2)	54(6.8)	11.1(7.9)†	<0.0001
PO Mean (SD)	47.3 (11)	53(9.6)	5.8 (8.3)†	0.035
IV versus PO comparison for SIBDQ improvement				0.08†

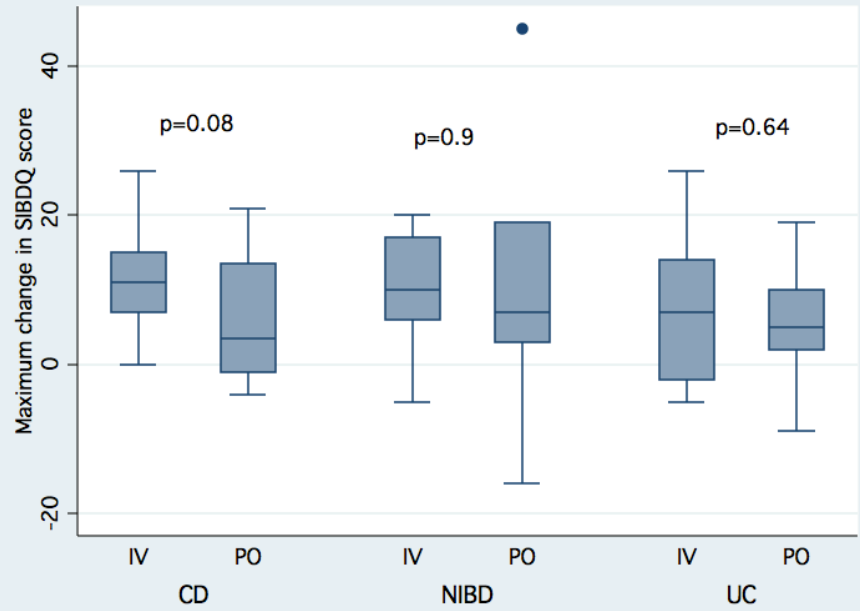
4.7.4.2.2 Crohn's Disease Group *(Table 4.11, Figure 4.13)*

The best mean SIBDQ score during PO iron therapy was 53 (SD 9.6), with a median gain of 5.8 (SD 8.3), p=0.035.The best mean SIBDQ score achieved during IV iron therapy was 52.6 (SD 7.2), with a statistical significant median gain of 11 (SD 7.9), p<0.0001. There was no statistical difference in the best mean SIBDQ achieved during the study between the two groups. There was no statistical difference in the magnitude of SIBDQ score gain during the study between the two groups. P=0.08.

Table 4.11 Best quality of life score achieved– Maximum SIBDQ achieved in Crohn's disease patients

Route	SIBDQ (total score is 70)			
	Base line	Maximum gained	Improvement in SIBDQ	P value*
IV Mean (SD)	44 (11)	54(8.8)	10(8.4)†	<0.0001
PO Mean (SD)	44.7(12)	50(11.6)	5.7(8.2)†	0.003
IV versus PO comparison for SIBDQ improvement				0.07†

Figure 4-13 Maximum changes in the SIBDQ score during the study period for UC and CD.



4.7.4.2.2 Ulcerative colitis Group *(Table 4.12, Figure 4.12)*

The best mean SIBDQ score during PO iron therapy was 47.5 (SD 13), with a statistical significant median gain of 5.7 (SD 8.6), p=0.05. The best mean SIBDQ score achieved during IV iron therapy was 54 (SD 12.8), with a median gain of 7.8 (SD 9.7). There was no statistical difference in the best mean SIBDQ achieved during the study between the two groups. There was no statistical difference in the magnitude of SIBDQ score gain during the study between the two groups. P=0.64. There is a trend that iron therapy significantly improved the SIBDQ score in UC patients. The magnitude of the maximum SIBDQ score gained between IV and PO was comparable, p=0.64.

Table 4.12 Best quality of life score achieved– Maximum SIBDQ achieved in ulcerative colitis patients

Route	SIBDQ (total score is 70) -UC			
	Base line	Maximum gained	Improvement in SIBDQ	P value*
IV Mean (SD)	46.5 (18.2)	54(12.8)	7.8(9.7)†	0.06
PO Mean (SD)	41.8 (12)	47.5(13)	5.7 (8.6)†	0.05
IV versus PO comparison for SIBDQ improvement				0.64†

4.7.5. Quality of Life assessment by Euro Quality 5 Dimension Visual Analogue Scale (EQ5D VAS) -out of 100 points

4.7.5.1 Non Inflammatory Bowel Disease Group (Table 4.13, Figure 4.14)

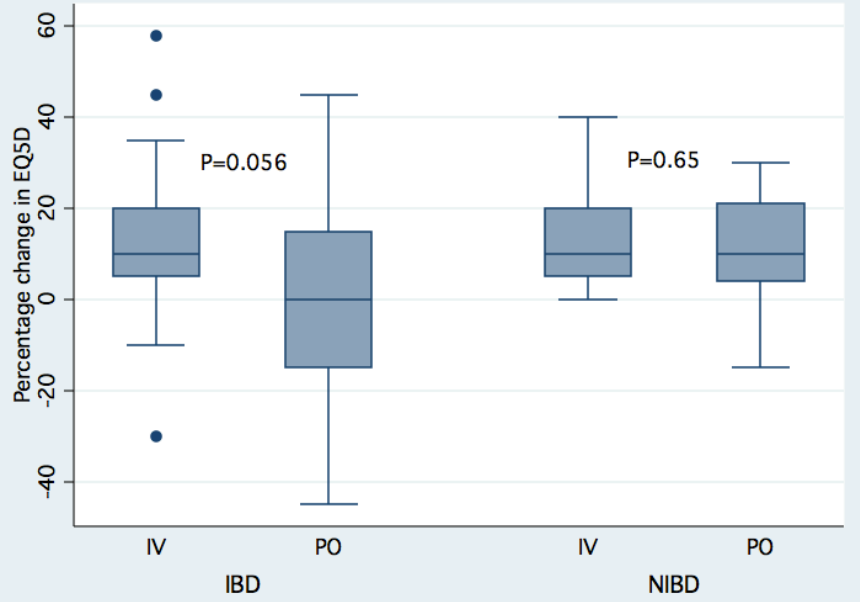
The mean EQ5D VAS score at 3 months for PO iron therapy was 74.7 (SD 14.8) with a median gain of 10.6 (SD 14), which was not statistically significant, p=0.07. The mean EQ5D VAS score at 3 months for IV iron therapy was 83.3 (SD 9.9), with a median gain of 13.4 (SD 12.2), which was statistically significant, p=0.005. However, there was no statistical difference in the magnitude of EQ5D VAS score gained during the study between the two groups. (p=0.65)

Table 4.13 ED 5D VAS in NIBD patients

Route	EQ5D VAS (Maximum score is100) -NIBD			
	Baseline	3 month	Improvement in EQ5D	P value*
IV Mean (SD)	69.9(18.3)	83.3(9.9)	13.4(12.2)†	0.005
PO Mean (SD)	63.4(17.2)	74.7(14.8)	10.6(14)†	0.07
IV versus PO comparison for EQ 5D VAS improvement				0.65†

Legend: EQ5D VAS: Euro-Quality 5-Dimension Visual Analogue scale;
IV: Intravenous, PO: Oral; NIBD: Non inflammatory bowel disease.

Figure 4-14 Change in EQ5D VAS during iron therapy in patients with and without IBD.



4.7.5.2 Inflammatory Bowel Disease Group (Table 4.14, Figure 4.14)

Table 4.14 ED 5D VAS in IBD patients

Route	EQ5D VAS (Maximum score:100) -IBD			
	Baseline	3 month	Improvement in EQ5D	P value*
IV Mean (SD)	64.2(18)	77(14.9)	13(17.5)†	0.0009
PO Mean (SD)	61.2(15.4)	64.2(19.5)	2(21)†	0.68
IV versus PO comparison for EQ 5D VAS improvement				0.056†

The mean EQ5D VAS score at 3 months for PO iron therapy was 64.2 (SD19.5) with a median gain of 2 (SD 21), which was not statistically significant, p=0.68. The mean EQ5D VAS score at 3 months for IV iron therapy was 77 (SD 14.9), with a statistical significant median gain of 13 (SD 17.5), p=0.0009. Overall there was a statistical trend favouring IV iron group. (p=0.056)

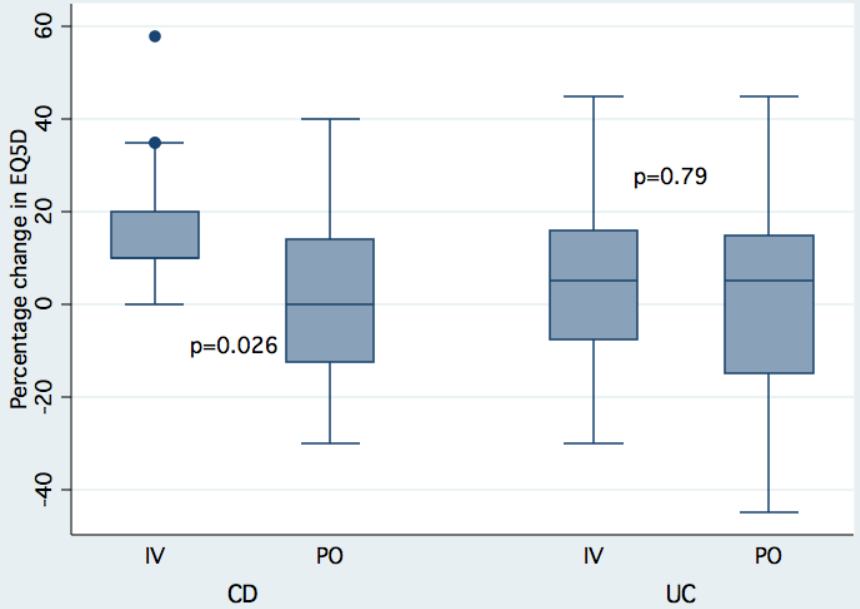
4.7.5.2.1 Crohn's Disease Group *(Table 4.15, Figure 4.54)*

Iron replacement therapy significantly improved the EQ5D score in CD patients, p= 0.026. This was driven by patients received IV iron replacement, p=0.0002. The mean EQ5D VAS score at 3 months for PO iron therapy was 67.3 (18.5), with a statistically non significant median gain of 1.7 (SD 19.7), p=0.77. The mean EQ5D VAS score at 3 months for IV iron therapy was 79.8 (SD 10), with a statistically significant median gain of 16.3 (SD 14), p=0.0002. Overall, IV iron therapy was superior to PO iron therapy in improving EQ5D VA score. (p=0.026)

Table 4.15 ED 5D VAS in Crohn's disease patients

Route	EQ5D VAS (Maximum score: 100) -CD			
	Baseline	3 month	Improvement in EQ5D	P value*
IV Mean (SD)	59.9(21.7)	79.8(10)	16.3(14)†	0.0002
PO Mean (SD)	65.6(16.9)	67.3(18.5)	1.7(19.7)†	0.77
IV versus PO comparison for EQ 5D VAS improvement				0.026†

Figure 4-15 Change in EQ5D VAS during iron therapy in patients with CD and UC



4.7.5.2.2. Ulcerative colitis Group *(Table 4.16, Figure 4.14)*

The mean EQ5D VAS score at 3 months for PO iron therapy was 60.9 (SD 20.9), with a median gain of 2 (SD 24), which was not statistically significant, p=0.78 The mean EQ5D VAS score at 3 months for IV iron therapy was 71 (SD 22.3), with a median gain of 5 (SD 22), which was not statistically significant, p=0.78. The difference in the magnitude of EQ5D VAS score gained during the study between the two groups was not statistically different, p=0.79.

Table 4.16 ED 5D VAS in ulcerative colitis patients

Route	EQ5D VAS (Maximum score:100) - UC			
	Baseline	3 month	Improvement in EQ5D	P value*
IV Mean (SD)	65.8(21)	71(22.3)	5(22)†	0.5
PO Mean (SD)	57.3(12.7)	60.9(20.9)	2(24)†	0.78
IV versus PO comparison for EQ 5D VAS improvement				0.79†

4.7.6 Disease activity by CRP

4.7.6.1 Non Inflammatory Bowel Disease Group (Table 4.8, Figure 4.2)

The mean CRP after 3 months of PO iron therapy was 5.5 (SD 7.3) and the mean CRP after 3 months of IV iron therapy was 2.2 (SD1.8). There was no statistical difference.

4.7.6.2 Inflammatory Bowel Disease Group (Table 4.8, Figure 4.2)

The mean CRP after 3 months of PO iron therapy was 7.2 (SD 10.8) and the mean CRP after 3 months of IV iron therapy was 6.8 (SD 8.4). There was no statistical difference.

4.7.6.2.1 Crohn's Disease Group *(Table 4.8, Figure 4.2)*

The mean CRP after 3 months of PO iron therapy was 10.6 (SD 13.8) and the mean CRP after 3 months of IV iron therapy was 4.8 (SD 4.8). There was no statistical difference.

4.7.6.2.2 Ulcerative colitis Group *(Table 4.8, Figure 4.2)*

The mean CRP after 3 months of PO iron therapy was 3.5 (SD 4.3) and the mean CRP after 3 months of IV iron therapy was 11.3 (SD 12.6). The difference in CRP was not statistically significant.

4.7.7 Disease activity by clinical disease activity index (Figure 4.11)

4.7.7.1 Crohn's Disease Group (Table 4.17, Figure 4.16)

<< HBI <5 indicates clinical remission **>>**

The median Harvey Bradshaw Index (HBI) after 3 months of PO iron therapy was 5 (IQR 3.5) with a trend towards statistical significant median HBI reduction of 2.5 (IQR 5), P=0.056. The median HBI after 3 months of IV iron therapy was 3.5 (IQR 4) with a statistical significant median HBI reduction of 2 (IQR 3), p=0.0002. There was no statistical difference in the magnitude of HBI change between IV and PO group during the 3 months study period, p=0.72.

Table 4.17 Influence of iron replacement on disease activity index at 3 months

	Crohn's Disease				Ulcerative Colitis			
	HBI Wk 0	HBI Wk 12	ΔHBI	P value*	PMS Wk 0	PMS Wk 12	ΔPMS	P value*
IV Median (IQR)	6 (3)	3.5 (4)	-2 (3)†	0.0002	1.5 (5)	2 (4)	0(1) ‡	0.94
PO Median (IQR)	6.5 (6.5)	5 (3.5)	-2.5 (5)†	0.056	3 (4)	2 (3)	0(3) ‡	0.11
PO versus IV in change of disease activity index				0.72†				‡0.31

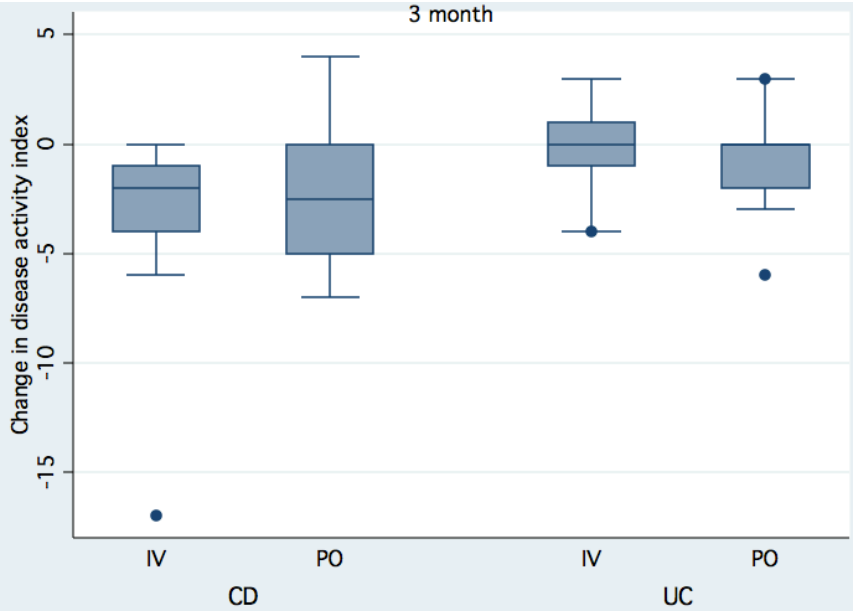
***Wilcoxon Sign rank test**
†Mann-Whitney test used for a two sample comparison.

4.7.7.2 Ulcerative colitis Group (Table 4.17, Figure 4.16)

<< PMS <2 indicates clinical remission **>>**

The median partial Mayo clinic score (PMS) after 3 months of PO iron therapy was 2 (IQR 3) and the median PMS after 3 months of IV iron therapy was 2 (IQR 4). There was no change in the PMS during the study period. There was no statistical difference in the PMS at 3 months between IV and PO group.

Figure 4-16 Change in clinical disease activity index at 3 months.



Legend: IV: Intravenous; PO: Oral; CD: Crohn's disease; UC: ulcerative colitis.

4.7.8 Reasons for incomplete follow up (Table 4.18)

Definition of *lost to follow up* – did not return for the end of study visit despite multiple phone calls and direct contact.

4.7.8.1 Non Inflammatory Bowel Disease

The only reason for incomplete follow up was lost to follow up. This occurred in 3 out of 11 patients in the IV group and 4 out of 9 in the PO group.

4.7.8.2 Crohn's disease

The reasons for incomplete follow for the IV group were pregnancy (1) and small bowel resection for structuring disease (1). In total, 2 out of 18 patients did not complete the study.

The reasons for incomplete follow up in the PO group were –lost to follow up (6), unable to travel from Fort Mcmurray for the clinic visit (1) and intolerant to oral iron and self-withdrawn from the study (1). In total, 8 out of 12 patients did not complete the study.

4.7.8.3 Ulcerative Colitis

There were 2 out of 8 patients in the IV group lost to follow up. In the PO group, there were 3 out of 11 patients lost to follow up.

Table 4.18 Reasons for incompletion

Number of occurrence (n)	Intravenous				Oral			
	CD (18)	UC (8)	NIBD (11)	Total (37)	CD (12)	UC (11)	NIBD (9)	Total (32)
Lost to follow up	0	2	3	5	6	3	4	13
Pregnant	1	0	0	1	0	0	0	0
Active disease required surgery	1	0	0	1	0	0	0	0
Geographical distance	0	0	0	0	1	0	0	1
Intolerant to therapy	0	0	0	0	1	0	0	1
TOTAL	2	2	3	7	8	3	4	15

4.7.8 Adverse effects (AE) (Table 4.19)

Number of subjects reported adverse event was same between the two routes of therapies: 7/ 46 subjects in IV and PO.

4.7.9.1 *Crohn's Disease*

In the IV group, adverse effects reported included gastrointestinal related (2); generalized arthralgia (1); diversion ileostomy for the management of severe perianal Crohn's disease (1); ileal resection for a fibrostenotic stricture (1); below knee amputation for arterial thrombosis (1) and headache (1). Four patients accounted for these AEs. In the PO group, the adverse effects were dominated by gastrointestinal origin (4); ileal resection for stricturing disease (1) Pelvic rami fractures from a simple mechanical fall (1). Five patients accounted for these AEs.

4.7.9.2. Ulcerative colitis

In the IV group, adverse events included left elbow bursitis (1) and total colectomy for fulminate ulcerative colitis (1). Two patients accounted for these AEs. In the PO group, one patient reported nausea during the first 2 weeks of oral iron replacement.

4.7.9.3 Non Inflammatory Bowel Disease

One patient developed abdominal cramp during the first iron sucrose infusion which subsequently resolved and did not recurred with the subsequent infusions. One patient in the oral iron group suffered from constipation during the treatment period.

Table 4.19 Frequency of adverse effects reported during the treatment period.

	IV			PO		
Adverse Event frequency	CD	UC	NIBD	CD	UC	NIBD
Gastrointestinal (total)						
Nausea	1	0	0	1	1	0
Cramps	1	0	1	2	0	0
Altered bowel habits	0	0	0	1	0	1
Arthritis/arthralgia	1	1	0	0	0	0
IBD related surgery						
Ileostomy	1	0	0	0	0	0
Colectomy	0	1	0	0	0	0
Fibrostenotic disease for resection	1	0	0	1	0	0
Others						
Fracture pelvic rami	0	0	0	1	0	0
Below Knee Amputation	1	0	0	0	0	0
Headache	1	0	0	0	0	0
Number of patients with AE	4	2	1	5	1	1

Legend: IV: Intravenous, PO: Oral, CD: Crohn's Disease, UC: Ulcerative colitis, NIBD: Non inflammatory bowel disease.

4.8. Summary

Intravenous iron therapy was associated with statistically significant higher serum ferritin at 2 and 3 months compared to oral iron therapy. The improvement in the quality of life scores at 2 and 3 months were statistical significant in the IV group but not in the PO group. The magnitude of improvement in the quality of life scores were greater in the IV group compared to the PO group, with a trend towards statistical significance in the IBD and the CD groups, but not in the UC group. These near statistical significance may be due to the small sample size.

There was no worsening of median disease activity indices at 2 and 3 months for the CD and the UC group with IV or PO iron replacement. At 3 months, statistical significant improvement in the HBI was noted in the IV group ($p=0.0002$) and a trend towards statistical significance in the PO group ($p=0.056$). HBI improvement was mirrored by the lower serum CRP levels at 3 months. In contrast, persistent active disease activity in the IV group of UC subjects was evident by the persistently elevated CRP during the study, increased median PMS at 3 months and a patient underwent total colectomy. Unremarkable change in the PMS and CRP levels was noted in UC subjects receiving oral iron. The apparent selection bias of having more active ulcerative colitis subjects in the IV group and the small overall sample size could significantly bias the results. This selection bias may have occurred at the randomization stage where subjects with active ulcerative colitis systematically declined oral iron therapy were they randomized into it.

Adherence to oral iron therapy was poor compared to the IV group and more subjects in the PO group failed to return for follow up.

Gastrointestinal symptoms were the most common adverse effect in the PO group. In contrast, more serious adverse effects were noted in the IV group, included arthralgia, headache and a subject with right leg ischemia resulted in an above knee amputation. No causal link was established with iron sucrose.

5 Chapter 5 THE INFLUENCE OF IRON THERAPY ON COLONIC BACTERIAL ECOLOGY

5.1. Background

Crohn's disease (CD) and ulcerative colitis (UC) are collectively referred to as inflammatory bowel disease (IBD). They are characterized by chronic inflammation of the gastrointestinal tract of relapsing and remitting or rapidly progressive course. It is widely accepted that the pathogenesis of IBD is the result of aberrant host immune response to intestinal microbe dysbiosis (a significant change in the intestinal microbiota composition from normal) in a genetically susceptible individual (1,115,116). Although many bacteria have been suggested historically, such as *Myocbacterium avium* and *Yersinia enterocolitica* for examples, none have been confirmed as the causative microbe in the pathogenesis of IBD (117,118). A two-year prospective double-blinded clinical trial using anti- *Mycobacterium avium paratuberculosis* antibiotic therapy did not demonstrate its efficacy in inducing and maintaining CD remission (119). The role of *Adherent-Invasive .E Coli* (AIEC) in CD is still under investigation. (85,89,120) More importantly, the paradigm has shifted from finding a causative bacterium to examining the change in the intestinal microbial community as whole, the microbiota (121,122). Culture independent molecular finger printing technique base on bacterial DNA polymerized chain reaction such as terminal restriction fragment length polymorphism (TRFLP) can be used to profile the unknown microbiome community.

Animal models indicated exposure to environmental bacteria is critical in the development of colitis and mice in a germ free environment do not develop colitis (62, 71, 123, 124).

5.2. Dysbiosis in Human IBD

Dysbiosis is well recognized in IBD with a significant reduction in the microbial diversity during the active disease state. Reduction in the 'good bacteria' such as Firmicutes, mostly Clostridium XIVa and IV groups, species *Faecalibacterium prausnitzii* (125-127) and butyrate producing Bacteroides (82) and increase in Proteobacteria and Actinobacteria are commonly seem in patients with IBD (128). Furthermore, higher post-operative recurrence of CD is seen in patients with reduced density of *F. prausnitzii* in their intestinal mucosa (129). An increase in Enterobacteriaceae such as *E. coli* in Crohn's disease (125,126) and sulfate reducing bacteria *Desulfobrio piger* (130) have been shown in patients with IBD when compared to control (56,85).

These sulfate-reducing bacteria enhance colonic hydrogen sulfide production and prevent colonocytes utilization of butyrate. (53) Butyrate is a short chain fatty acid produced by intestinal bacteria and it is an important source of energy for the colonic epithelial cells. It enhances the epithelial barrier function and modulates the intestinal immune response.

Diversion of fecal stream with a 'ostomy' in human has been shown to prevent post-operative recurrence Crohn's colitis and improve perianal disease, and recurrence of disease is noted with the re-constitution of faecal stream. (131) Human antibiotic and probiotic clinical trials in IBD patients have been encouraging. Meta-analysis by Doherty et al confirmed good efficacy of nitroimidazole antibiotics in reducing the risk of post-operative recurrence of ileal CD and the number needed to treat in order to prevent one recurrence was 4. A more recent meta-analysis on antibiotic therapy in IBD confirmed the role of antibiotics in IBD management (132). Clinical trials outcome on probiotic use in IBD is conflicting and is reflected in its limited clinical utility (133).

5.3. The effect of iron on the intestinal epithelia and colonic bacterial microbiota

Several animal models have demonstrated deleterious effects of oral iron replacement therapy on colonic mucosa. Firstly, oral iron replacement therapy is associated with increased intestinal mucosal oxidative stress (54,55,85), higher histological score for inflammation (134) and it could promote carcinogenesis (135). Secondly, oral iron replacement is associated with reduction in the intestinal microbiota biodiversity (136). Manipulation of infant formula or cow's milk by additional iron supplement in 10 month olds has been shown to alter faecal microbiota (137). Animal study suggested a low iron diet promotes the colonization of 'beneficial' bacteria such as Lactobacillus and Enterococcus and a high iron diet promotes the colonization of the more pathogenic bacteria such as the coliforms (138). Moreover, iron deficient diet has been shown to prevent the development of Crohn's like ileitis in an animal model (55). These animal model findings have major implications in the management of IBD patients with iron deficiency. Current literature is lacking on the impact of iron replacement therapy on the human intestinal microbiota.

5.4. Determining Diversity of the intestinal bacterial microbiota

5.4.1. Terminal Restriction Fragment Length Polymorphism (TRFLP)

TRFLP analysis is a culture independent method of studying a collective set of intestinal microbial genetic composition termed microbiome. This molecular fingerprinting approach to the intestinal bacterial community nucleic acid profiling overcomes known biases of traditional bacteria cultured based analysis as many bacteria are anaerobes and fastidious to grow. TRFLP enables rapid inference of plausible gut microbiota by comparing the digested DNA fragment lengths against a web based database (139,140). This has been shown to be a fast and effective method for rapid and accurate examination of human gut microbiome in the setting of interventional or population based observational study (141).

5.4.2. Measures to describe the microbiome (Also refer to chapter 3, section 3.3.1 and 3.3.2)

TRFLP analysis enables qualitative descriptive comparison of the intestinal microbiome composition changes attributed to an intervention rather than identifying and quantifying individual bacterial species. BioNumerics software (Applied Maths, Belgium) mathematically correlates species richness, species evenness, and proportional abundance based on the TRFLP electropherogram to determine Shannon Weiner's diversity index (SWI). It is an ecological concept that describes the 'richness' - number of different species, the relative evenness in the number of each species and 'abundance' – the total number of each species in an ecological system (142,143). Having a high number of unique species in combination with relative even abundance indicates high index of diversity. Where as either a high number of species with uneven distribution or low total number of species are indicative of low diversity.

5.4.3. Limitations of TRFLP include

5.4.3.1 Polymerase Chain Reaction amplification of bacterial DNA has inherent bias because of different primer amplification efficiency. (Chapter 3, section 3.2.4).

5.4.3.2 The inter and intra-operator variation in specimen handling during DNA clean up (Chapter 3, section 3.2.6)

5.4.3.3 Variable enzymatic activities according to the 'age' of the enzyme - new batch vs an old yet not expired batch of enzymes. (Chapter 3, section 3.2.7).

5.4.3.4 Different bacterial DNA sequences could sharing the same restriction site for a particular restriction enzyme, therefore, it would appear as one band on the electrophoresis.

5.4.3.5 Inability of TRFLP to definitively identify or correlate a peak on electropherogram as a particular or multiple bacteria species. TRFLP correlates a restriction fragment length with a database of known bacteria species and its corresponding fragment length for a given restriction enzyme(s). TRFLP is analogous to identifying a possible bacteria species by the shadow its casts.

5.4.3.6 Variation in the sample processing on different days by different operators.

5.4.3.7 Variation in the number copies of 16S DNA in each bacterium.

5.5. Hypothesis

Oral and intravenous iron replacement therapies have different effects on the colonic bacterial ecology in iron deficient patients.

*5.*6. Aim

5.6.1 To determine the mucosal and faecal bacterial microbiome diversity index in patients receiving oral (PO) or intravenous (IV) iron replacement therapy.

5.6.2 To describe the mucosal and faecal bacterial microbiome composition at **phylum level** in patients receiving oral (PO) or intravenous (IV) iron replacement therapy.

*5.*7. Results

Total of 39 paired (18 sigmoid mucosal biopsy and 21 faecal) specimens were successfully examined by TRFLP. Of the 18 mucosal biopsies – 11 were IBD (5CD IV, 4UC PO, and 2 UC IV) and 7 NIBD (4 PO, 3 IV). In the 21 pairs of faecal specimen analysed, 12 were IBD (2 CD PO, 3 CD IV, 4 UC PO, and 3 UC IV) and 9 were NIBD (5 PO, 4 IV). (Table 5.1)

Table 5.1 Number of mucosal and Faecal bacterial Microbiome analysed

Mucosal microbiome: 18						**Faecal Microbiome: 21**					
IBD: 11				**NIBD: 7**		**IBD: 12**				**NIBD: 9**	
CD: 5		**UC: 6**				**CD: 5**		**UC: 7**			
PO:0	**IV: 5**	**PO:4**	**IV: 2**	**PO:4**	**IV: 3**	**PO: 2**	**IV: 3**	**PO:4**	**IV: 3**	**PO:5**	**IV: 4**

Legend: IBD: Inflammatory Bowel Disease, NIBD: Non IBD; UC: Ulcerative colitis; CD: Crohn's disease; PO: Oral; IV: Intravenous

5.7.1 Inflammatory bowel disease

5.7.1.1 Mucosal bacterial microbiome diversity (Table 5.2)

The median baseline mucosal Shannon Weiner diversity index (SWI) was 3.1 in the IV group and 1.9 in the PO group without statistical significant difference, p=0.08. At 3 month, comparable gain in the median diversity index was noted, 0.5 and 0.4, for PO and IV respectively, p=0.9.

Table 5.2 Mucosal Shannon Weiner diversity index (SWI) in IBD

Median (IQR)	Mucosal SWI-IBD		
Route (N)	Oral (4)	Intravenous (7)	P value
Baseline	1.9 (1.5)	3.1(1)	0.08
3 months	2.2 (1.1)	3.3 (1.3)	0.09
Change	0.02 (2.4)	0.4 (1.7)	0.9

P values were determined by Wilcoxon signed rank test for one sample and Mann-Whitney Test for 2-sample comparison.

5.7.1.2 Mucosal bacterial microbiome composition at phyla level (Figure 5.1, 5.2)

A dramatic change in the mucosal bacterial Microbiome composition was noted with *IV iron therapy*. It was associated with increased Firmicutes and Bacteroidetes abundance and reduced Actinobacteria and Deferribacteres abundance. In contrast, oral iron therapy was associated with further dysbiosis characterized by reduced Firmicutes and Actinobacteria abundance and increased Proteobacteria and unclassified bacteria abundance. None of the included subject was on antibiotics.

Figure 5-1 Mucosal bacterial microbiome composition at Phylum level: IBD group before and after IV iron.

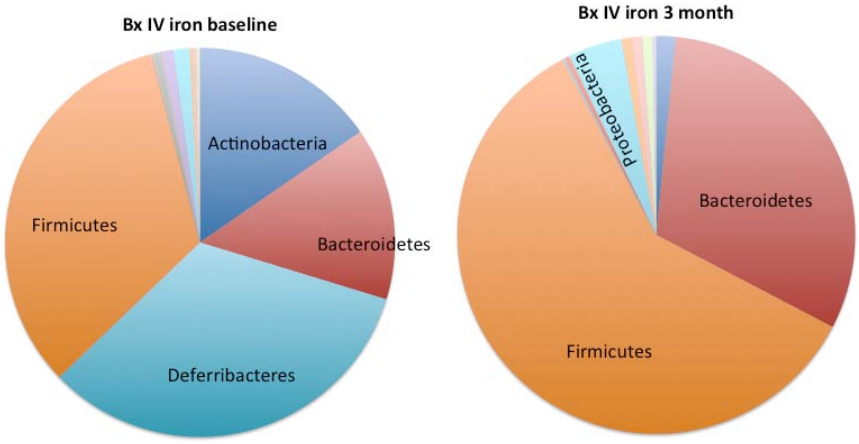
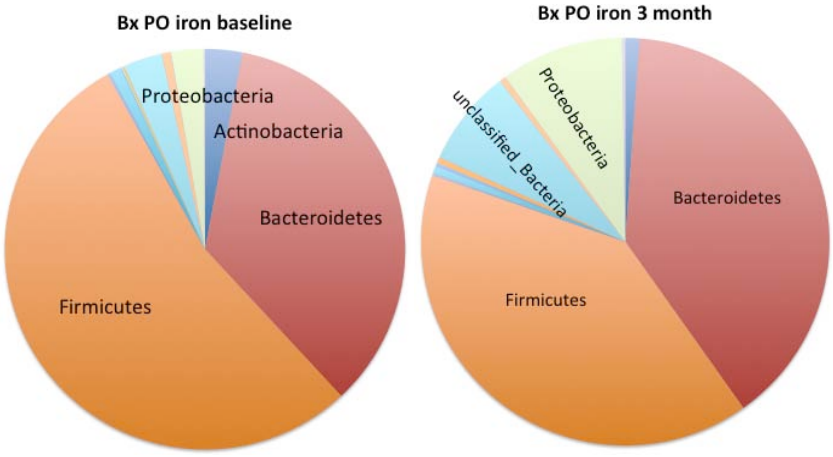


Figure 5-2 Mucosal bacterial microbiome composition at Phylum level: IBD group (UC) before and after PO iron.



5.7.1.3 Faecal bacterial microbiome diversity (Table 5.3)

The median baseline faecal diversity index was 2.4 in the IV group and 3.2 in the PO group, which was not statistically significant different, p=0.2. At 3 month, a median reduction of diversity index was noted in the PO iron group (-0.5) whereas a median gain in diversity index was noted in the IV iron group (0.29), with a trend towards statistical significant, p= 0.07. None of the included subject was on antibiotics.

Table 5.3 Faecal Shannon Weiner diversity index (SWI) in IBD

Median (IQR)	Faecal SWI- IBD		
Route (N)	Oral (6)	Intravenous (6)	P value
Baseline	3.2 (1.2)	2.4(0.74)	0.2
3 months	2.7 (1.4)	2.77 (1.1)	0.7
Change	-0.5 (1.5)	0.29 (0.6)	0.07

P values were determined by Wilcoxon signed rank test for one sample and Mann-Whitney Test for 2-sample comparison.

5.7.1.4 Faecal bacterial microbiome composition at phyla level (Figure 5-4, 5-5)

Intravenous iron therapy did not change the stool Microbiome composition. The dominance of Firmicutes and Baceroidetes persisted.

Oral iron had minimal affect on the stool microbiome composition. The dominance of Firmicutes and Bacteroidetes remained unchanged after 3 months of oral iron ingestion.

Figure 5-3 Faecal bacterial microbiome composition at phylum level: IBD group before and after IV iron

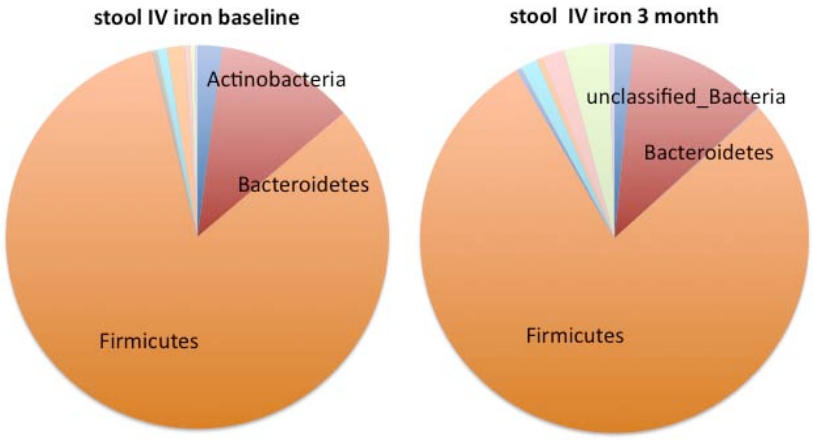
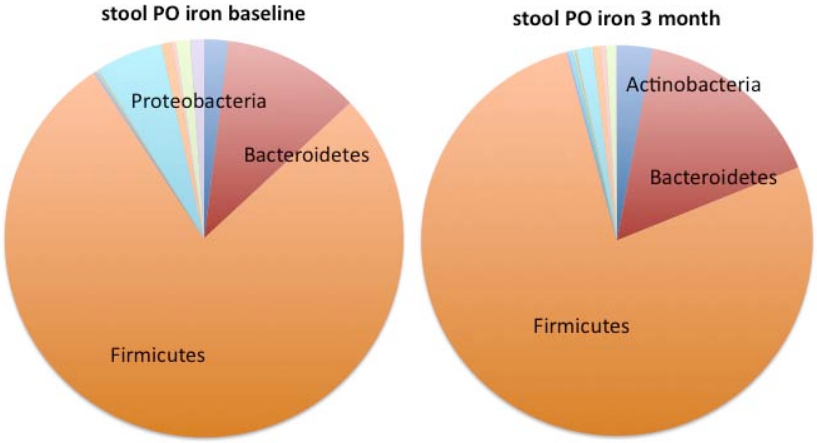


Figure 5-4 Faecal bacterial microbiome composition at phylum level: IBD group before and after PO iron.



5.7.1.5 Mucosal bacterial microbiome similarity index (SI)

SI describes how similar the terminal restriction fragment profiles are at baseline and at 3 month for an individual. Higher SI was noted in the IV group 61%, compared to 44% in the oral group. There was no statistical difference by Mann-Whitney test, p=0.7. This indicates the terminal restriction fragment profiles at 3 months and baselines were *more* similar in the IV group compared to the PO group.

5.7.1.6 Faecal bacterial microbiome similarity index

Higher median similarity index was noted in the IV group 83%, compared to 68% in the oral group. There was no statistical difference by Mann-Whitney test, p=0.5.

5.7.2 Crohn's disease (CD)

5.7.2.1 Mucosal bacterial microbiome diversity Index (Table 5-4.)

No result for oral iron treated CD patient was available at the time of thesis write up. The median diversity index in the IV treated group was 2.6 at baseline with a median gain of 0.6 diversity index at 3 month.

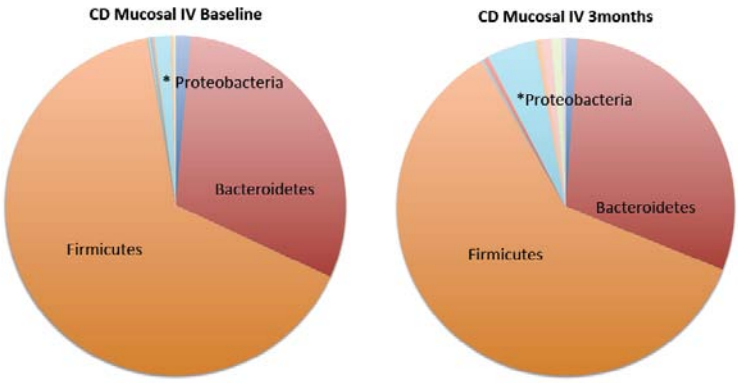
Table 5.4 Mucosal Shannon Weiner diversity index (SWI) in CD

Median (IQR)	Mucosal SWI-CD		
Route (N)	Oral (0)	Intravenous (5)	P value
Baseline	--	2.6 (0.7)	--
3 months	--	3.3 (0.8)	--
Change	--	0.6 (0.53)	--

5.7.2.2 Mucosal bacterial microbiome composition at phyla level (Figure 5.5)

Intravenous iron therapy had minimal impact on the mucosal bacterial microbiome composition. No result was available for the corresponding PO group at the time of thesis writing.

Figure 5-5 mucosal bacterial Microbiome composition at phyla level: CD group before and after IV iron.



5.7.2.3 Faecal bacterial microbiome diversity (Table 5.5)

The median baseline faecal diversity index was 2.8 in the IV group and 2.4 in the PO group, which was not statistically significant different, p=0.6. At 3 month, a median reduction of diversity index was noted in the PO iron group (-0.6) whereas a median gain in diversity index was noted in the IV iron group (0.1) with a trend towards statistical significant, p= 0.08.

Table 5.5 Faecal Shannon Weiner diversity index (SWI) in CD

Median (IQR)	Faecal SWI-CD		
Route (N)	Oral (2)	Intravenous (3)	P value
Baseline	2.8 (1)	2.4(0.6)	0.6
3 months	2.2 (1.2)	3.1 (1.2)	0.2
Change	-0.6 (-0.3)	0.1 (0.9)	0.08

P values were determined by Wilcoxon signed rank test for one sample and Mann-Whitney Test for 2-sample comparison.

5.7.2.4 Faecal bacterial microbiome composition at phyla level (Figure 5-6, 5-7)

Intravenous iron replacement therapy was associated with a reduction in the Firmicutes abundance and an increased in Bacteriodetes and unclassified bacteria abundance in the faeces of CD patients. This was unexpected. One patient in the IV group and no one in the PO group took antibiotics during the study period. Oral iron replacement therapy was associated with dramatic increased Firmicutes abundance and it became the major phylum at 3 months. These

results are unexpected and need to be interpreted with caution as the sample size was small (2 for the PO group and 3 for the IV group).

Figure 5-6 Faecal bacterial microbiome composition at phyla level: CD group before and after IV iron

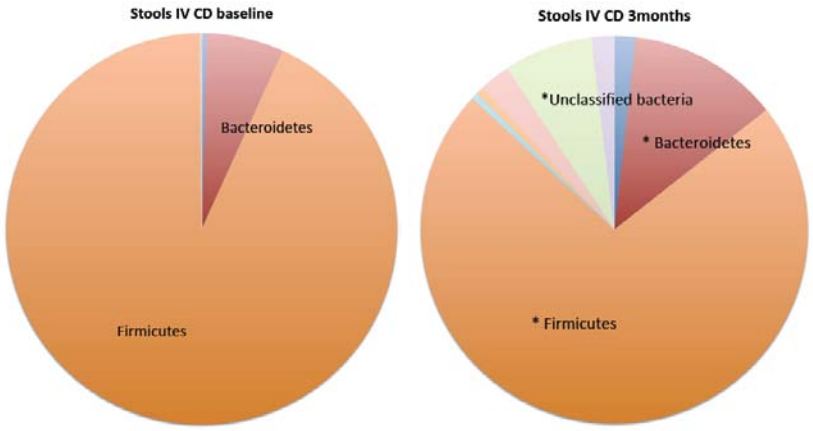
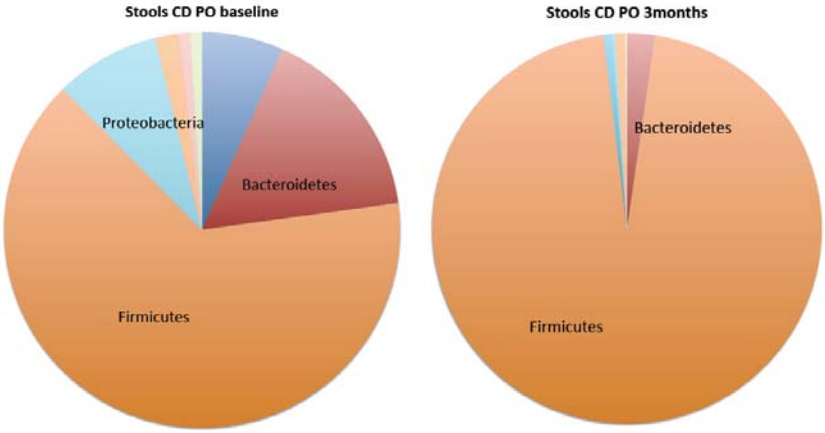


Figure 5-7 Faecal bacterial microbiome composition at phyla level: CD group before and after PO iron



5.7.2.5 Mucosal bacterial microbiome similarity index (SI)

No mucosal bacteria microbiome SI result for CD patient in the PO group was available for inclusion in thesis. The median SI in the IV treated group was 56%.

5.7.2.6 Faecal bacterial microbiome similarity index (SI)

Higher median Faecal bacterial Microbiome SI was noted in the IV group, 85%, compared to 51% in the oral group. There was no statistical difference by Mann-Whitney test, p=0.1.

Caution is needed when interpreting the results because of small sample size.

5.7.3 Ulcerative Colitis (UC)

5.7.3.1 Mucosal bacterial microbiome diversity Index (Table 5-6)

The median baseline mucosal diversity index was 3.56 in the IV group and 1.9 in the oral iron treatment group, with a trend towards statistical significance, p=0.06. At 3 month, IV group was associated with a median diversity index reduction of 0.58 where as a 0.02 gain in the median diversity index was noted in the PO group, p=0.35. As mentioned in Chapter 4 (page 25, 6.4.2.2), there were more patients in the IV group with active ulcerative colitis than in the PO group.

Table 5.6 Mucosal Shannon Weiner diversity index (SWI) in UC

Median (IQR)	Mucosal SWI-UC		
Route (N)	Oral (4)	Intravenous (2)	P value
Baseline	1.9 (1.5)	3.56(0.03)	0.06
3 months	2.2 (1.1)	3 (1.3)	0.9
Change	0.02 (2.4)	-0.58 (1.4)	0.35

5.7.3.2 Mucosal bacterial microbiome composition at phyla level (Figure 5.2, Fig 5.7)

Oral iron therapy was associated with reduced Firmicutes abundance and increased Proteobacteria and Unclassified bacteria abundance. IV iron was associated with reduced Firmicutes abundance and a corresponding increase in Bacteroidetes abundance.

This is the same pie chart as the IBD group received PO iron therapy (Figure 5.2) because there was no CD patient in the PO group for this section. One person in the IV group and no one in the oral group took antibiotics.

Figure 5-8 5-2 Mucosal bacterial mcrobiome composition at phyla level: UC group before and after PO iron

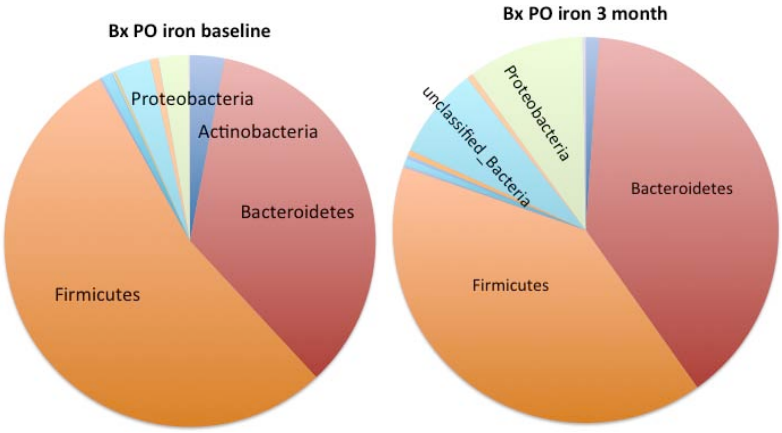
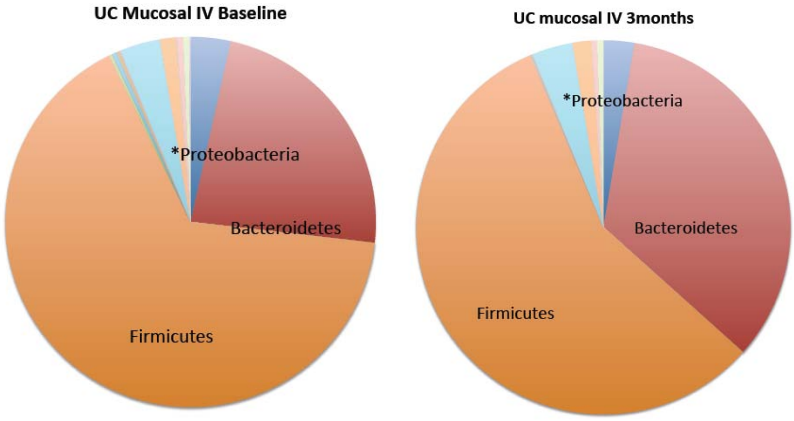


Figure 5-9 Mucosal bacterial microbiome composition at phyla level: UC group before and after IV iron



5.7.3.3 Faecal bacterial microbiome diversity Index (Table 5-7)

The median baseline mucosal SWI was 2.5 in the IV group and 3.3 in the oral iron treatment group, which was not statistically significant, p=0.3. At 3 month, IV group was associated with median diversity index gain of 0.46 where as a 0.5 reduction in the median diversity index was noted in the PO group, p=0.47.

Table 5.7 Faecal Shannon Weiner diversity index (SWI) in UC

Median (IQR)	Faecal SWI-UC		
Route (N)	Oral (4)	Intravenous (3)	P value
Baseline	3.3 (0.9)	2.5(1.3)	0.3^
3 months	2.8 (1.7)	2.4 (2.8)	0.7^
Change	-0.5 (-0.78)	0.46 (3.6)	0.47*

P values were determined by ^Wilcoxon signed rank test for one sample and *Mann-Whitney Test for 2-sample comparison.

5.7.3.4 Faecal bacterial microbiome composition at phyla level (Figure 5-8, 5-9)

At 3 months, oral iron therapy was associated with a slight reduction in Firmicutes abundance and slight increase in the Bacteroidetes abundance. IV iron was associated with persistent dominate Firmicutes abundance; however, there was an emergence of Tenericutes phylum which includes bacteria such as Mycoplasma and Chlamydia. None of the included patients was on antibiotics.

Figure 5-10 Faecal bacterial microbiome composition at phyla level: UC group before and after PO iron

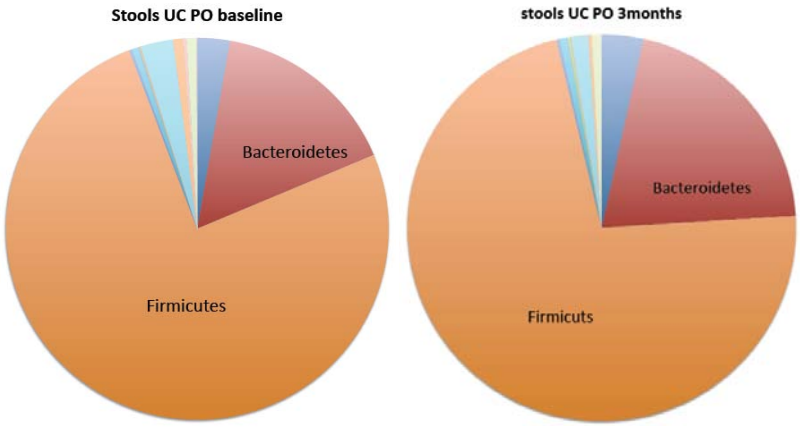
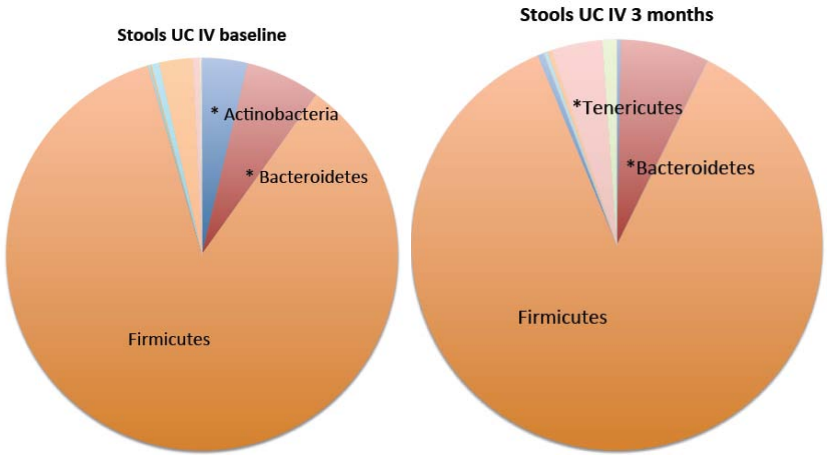


Figure 5-11 Faecal bacterial microbiome composition at phyla level: UC group before and after IV iron



5.7.3.5 Mucosal bacterial microbiome similarity index (SI)

Higher median Faecal bacterial Microbiome SI was noted in the IV group, 78%, compared to 44% in the oral group but it was not statistical significant by Mann-Whitney test, p=0.2.

5.7.3.6 Faecal bacterial Microbiome similarity index (SI)

Higher median Faecal bacterial Microbiome SI was noted in the IV group, 81%, compared to 72% in the oral group but it was not statistical significant by Mann-Whitney test, p=1.0.

The small sample size limits generalization of the data.

5.6.4 Non Inflammatory bowel disease

5.7.4.1 Mucosal bacterial microbiome diversity index (Table 5-8)

The median baseline mucosal SWI was 2.8 in the IV group and 2.2 in the PO iron treatment group, which was not statistically significant, p=0.4. At 3 month, a greater reduction in the diversity index occurred in the IV group (-1.2) compared to PO group (0.02), p=0.06. This is in

contrast to IBD group where IV iron was associated with increased mucosal bacterial microbiome diversity.

Table 5.8 Mucosal Shannon Weiner diversity index (SWI) in NIBD

Median (IQR)	Mucosal SWI-NIBD		
Route (N)	Oral (4)	Intravenous (2)	P value
Baseline	2.2 (1.3)	2.8(0.2)	0.4
3 months	2.6 (0.8)	1.7 (0.7)	0.2
Change	-0.02 (2)	-1.2 (0.9)	0.06

P values were determined by Wilcoxon signed rank test for one sample and Mann-Whitney Test for 2-sample comparison.

5.7.4.2 Mucosal bacterial microbiome composition at phyla level (Figure 5.10, 5.11)

Similar to what is observed in IBD patients on IV iron replacement, an increase in Firmicutes abundance was noted in the NIBD group with significant reduction in the abundance of other major phyla such as Bacteroidetes and Proteobacteria. Moreover, as in IBD patients, PO iron was associated with a reduction in Firmicutes abundance and an increase in Proteobacteria abundance. This implies that the alteration in the *mucosal bacterial microbiome composition* is strongly influenced by the route of iron replacement rather than diagnosis.

Figure 5-12 Mucosal bacterial microbiome composition at phyla level: NIBD group before and after IV iron.

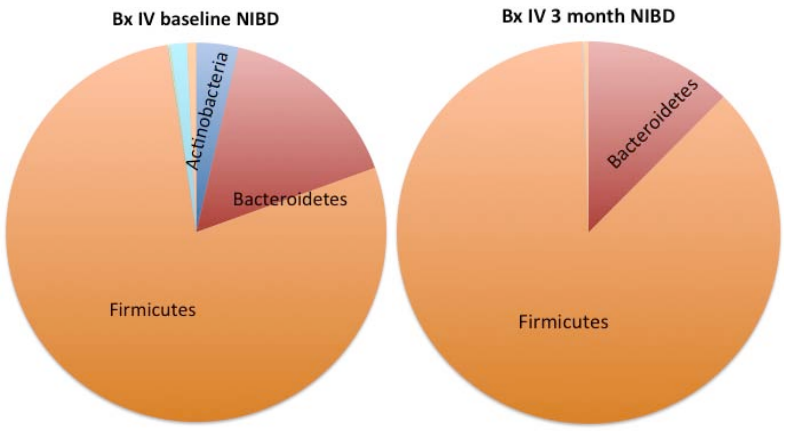
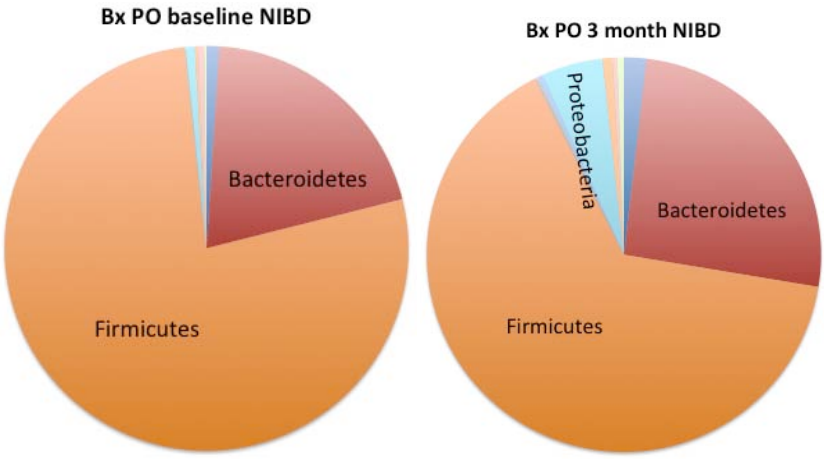


Figure 5-13 Mucosal bacterial microbiome composition at phyla level: NIBD group before and after PO iron.



5.7.4.3 Faecal bacterial microbiome diversity (Table 5.9)

The median baseline faecal diversity index was 3.8 in the IV group and 3.2 in the PO group, which was no statistically significant, p=0.2. At 3 months, a median reduction of 0.1 in the diversity index was noted in the PO group and a median 0.3 gain in diversity index was noted in the IV iron group, but there was no statistical significant difference, p= 0.6.

The reduction in faecal diversity index in the PO iron and an increase in the IV group were consistent with the observations in the IBD group. A stronger statistical trend towards a significant reduction in the faecal diversity index in the PO group was seen in the IBD group. This implies that the alteration in the ***faecal diversity index*** is strongly influenced by the route of iron replacement rather than diagnosis. However, the major limitation with the data presented is the small sample size.

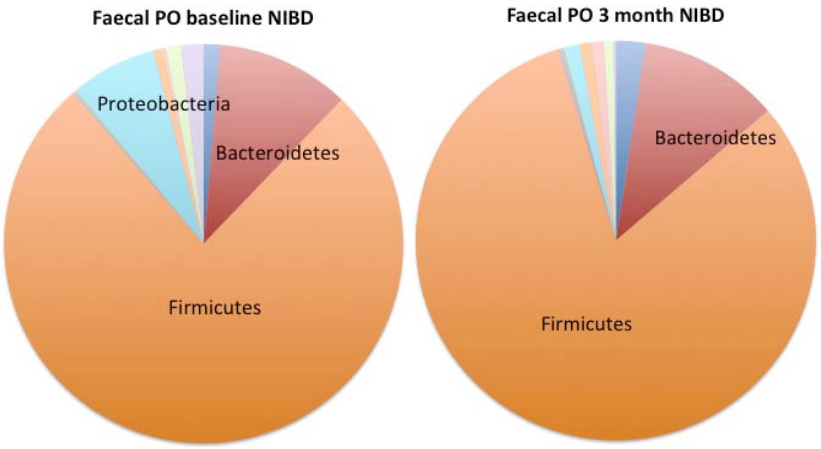
Table 5.9 Faecal Shannon Weiner diversity index (SWI) in NIBD

Median (IQR)	Faecal SWI-NIBD		
Route (N)	Oral (6)	Intravenous (3)	P value
Baseline	3.2 (1.3)	3.8(1.2)	0.2
3 months	3.2 (1.3)	4 (1.6)	0.2
Change	-0.12 (0.3)	0.3 (2.8)	0.6

5.7.4.4 Faecal bacterial microbiome composition at phyla level (Figure 5.12, 5.13)

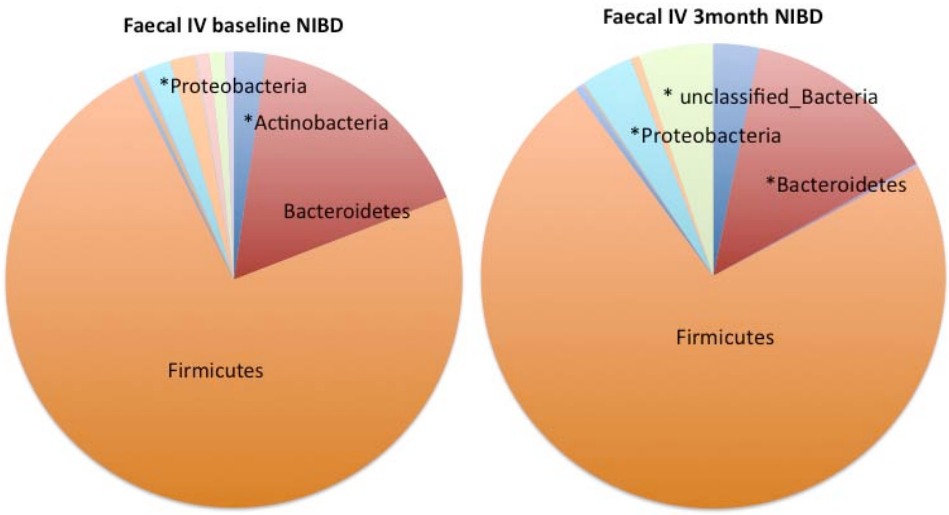
Oral iron therapy did not change the stool Microbiome composition significantly at phyla level. The dominance of Firmicutes and Bacteroidetes persisted and Proteobacteria abundance was reduced. This is similar to what is observed in the IBD group. Oral iron appears to have similar effect on IBD and NIBD ***Faecal bacterial Microbiome*** composition at phyla level.

Figure 5-14 Faecal bacterial Microbiome composition at phyla level: NIBD group before and after PO iron.



IV iron had minimal affect on the stool microbiome composition in the NIBD group. The dominance of Firmicutes and Bacteroidetes remained unchanged; however, there is increase abundance of Proteobacteria, Actinobacteria and unclassified bacteria. This is consistent with IBD patients.

Figure 5-15 Faecal bacterial microbiome composition at phyla level: NIBD group before and after IV iron.



5.7.4.5 Mucosal bacterial microbiome similarity index (SI)

Median similarity index was 75.5% in the IV group and 67.5% in the oral group. There was no statistical difference by Mann-Whitney test, p=0.4. This is consistent with IBD group.

5.7.4.6 Faecal bacterial microbiome similarity index

The median similarity index was comparable between IV and PO group, 83% and 79.5% respectively, p=0.8. This is consistent with IBD group.

5.8. Summary

IV iron therapy was associated with a higher similarity index than PO iron therapy in IBD patients but not statistical significant. This suggests that IV iron therapy may have a lesser impact on the gut microbiome.

Although statistical significance was not reached when comparing the diversity index between PO and IV group, there was a consistent trend towards a significant reduction in the faecal diversity index with PO iron use and a consistent gain in the faecal diversity with IV iron use. Oral

iron was associated with a significant change in the mucosal bacterial microbiome composition at phyla level but not in faecal bacterial microbiome composition. In general, where there were changes in the intestinal bacterial ecology, less dysbiosis was noted in the IV group with an increase in the Firmicutes and Bacteroidetes abundance. In contrast, PO iron was associated with a reduction in Firmicutes abundance and increase in Proteobacteria and unclassified bacteria abundance, suggesting a progression of bacterial dysbiosis.

6 URINARY METABOLOMICS

6.1. Background

6.1.1 Iron and cellular function

Iron plays a critical role in cellular metabolism – as a cofactor in Krebs cycle, mitochondrial respiratory chain reaction in the production of adenosine triphosphates (ATP) or its incorporation into the haeme component of red cells and myoglobin of myocytes to carry oxygen. Iron could accept an electron and be reduced from ferric form (Fe^{3+}) to ferrous form (Fe^{2+}) or oxidised from Fe^{2+} to Fe^{3+} by donating an electron. Through peroxide, free radicals may be generated and mediate cellular damage at the protein or DNA level. Iron has been implicated as a possible cause of many chronic illnesses ranging from neurodegenerative disease to the formation of atherosclerotic plaques in coronary artery disease (144).

Iron is critical to the growth and survival of gut microbes and as such many have developed evolutionary adaptations in order to capture the limited free iron available in the host gut lumen. An animal model demonstrated that the route of iron therapy, oral versus parental, has a differential effect on gut microbiota composition (55). The interaction between the host and gut microbial metabolism is complex and it would be difficult to dissect out host and bacterial metabolic pathways individually in an attempt to determine which metabolic product is associated with a disease state and this is where our metabolomics study fits in (145). An all-inclusive approach to examine the impact of iron replacement therapy on this complex ecosystem examines the final metabolic products in urinary metabolomics analysis.

6.1.2 What is Nuclear Magnetic Resonance based urinary metabolomics?

Metabolomics is the study of metabolic pathway and unique biochemical molecules created in a living system. Through the use of Nuclear Magnetic Resonance (NMR), various ‘metabolites’ can be identified against a known database and quantified. With it, one could compare the impact of an intervention such as giving iron replacement therapy either orally or intravenously, and examine its impact on the host-microbial metabolism. An alternate method of identifying these metabolites is using mass spectrophotometry, which has the ability to identify individual metabolites, including those that are unknown to the database.

NMR was chosen over mass spectrophotometer because NMR required 1) minimal sample preparation, 2) specimens are preserved and are able to be reused in other analyses, 3) NMR is able to detect multiple metabolites within a single experiment and 4) NMR results are reproducible. Moreover, the National High Field Nuclear Magnetic Resonance Centre (NANUC) is located on the University of Alberta campus and is home to Varian 500Hz, 600Hz, and 800Hz NMR spectrometers and is equipped with highly skilled and knowledgeable personnel. For this study, the 600Hz spectrometer was used in conjunction with a Varian 768 AS sample handling robot as it was the most cost-effective. Urine specimen was chosen, as it was the easiest to acquire from the patient. NMR based urinary metabolomics analysis is a novel approach in describing the effect of iron therapy in iron deficient patients with IBD which has not been widely described.

6.1.3. NMR-based faecal metabolomics

NMR-based faecal metabolomics analysis of both CD and UC patients revealed reduced levels of butyrate (bacterial origin), acetate, methylamine (break down product of ammonia) and trimethylamine (product of animal and plant putrefaction) in comparison with a control population, suggesting changes in the gut microbial community (146). Moreover, higher quantities of essential amino acids such as isoleucine, leucine, lysine and valine were present in the faece from CD than UC group and lowest in the control group implying malabsorption caused by the inflammatory disease. Metabolic differences in faecal profiles were more marked in the CD group.

6.2. Objectives

6.2.1 To describe the metabolite profile of iron deficient IBD patients undergoing iron replacement therapy.

6.2.2 To compare the metabolite profile of iron deficient IBD patients receiving IV and PO iron therapy.

6.3. Results

NMR spectra were acquired in 39 IBD patients, 21 received IV iron and 18 received oral iron replacement. The baseline characteristics were comparable in terms of mean age (p=0.48), males to females proportion (p=0.075), CD: UC ratio (p=0.28), proportion of patient with CRP >8 at baseline (p=0.45) and at 3-month (p=0.7), proportion of patient used concurrent antibiotic/probiotic/prebiotic (p=0.8) and concurrent use of immunosuppressant +/- biological drug (p=0.09).

The unsupervised principal component analysis (PCA) on the metabolites concentrations generated unsatisfactory model scores for model description (R^2)
and predictivity (Q^2). (See chapter 3, page 23, section3.7) Creatinine normalization and logarithmic transformation of the metabolites concentrations did not generate a satisfactory model score.

Table 6.1 Baseline characteristics

Inflammatory Bowel Disease (Number)	Intravenous 21	Oral 18	P value*
Age, years (mean, SD)	40.6 (16.2)	41(16.7)	0.48^
Sex M: F (% male)	13:8 (62)	6:12 (33)	0.075
CD: UC (% CD)	13:8 (62)	8:10 (44)	0.28
CRP >8 at baseline (%)	8:13 (38)	9:9 (50)	0.45
CRP >8 at 3 months (%)	7:14 (33)	5:13 (28)	0.7
Use of antibiotic/probiotic/prebiotic (%)	4 (19)	4 (22)	0.8
Use of immunosuppressant +/-biological drug (%)	15 (71)	8 (44)	0.09

***Chi square proportion test, ^ 2 sample t-test**

Legend: M- male, F- female, CD- Crohn's disease, UC – Ulcerative colitis

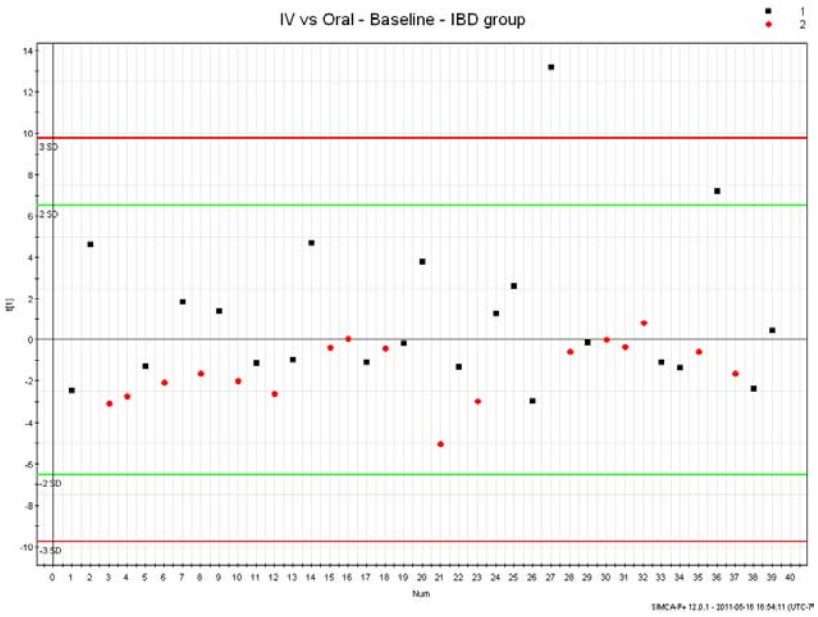
6.3.1. Inflammatory Bowel Disease (Table 6.1)

6.3.1.1. Building the Models

6.3.1.1.1 Baseline (figure 6.1)

Using two-component separation, a supervised orthogonal partial least squares (OPLS) model was built with R^2Y of 0.162 and Q^2 -0.0173 (not predictive). Exploratory data analysis of the OPLS scatter plot showed no separation between the IV and the PO group indicating the lack of statistical significant difference in the metabolite profiles between the two groups.

Figure 5-1 OPSL scatter plot of the baseline IBD group.

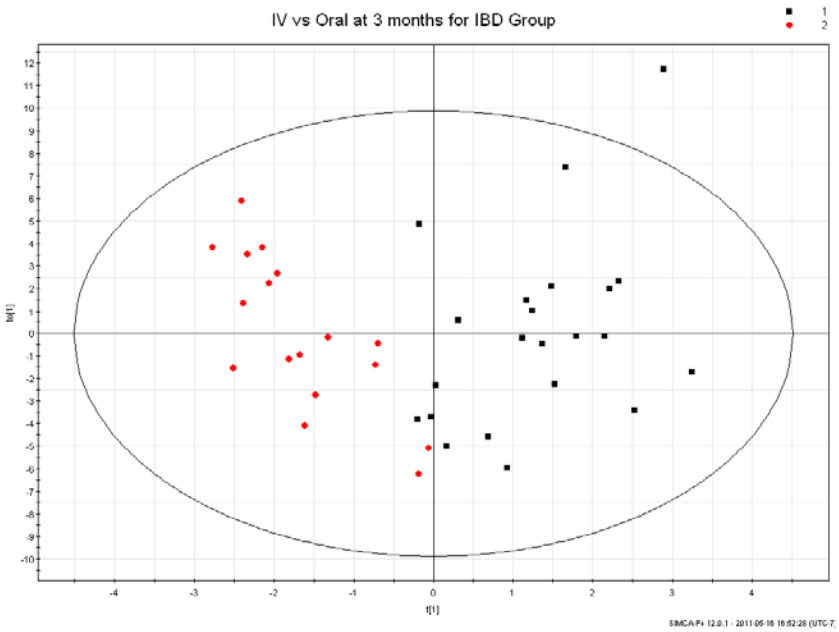


Legend: Black squares = IV, red dots = PO

6.3.1.1.2 Three months (Figure 6.2)

Using two-component separation, a supervised orthogonal partial least squares (OPLS) model was built with R^2Y of 0.722 (well fitted model) and Q^2 of 0.144 (but poor predictability). Exploratory data analysis showed clear separation of the samples into two groups. The lack of separation at baseline implies similarity in the metabolic profile at baseline making the clear separation between the two groups at 3 month significant. One possible interpretation is that PO and IV iron had differential effects on the subjects and their gut microbial metabolism.

Figure 6-2 OPLS scatter plots of IBD group at 3 months.



Legend: Black squares: IV group; red dots: PO group.

6.3.1.2 Metabolites

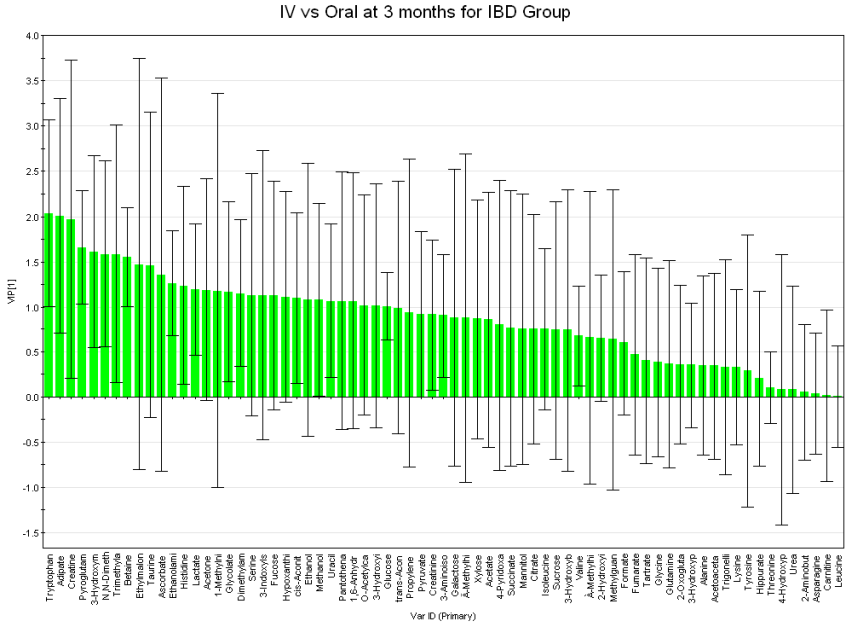
6.3.1.2.1 Variable Importance Plot (VIP)

The variable importance plot summarises the weight of each metabolite's contribution to the separation between the two groups. The VIP score is an absolute value representing the impact a metabolite has in driving the separation between the two groups. VIP was not generated for the baseline comparison between the IV group and the PO group because there was no separation.

6.3.1.2.1.1 Three months (Figure 6.3)

The top 10 metabolites that contributed to the separation between the IV and the PO groups were (in order of importance): Tryptophan, Adipate, Creatine, Pyroglutamate, 3-Hydroxymandelate, N,N-Dimethylglycine, Trimethylamine N-oxide, Betaine, Ethylmalonate, and Taurine.

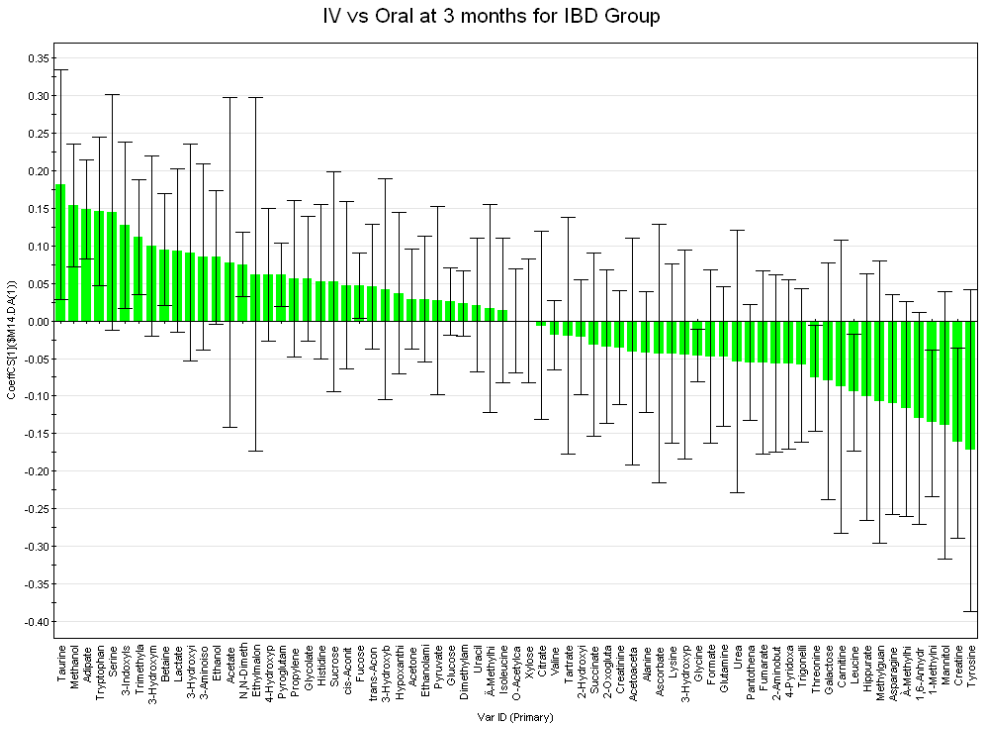
Figure 6-3 **Variable Importance Plot for IBD group at 3 months, IV versus PO.**



6.3.1.3.1 Coefficient Plot (Figure 6-4)

The coefficient plot below separated the metabolites into two groups. Metabolites listed on the left hand side of the plot were found at a higher concentration in the IV group than in the PO group, these included Taurine, Methanol, Adipate, Tryptophan, and Serine. The metabolites on the right side were found at a higher concentration in the PO group than the IV group, these included Tyrosine, Creatine, 1-Methylnicotinamide, 1,6-Anhydro-²-D-glucose, and Ä-Methylhistidine.

Figure 6-4 Coefficient Plot



6.3.2 Ulcerative Colitis (Table 6.2)

NMR spectra were acquired in 18 UC patients, 8 received IV iron and 10 received oral iron replacement. Relative to the PO group (30%), there were more males in the IV group (75%). The mean age in the IV group was 11 years older than the PO group. Higher proportion of subjects in the IV group had elevated CRP at baseline and 3 months compared to PO group. There was more concurrent immunosuppressant and/or biological therapy usage in the IV group. However, antibiotics, probiotics or prebiotics use were higher in the PO group. Despite these numerical differences, they were not statistical significant. This may be due to the small sample size.

Table 6.2 Ulcerative colitis patient characteristics

Ulcerative Colitis (Number)	Intravenous 8	Oral 10	P value*
Age (year, SD)	51 (18)	40(19)	0.26^
Sex M: F (% male)	6:2 (75)	3:7 (30)	0.06
CRP >8 at baseline (%)	4:4 (50)	3:7 (30)	0.4
CRP >8 at 3 months (%)	3:5 (38)	2:8 (20)	0.4
Use of antibiotic/probiotic/prebiotic (%)	1 (12.5)	4 (40)	0.2
Use of immunosuppressant +/-biological drug (%)	4 (50)	3 (30)	0.38

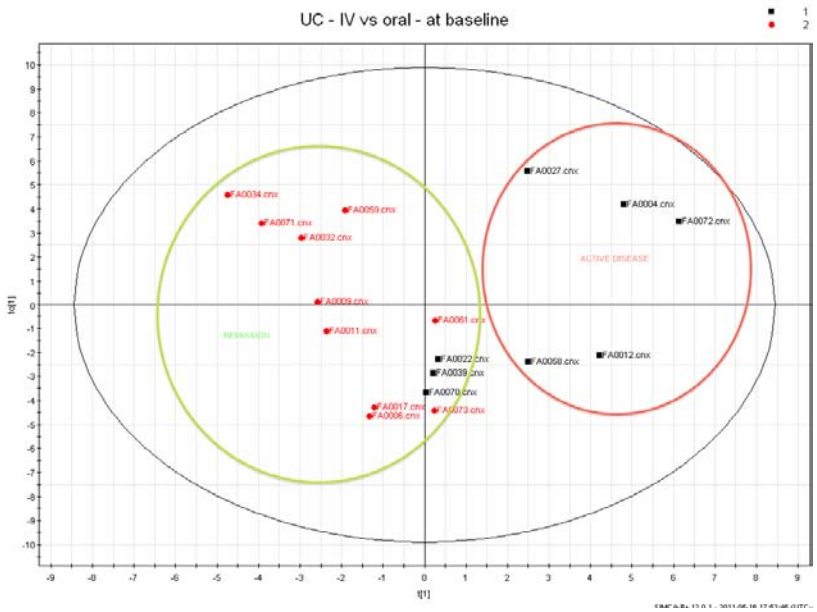
^ 2 sample t test, * chi square

6.3.2.1 Building the Models

6.3.2.1.1 Baseline (figure 6.5)

Using two-component separation, a supervised orthogonal partial least squares (OPLS) model was built with R^2Y of 0.611, and Q^2 of 0.233. Exploratory data analysis of the OPLS scatter plot showed a clear separation between the two groups. Possible reasons for the separation include 1) sex ratio imbalance: male predominance in the IV group (75%) and female predominance in the PO group (70%), though it was not statistical significant, p=0.06. 2) More patients in the IV iron group had active UC (4/8) at enrolment and started on anti-TNFa therapy +/-intravenous corticosteroids (circled in red), compared to 1/10 in the PO group, and 3) The mean serum CRP was elevated (>8mg/L) at baseline and remained elevated at 3 months in the IV group confirming persistent active inflammatory state. (Chapter 4, page 25, Figure 3.10) Majority of the patients in the PO iron group had milder disease or were in fact in clinical remission (circled in green).

Figure 6-5 OPLS scatter plot for UC patients at baseline.

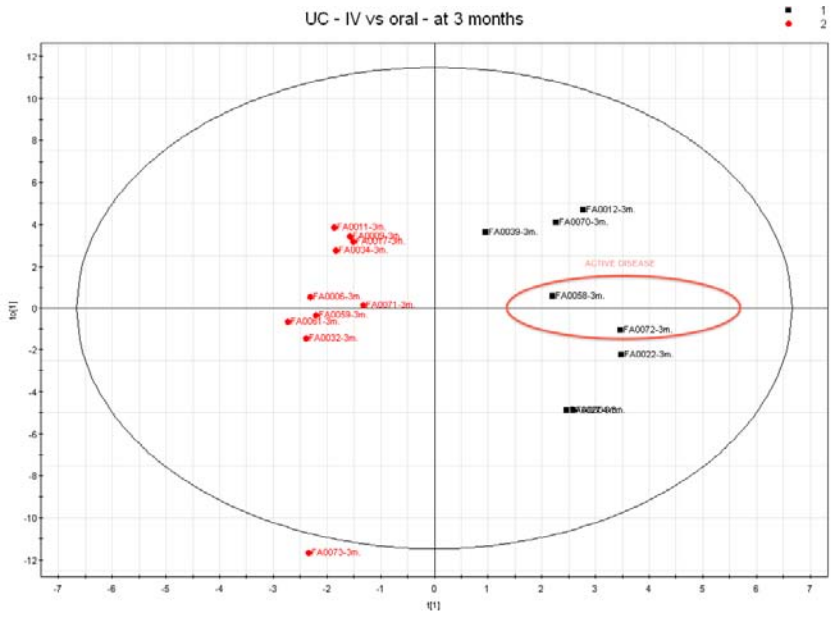


Black squares: IV group; Red dots: PO group

6.3.2.1.2 Three months (Figure 6.6)

Using two-component separation, a supervised orthogonal partial least squares (OPLS) model was built with R^2Y of 0.935, and Q^2 of 0.446. Exploratory data analysis of the OPLS scatter plot showed a closer clustering within the PO and the IV group, which resulted an increased separation between the two groups. The two samples inside the red circle of Figure 6.6 represent one subject underwent colectomy after completion of the study and the other subject had ongoing moderately severe colitis.

Figure 6-6 OPLS scatter plot for UC patients at 3 months



6.3.3 Crohn's Disease (Table 6.3)

NMR spectra were acquired in 21 CD patients, 13 received IV iron and 8 received oral iron replacement. Patients in the oral group (45.3 years old) were significantly older than those in the IV group (36.5 years old), p=0.02 and with significantly higher proportion of patients with CRP>8g/L (75%) compared to the IV group (53%), p=0.04. Other baseline characteristics were comparable in terms of males to females proportion (p=0.8), proportion of patient with CRP >8 at 3-month (p=0.13), the proportion of patient used concurrent antibiotic/probiotic/prebiotic (p=0.14) and the proportion of patient used concurrent immunosuppressant +/- biological drug (p=0.55).

Table 6.3 Crohn's disease patient characteristics

Crohn's Disease Number	Intravenous 13	Oral 8	P value*
Age (year, SD)	36.5 (13.7)	45.3(14.5)	0.02^
Sex M: F (% male)	7:6 (53)	3:5 (37.5)	0.8
CRP >8 at baseline (%)	7:6 (53)	6:2 (75)	0.04
CRP >8 at 3 months (%)	2:11 (15)	3:5 (20)	0.13
Use of antibiotic/probiotic/prebiotic (%)	3 (23)	0 (0)	0.14
Use of immunosuppressant +/-biological drug (%)	10 (77)	7 (87.5)	0.55

^ 2 sample t test, * chi square

6.3.3.1 Building the Models

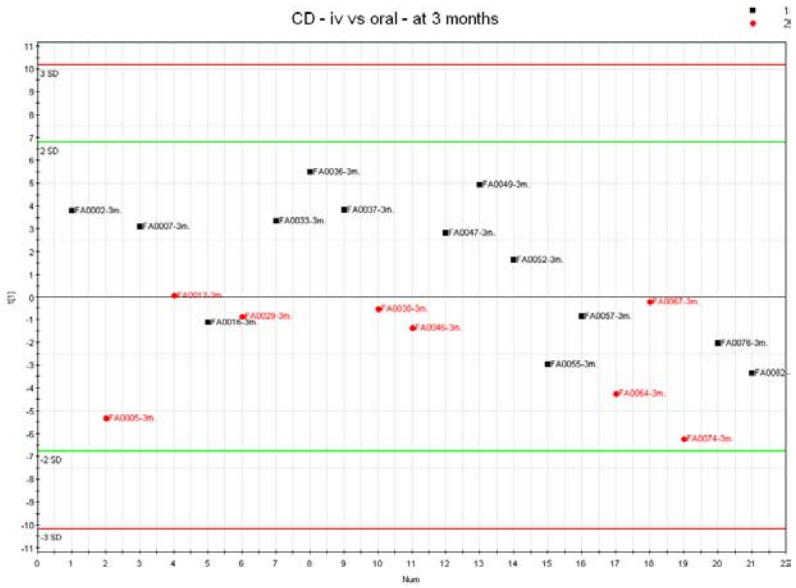
6.3.3.1.1 Baseline

Using two-component separation, a supervised orthogonal partial least squares (OPLS) model could not be built. This implies the lack of statistical significant difference in the metabolite profiles between the two groups or a more likely explanation is the small sample size.

6.3.3.1.2 Three months (Figure 6-7)

Using two-component separation, a supervised orthogonal partial least squares (OPLS) model was built with R^2Y of 0.306, and Q^2 of 0.0203. Exploratory data analysis of the OPLS scatter plot showed no separation between the IV and the PO groups suggesting the lack of statistical significant difference in the metabolite profiles between the two groups. This may simply due to the small sample size.

Figure 6-7 **OPLS scatter plot of CD group at 3 months.**



Black squares: IV group; Red dots: PO group.

6.4. Summary

In the IBD group, the baseline metabolic characteristic was similar between the PO and IV group as indicated by the lack of separation on OPLS scattered plot. This makes the observed separation at 3 month significant and suggestive of differential effect of oral versus intravenous iron therapy.

In the ulcerative colitis group, clear separation between the PO and IV group was seen in the OPLS scatter plot at baseline and 3 months. The separation at baseline may be due to higher proportion of patients with active inflammation and/or higher proportion of male sex and/or older mean age in the IV group compared to PO group. The small sample size with multiple baseline characteristic differences could account for the separation between the IV and the PO group independent of differential iron intervention.

7 DISCUSSION

Clinical outcomes

Biochemical parameters such as haemoglobin, iron saturation and CRP were comparable between the PO and the IV group at the end of the study period. However, IV iron was superior to PO iron in improving serum ferritin. In terms of quality of life, a statistical trend of superior improvement with IV route was noted when compared to PO route. The disease activity indices were comparable between IV and PO routes. However, more subjects underwent bowel resection in the IV group compared to the PO group.

7.1. Association between improved serum ferritin level and improved QoL score

It is not surprising that significantly higher serum ferritin level was achieved in the IV group compared to the PO group given the fact that the IV route bypasses the requirements of gastric and duodenal digestion and small bowel iron absorption. This finding is consistent with previous published studies. (10,99,122) What is novel in this study is the higher quality of life (QoL) score, as determined by the Short form Inflammatory Bowel Disease Questionnaire (SIBDQ) and Euro-Quality 5 Dimension Visual Analogue Scale (EQ5D VAS), reported by IBD patients in the IV group. While beyond the objectives of this thesis, the possible mechanisms for the improved QoL in the IV-treated group include the improved iron stores enabling a more efficient mitochondrial respiratory chain reaction which in turn increases ATP production and enhanced ATP availability as well as an improved neuronal and neuronal synaptic function (22,23).

One potential confounder accounting for the observed greater QoL score in IBD patients is the concurrent use of biological therapy. Patients receiving schedule maintenance anti-TNFα therapy such as infliximab for active IBD would have higher QoL because of better disease control (147,148). Secondly, anti-TNFα therapy has been shown to reduce fatigue and depression scores as well as improve quality of life in patients with Crohn's disease (149). Increased serum pro-inflammatory cytokines such as TNFa, IL 1 and IL 6 have been associated with bipolar depression (150). Although there were more patients in the IV group receiving concurrent biological therapy (10/26, 38%) than in the PO group (6/23, 26%), it was not statistical significant (p=0.36). Therefore it is plausible that the improved serum ferritin alone was not the sole mediator of the improved QoL.

7.2 Intravenous iron therapy was associated with more severe adverse events

7.2.1 More subjects in the IV group underwent bowel surgery

Three IBD subjects in the IV group had severe adverse events characterized by IBD related bowel surgery compared to one subject in the PO group. This may in part be due to selection bias as the IV group had a higher mean baseline CRP 15.9 mg/L compared to the PO group 9.2mg/L, though not statistically significant (p=0.34) and more subjects in the IV group had severe disease as indicated by more concurrent biological therapy usage (10/26, 38%) compared to the PO group (6/23, 26%). It is unclear how intravenous iron could worsen IBD activity. Iron is known to regulate lymphocytes proliferation and differentiation. In hereditary haemochromatosis, iron overload promotes the expansion of cytotoxic T cells (CD8+) over T-helper CD4+ cells (151). In this context it is possible to hypothesize that intravenous iron therapy may induce a rapid and transient iron overload state in which the cytotoxic T cells proliferated and its activities were exaggerated leading to worsening of bowel disease. However, were this is to be the case, we would expected to see uniformly increased disease activity index in the IV group compare to the PO group during the study and it was not the case.

7.2.2 Arterial thrombosis

One subject in the IV group had a below knee amputation secondary to arterial thrombosis during the study period. He is 53 years old with a history of rheumatoid arthritis on Methotrexate and prednisone, hypercholesterolaemia, hypertension and severe active Crohn's colitis. He presented with an ischemic right leg. The CT abdomen confirmed 30% stenosis of the distal abdominal aorta with a possible mural thrombus, 95% stenosis of right common iliac artery complicated by acute thrombus on plaque. He had occlusion of the right distal popliteal artery and anterior tibial, peroneal and post tibial arteries. Surprisingly he had no history of intermittent leg claudication or a cardiac history. He had two unsuccessful embolectomies and revascularization procedures, which eventually led to above knee amputation. His pro-thrombophilia screen was negative, including negative anti-cardiolipin antibody and lupus anticoagulant. His anti-thrombin III, protein S and C levels were within the normal range.

Unfortunately, he did not respond to infliximab and after infusion number seven, he had total colectomy with end ileostomy. He has received further iron sucrose infusions with no issue. Below outlined the potential mechanisms that IBD and IV iron may have played in the development of arterial thrombosis:

7.2.2.1 Hypercoagulable state due to the underlying severe Crohn's colitis

The incidence of thromboembolism (TE) in patients with IBD is 6.5% with a threefold increase in the risk of TE compared to the general population (152). Most common TE events were deep venous thrombosis of the lower limb and pulmonary embolism. One case series reported six young (24-48 years old) CD patients with arterial occlusive disease between 1985 and 1994 (pre-biological era), five presented with ischemic leg required revascularization. Most of these patients had at least one risk factor for vascular disease (153).

The hypothesized mechanisms for vascular thrombosis in patients with IBD include hypercoagulable state induced by inflammatory cytokines such as TNFa and IL-6 which enhanced expression of tissue factor (activates prothrombin to thrombin to initiate coagulation), reduced endothelial protein C receptors (protein C is an anticoagulant) and inhibited fibrinolysis on endothelial cells (154-156). Interestingly, the use of anti-TNFa significantly blunted the thrombosis response in chemical induced colitis mice model (157).

7.2.2.2 Adverse effect due to anti-TNFa

A retrospective review of the French adverse drug reaction reporting system database identified 85 (4.5% of all the anti-TNFa related AE) spontaneous reports of TE during 2000-6 in association with anti-TNFa usage in patients with rheumatoid arthritis (158). Adalimumab and Infliximab were equally presented in terms of frequency of TE events. The authors suggested no causal link.

In another case series, the formation of anti-adalimumab antibodies was detected in 76 of 272 patients in an outpatient rheumatology practice. Eight cases of thromboemoblic events were found and 4 were of arterial origin. One of these four arterial thromboembolic events was associated with anti-adalimumab and dsDNA antibodies. Therefore a causal link between anti-adalimumab antibody formation and arterial thrombosis could not be suggested (159).

7.2.3 Adverse effect due to iron sucrose

Animal models have demonstrated intravascular iron behaving as a pro-oxidation cofactor and inducing the progression of atherosclerosis and accelerating the thrombotic response (160,161). Conversely, iron chelation with deferoxamine improved nitric oxide-mediated, endothelium-dependent vasodilation in patients with coronary artery disease (162). These animal data provides plausible mechanism of actions for the role of IV iron in the initiation of arterial thrombosis. Nevertheless, an extensive search of the published literature failed to find a single human case of arterial thrombosis related to IV iron therapy.

In summary, it is thus unlikely that infliximab or iron sucrose caused the arterial thrombosis in this subject. It is more likely that he has underlying progressive peripheral vascular disease, which in combination with inflammation induced a hypercoagulable state and in turns resulted in the catastrophic outcome of above knee amputation.

7.3 Adherence to medication

Non-adherence to medication was a major issue among subjects in the PO group. Oral iron therapy was associated with statistical significant higher risk ratio of non-adherence relative to IV iron therapy (risk ratio ranged from 1.7 to 2.5 depending on the subgroups) and it may have contributed to both the lower ferritin level and the poorer quality of life scores compared to the IV group. Poor medication adherence is seen in many chronic illnesses such as psoriasis (163), epilepsy (164) and hypertension (165) with resultant poorer quality of life and adverse outcomes. Moreover, poor adherence to asthmatic medication may have contributed to 48% of asthma deaths, 80% increase in the diabetes related deaths and increased risk of cardiac death one year post myocardial infarction (166). Although non-adherence to oral iron therapy may not result in death, nevertheless, it is associated with poorer quality of life as demonstrated in this study. The financial cost of medication non-adherence is also considerable, estimated at $100 billion in the United States in 2007 (76). These include direct costs such as unfilled or unused medication, extra- physician visits and hospital visits as well as indirect costs such as time off work, loss of productivity and use of non-prescription drugs (163). This should be taken into account when considering cost benefit analysis as iron sucrose is inherently more expensive than iron sulfate pills. For the reason of poor medication adherence, IV iron is a more reliable alternative.

7.4 Small sample size

The targeted 100 IBD subjects (50 IV group and 50 PO group) and 100 NIBD subjects (50 IV group and 50 NIBD group) were not met, therefore the clinical results are underpowered and careful interpretation is needed. Challenges faced with subject recruitments include 1) different expectation between subject's preference for the IV or the PO route versus randomized route. 2) Intensive specimen collection process - blood, urine and stools samples were collected at baseline and three months. 3) Optional participation in having two unsedated flexible sigmoidoscopy 3 months apart, and 4) significant distance to travel for study review – up to 5 hours each way for some subjects.

Despite of the small sample size, these results can be served as a pilot study. Moreover, limited human literature is available on the impact of iron therapy on patients with IBD and the finding from this study will assist physicians in patient care.

Colonic Microbiome

The microbiome composition were analysed at the phylum level in order to minimize the number of assumptions made given the intrinsic limitation with TRFLP. This would limit the ability of the study in detecting changes at a more specific level such as changes in the species composition.

7.5. Oral iron was associated with unfavourable change in the mucosal microbiome composition

Oral iron therapy had a significant impact on the mucosal microbiome composition with reduction in Firmicutes and Bacteroidetes abundance and increase in Proteobacteria abundance, implying a more "pathogenic" luminal bacterial profile. In contrast, the mucosal bacterial microbiome composition with IV iron therapy was associated with increased abundance of the less "pathogenic" phyla - Firmicutes and Bacteroidete. This observation is consistent with the murine model of Crohn's like ileitis described by Werner et al. Mice on iron sulfate free diet were associated with reduced pathogenic sulfur producing species such as Desulfovibrio and increased abundance of probiotic bacteria such as bifidobacteria (55). Furthermore, an iron deficient diet prevented the development of Crohn's disease like ileitis in this animal model.

Clostridium, a genus of Firmicute phylum and Bifidobacterium, a genusof Actinobacteria phylum, are examples of 'good' (ie, less "pathogenic") bacteria because they are efficient short chain fatty acid (SCFA) producers. SCFA such as butyrate has immunomodulatory effect – suppresses epithelial secretion of inflammatory cytokines. It has bacteriostatic property either directly or by reducing luminal pH and also serves as fuel for epithelial cells. B thetaiotaomicron, a species of Bacteroidetes phylum is able to dampen inflammatory signals by inhibiting NF-kB pathways (167). Therefore reduction in the abundance of these favourable bacteria by oral iron therapy could cause or contribute to the ongoing pro-inflammatory state.

The unfavourable changes in the mucosal bacteria composition is a concern because mucosal associated bacteria are considered immunologically significant compared to the faecal bacteria. Mucosal bacteria are able to interact with host immune cells such as dendritic cells. Commensal and probiotic bacteria such as Lactobacillus and Bifidobacterium are capable of inducing the production of regulatory
cytokines such as IL 10 and subsequently influence lymphocytic differentiation and generation of regulatory T cells (168).

7.6. Oral iron reduced faecal Microbiome diversity index

Oral iron replacement was associated with a statistical trend towards reduced *faecal* microbiome diversity index with a corresponding lower faecal microbiome similarity index. This may be due to ferric iron's ability to increase the redox potential in the colonic lumen, i.e. becomes a more acidic environment, thereby limiting the growth of certain bacteria population (50). It is generally accepted that a greater ecological diversity is desirable because of greater functional redundancy in the ecosystem to support both host's and the bacterial community's needs and nutrition. Moreover, higher microbiota diversity enables a more effective competitive exclusion of pathogens from colonizing the gut epithelial surface. For example, significantly less faecal microbiome diversity has been demonstrated in subjects with recurrent *C. difficile* infection compared to control patients and those with first episode of *C. difficile* infection (169). Therefore greater diversity is associated with greater functional stability, to contain species, or functional groups, that are capable of differential response (170). The lower diversity index with oral iron treatment may have detrimental effects on the gut microbial ecosystem and subsequently adversely affecting the host.

7.7. Emergence of pathogenic bacteria with PO iron

Oral iron was associated with higher mucosal abundance of the less desirable bacteria phylum – Proteobacteria, and reduced abundance of the desirable bacteria phylum- Firmicutes. The superior and more competitive iron acquisition mechanism of Proteobacteria may explain this, although it has not been described in the literature. Analogy may be drawn from studying pathogenic bacteria such as *Salmonella enterica* serotype Typhimurium, which has evolved to thrive in the bacteria-hostile environment such as an inflamed intestine. In the setting of intestinal inflammation, epithelial cells produce lipocalin-2, an antimicrobial protein that prevents gut luminal bacteria iron acquisition. However, *Salmonella enterica* acquired a genetic mutation resulted in its resistance to lipocalin-2 protein and thereby thrive in the setting of intestinal inflammation and becomes the dominate species (171). Similarly, uropathogenic E coli has a large armamentarium of iron acquisition proteins that confer its pathogenicity and dominance in the urinary tract (40).

7.8. Future directions

2.4.1 A significant difference in the microbiome composition and diversity index has been demonstrated between the PO and IV group using TRFLP (as outlined above). The next step of experimentation would be to confirm these differences by pyrosequencing and to determine the actual bacteria composition and abundance.

2.4.2 Quantifying the faecal iron content in the PO and the IV groups would be important in future studies. In conjunction with the Pyrosequencing results, one would be better able to correlate the actual bacterial composition in the setting of iron excess versus iron deficient colonic environment.

Urinary metabolomics

Examination of urinary metabolomic profiles in iron deficient IBD patients revealed significant changes from an iron deficient state to an iron sufficient state as well as between PO and IV route of replacement. The most influential metabolites identified in the study included those with anti-inflammatory properties, amino acids of human and bacterial origin and metabolites

relating to energy production. These are clear indications that iron as well as the route in which it was given had differential effects on the human and the gut microbial metabolisms. The changes in the urinary metabolites could also reflect the changes in the colonic bacteria composition (172).

The urinary metabolomics fingerprint is very different among the healthy control, Crohn's disease and ulcerative colitis subjects (173). The reduction in Clostridia spp. (Firmicutes phylum) is frequently seen in IBD and is associated with reduced urinary hippurate level. CD is associated with an increased mucosal E coli abundance. Moreover, E coli is a great producer of format, therefore it is not surprising that an increase in the urinary format concentration is seen in CD (174). However, neither metabolite contributed to the separation in the urinary metabolomic profiles in this study.

7.9. Reasons for the urinary metabolite profiles separation at 3 months

The lack of metabolites profile separation on the OPSL scatter plot at baseline implies similarity in the measured urinary metabolites profiles. The subsequent clear separation into two groups at 3 months is significant. It implies different route of iron therapy had different effects on the patients' and their gut microbial metabolisms at 3 months.

Other possible factors contributing to the separation include,

7.9.1 The differences in the sex ratio.

There were more males in the IV group (62%) compared to the PO group (33%). However, this is unlikely to be of clinical significance. In a study examining the urinary metabolite profile of healthy volunteers, sex difference did not have an impact on the urinary metabolite profile separation (175).

7.9.2. The differences in the proportion of subjects with active IBD.

There were more subjects in the IV group (33%) with elevated CRP compared to the PO group (28%), suggesting the IV group was a 'sicker' compared to the PO group. It is conceivable that active disease would have altered host and/or gut microbial metabolism and leading to changes in the urinary metabolite profile. However, an animal study demonstrated no difference in the urinary metabolite profile in the context of gut inflammation (176).

7.9.3. Disease activity status

There were more patients in the IV group (71%) using concurrent biological therapy compared to the PO group (44%) and whether this factor alone could contribute to the separation in the metabolite profiles is uncertain. A recent study demonstrated the lack of impact of IBD medications on the human urinary metabolomics profile when comparing medicated IBD subjects, non medicated IBD subjects to the healthy controls(174) . The significant difference in the metabolite fingerprints among healthy controls, subjects with UC or CD persisted when controlling for concurrent medication use.

7.9.4. Change in colonic microbiome

The resultant changes in the microbiome composition due to IV or PO routes of iron replacement may itself contributed to the separation of the metabolomics fingerprints. It has been demonstrated that changes in the gut microbial composition will change the urinary metabolomics profile due to the changes in urinary bacterial products of metabolism (174).

Having considered above confounders, the observed separation in the metabolite profiles at 3 months is most likely due to the interaction between the intervention – iron therapy and the metabolism – human host and their microbiota.

7.10 Assumptions

Assuming normal glomerular function, the interpretation of the measured urinary metabolites would still be challenging.

7.10.1 Would the presence of a metabolite at a higher urinary concentration compared to other measured metabolites indicate its excess in the body? Would the excess production of this particular metabolite due to the disease state related altered metabolism? Or would it confer protection if this were found in the disease free group?

7.10.2 Would the presence of a metabolite at a lower urinary concentration compared to other measured metabolites indicates its deficiency and possibly caused the disease process? Or, could it be due to an increased usage of this metabolite by other metabolic pathways as a result of the disease process?

These questions remained unanswered in the literature. The goal of studying these metabolites is an attempt to decipher which metabolic pathway(s) may be up or down regulated in the disease process and thus contributing to the understanding of disease pathogenesis.

7.11. Metabolites

The followings are the urinary metabolites identified to be most influential in driving the metabolomics fingerprints separation at 3 months in the IBD group. In addition, their postulated connections to IBD or inflammation are also presented here. KEGG: Kyoto Encyclopedia of Genes and Genomes (177) website was used to clarify where the metabolite of interest fits in the metabolic pathway as well as the origin of its production.

7.11.1 Urinary metabolites found at a higher concentration in the IV group compared to the PO group

Taurine is an amino acid of human origin with in vitro evidence of enterocytes protective function by reducing IL 8 production. Its prophylactic use in DSS treated mice limits intestinal inflammation (178,179). Interestingly, in a human colonic biopsy metabolomic study, a higher taurine concentration was associated with active UC (180). These conflicting findings may be a simple result of difference in species – human vs. mice. Taurine is involved in the Glutathione metabolism and bile acid synthesis.

Methanol is a simple alcohol and a byproduct of human urea cycle Polycyclic aromatic hydrocarbon degradation. Methanol extract from Patrinia scabiosaefolia has anti-inflammatory property. It reduces inflammation in DSS treated mice (181).

Adipate is of human origin and is involved in Urea cycle Polycyclic aromatic hydrocarbon degradation Amino sugar and nucleotide sugar. Its relevance in IBD and iron metabolism is not clear.

Tryptophan is an essential amino acid of human and bacterial origin. Elevated faecal tryptophan has been demonstrated in human CD and it may reflect malabsorption. An interesting association between elevated faecal tryptophan and E. coli abundance has been hypothesized, especially in the context of suspected pathogenic role of adherent invasive E coli in IBD (182). It would be interesting to see if an increase in the E coli abundance in the stools of PO group could be demonstrated in the future pyrosequencing .

Serine is a non-essential amino acid of human origin. Serine participates in many aspects of human metabolism, ranging from insulin signaling pathway, complements and coagulation cascades, purine and pyruvate metabolism. There has been no literature report regarding elevated serine in any biofluid and IBD.

7.11.2 Increased concentration in the PO group compared to the IV group

Tyrosine is a non-essential amino acid of human origin. Using faecal metabolite profiling in human, elevated tyrosine was associated with Crohn's disease compared to control subjects (182). However, its significance in the urine is not known.

Creatine is a nitrogenous organic acid that occurs naturally in vertebrates and help to supply energy to all cells in the body, primarily muscle, by increasing the formation of Adenosine triphosphate (ATP). Elevated creatine level was found in the colonic mucosa of animal model of colitis (183).

1 Methylnicotinamide is an inactive metabolite of nicotinamide of human origin. It has anti-inflammatory property (184). It is involved in nicotinate and nicotinamide metabolism and bile secretion. Its role in IBD and iron metabolism is not known.

1,6-Anhydro-D-glucose is a simple sugar. Its origin is not known to the KEGG website .This metabolite has not been discussed in the literature with regards to its significance in IBD or iron metabolism.

Methylhistidine is of human origin and its urinary concentration is increased in patient with IBD compared to healthy control (174). Its role in metavolism is not known.

Two potential anti-inflammatory metabolites – Taurine and methanol and one IBD associated metabolite– tryptophan were found to be elevated with IV iron use compared to PO iron use. In contrast, colitis associated metabolites such as Tyrosine, creatine and Methylhistdine were found at higher concentrations in the PO iron group compared to the IV group. It appeared that the urinary metabolomics profile was more favourable in the IV group – more anti-inflammatory components than the PO group. The oral group was associated with more colitis associated metabolites.

It is not surprising that butyrate was not one of the most influential metabolite found in the urinary metabolomics study. Butyrate is used by colonocytes as energy source therefore it would be unusual for butyrate to be absorbed into the systemic circulation and excreted renally. In a

large urinary metabolomics study involved subjects with CD (86), UC (60) and NIBD (60), butyrate was not mentioned as one of the key metabolite differentiating between IBD and NIBD patients (185). Our finding that Butyrate was not a key discriminate metabolite is consistent with the published literature.

Summary

The less profound colonic bacterial dysbiosis, higher diversity and similarity index with IV iron therapy in conjunction with the apparent more 'anti-inflammatory' urinary metabolites profile in the IV group is of great interest and worth further investigation. From the clinical endpoint perspective, the IV group had a higher serum ferritin levels and a trend towards better quality of life score with better medication adherence compared to the PO group. However, concerns exist with a higher number of IBD related surgeries in the IV group and the associated higher set up cost.

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Appendix 3.1 Iron sucrose infusion protocol

Capital Health

CARITAS HEALTH GROUP

Patient Care Orders
Iron Replacement Protocol
Iron Sucrose Infusion - Adult

1. All orders must be completed and signed by the physician. All co-signatures must be timed and dated within **24 hours.**
2. Orders may be deleted by stroking the order out, and initialing the entry or by leaving prompt blank (boxes and / or lines).

Allergies _____

_____

Approved indication of use within Capital Health:

☐ Documented hypersensitivity to Iron Dextran

☐ Intolerance to Iron Dextran

Date / Time	**Test dose not required for Iron Sucrose Infusion**
	Iron Sucrose Infusion:
	1. Formula to calculate dose of Iron Sucrose in mg (= total iron deficit in mg):
	Iron Sucrose dose (mg) = weight (kg) x (target Hgb g / L – actual Hgb g / L) x 0.24 + depot iron * (mg)
	* If weight ≤ 35 kg (and with target Hgb of 130 g / L), then iron depot = 15 mg / kg
	If weight > 35 kg (and with target Hgb of 150 g / L), then iron depot = 500 mg
	Note: maximum daily dose is 500 mg; maximum weekly dose is 1500 mg.
	2. Dose: Iron Sucrose _____ mg mixed in 500 mL 0.9% sodium chloride IV over 4 hours (125 mL / h).
	3. Take vital signs every 30 minutes until infusion is completed.
	4. Observe for delayed reactions: backache, muscle pain, chills, dizziness, fever, headache,
	nausea and / or vomiting.
	These may occur 24 – 48 hours after the onset of infusion and usually subside in 3 – 7 days.
	Iron Sucrose Reaction Protocol
	1. Stop infusion and disconnect IV tubing.
	2. Start IV 0.9% sodium chloride at 300 mL / hour.
	3. Diphenhydramine 25 mg IV push over 1 – 5 minutes.
	4. Notify attending physician.
	Physician's Signature _____, MD.

Appendix 3.2 European Quality 5 Dimension

EQ - 5D

Health Questionnaire

(Canadian English version)

By placing a check-mark in one box in each group below, please indicate which statements best describe your own state of health today.

Mobility

I have no problems in walking about ❑

I have some problems in walking about ❑

I am confined to bed ❑

Self-Care

I have no problems with self-care ❑

I have some problems washing or dressing myself ❑

I am unable to wash or dress myself ❑

Usual Activities (*e.g. work, study, housework, family or leisure activities)*

I have no problems with performing my usual activities ❑

I have some problems with performing my usual activities ❑

I am unable to perform my usual activities ❑

Pain/Discomfort

I have no pain or discomfort ❑

I have moderate pain or discomfort ❑

I have extreme pain or discomfort ❑

Anxiety/Depression

I am not anxious or depressed

❑

I am moderately anxious or depressed

❑

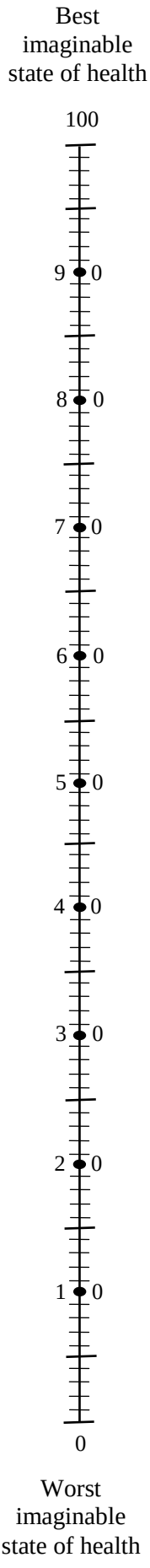
I am extremely anxious or depressed

❑

To help people say how good or bad their state of health is, we have drawn a scale (rather like a thermometer) on which the best state you can imagine is marked 100 and the worst state you can imagine is marked 0.

We would like you to indicate on this scale how good or bad your own health is today, in your opinion. Please do this by drawing a line from the box below to whichever point on the scale indicates how good or bad your state of health is today.

Your own state of health today



Appendix 3.3 Short form Inflammatory Bowel Disease Questionnaire

Short Quality of Life in Inflammatory Bowel Disease Questionnaire

This questionnaire is designed to find out how you have been feeling during the last 2 weeks. You will be asked about symptoms you have been having as a result of your inflammatory bowel disease the way you have been feeling in general, and how your mood has been.

1. How often has the feeling of fatigue or of being tired and worn out been a problem for you during the last 2 weeks? Please indicate how often the feeling of fatigue or tiredness has been a problem for you during the last 2 weeks by picking one options from:

   - ☐ All of the time
   - ☐ Most of the time
   - ☐ A good bit of the time
   - ☐ Some of the time
   - ☐ A little of the time
   - ☐ Hardly any of the time
   - ☐ None of the time

2. How often during the last 2 weeks have you had to delay or cancel a social engagement because of your bowel problem? Please choose an option from:

   - ☐ All of the time
   - ☐ Most of the time
   - ☐ A good bit of the time
   - ☐ Some of the time
   - ☐ A little of the time
   - ☐ Hardly any of the time
   - ☐ None of the time

3. How much difficulty have you had, as a result of your bowel problems, doing leisure or sports activities you would have liked to have done during the last 2 weeks? Please choose an option from:

   - ☐ A great deal of difficulty, activities made impossible
   - ☐ A lot of difficulty
   - ☐ A fair bit of difficulty
   - ☐ Some difficulty
   - ☐ A little difficulty
   - ☐ Hardly any difficulty
   - ☐ No difficulty; the bowel problems did not limit sports or leisure activities

4. How often during the last 2 weeks have you been troubled by pain in the abdomen? Please choose an option from:

   - ☐ All of the time
   - ☐ Most of the time
   - ☐ A good bit of the time
   - ☐ Some of the time
   - ☐ A little of the time
   - ☐ Hardly any of the time
   - ☐ None of the time

5. How often during the last 2 weeks have you felt depressed or discouraged? Pleases choose an option from:

   - ☐ All of the time
   - ☐ Most of the time
   - ☐ A good bit of the time
   - ☐ Some of the time
   - ☐ A little of the time
   - ☐ Hardly any of the time
   - ☐ None of the time

6. Overall, in the last 2 weeks, how much of a problem have you had with passing large amounts of gas? Please choose an option from:

   - ☐ A major problem
   - ☐ A big problem
   - ☐ A significant problem
   - ☐ Some trouble
   - ☐ A little trouble
   - ☐ Hardly any trouble
   - ☐ No trouble

7. Overall, in the last 2 weeks, how much of a problem have you had maintaining or getting to the weight you would like to be? Please choose an option from:

   - ☐ A major problem
   - ☐ A big problem
   - ☐ A significant problem
   - ☐ Some trouble
   - ☐ A little trouble
   - ☐ Hardly any trouble
   - ☐ No trouble

8. How often during the last 2 weeks have you felt relaxed and free of tension? Please choose an option from:

   - ☐ None of the time
   - ☐ A little of the time
   - ☐ Some of the time
   - ☐ A good bit of the time
   - ☐ Most of the time
   - ☐ Almost all of the time
   - ☐ All of the time

9. How much of the time during the last 2 weeks have you been troubled by a feeling of having to go to the bathroom even though your bowels were empty? Please choose an option from:

   - ☐ All of the time
   - ☐ Most of the time
   - ☐ A good bit of the time
   - ☐ Some of the time
   - ☐ A little of the time
   - ☐ Hardly any of the time
   - ☐ None of the time

10. How much of the time during the last 2 weeks have you felt angry as a result of your bowel problem? Please choose an option from:

    - ☐ All of the time
    - ☐ Most of the time
    - ☐ A good bit of the time
    - ☐ Some of the time
    - ☐ A little of the time
    - ☐ Hardly any of the time
    - ☐ None of the time

(Appendix from American Journal of Gastroenterology 1996; 91(8):1571-8.)

Appendix 3.4. Harvey Bradshaw Index

Patient, please complete Questions 1, 2 & 3.
Base your answers on how you felt yesterday.

1. General Well-being (see descriptors)
- Very well = 0
- Slightly below Par = 1
- Poor = 2
- Very Poor = 3
- Terrible = 4

2. Abdominal Pain (see descriptors)
- None = 0
- Mild = 1
- Moderate = 2
- Severe = 3

3. Number of Liquid or Soft Stools per day (Yesterday)

Physician, please complete Question 4

4. Additional Manifestations
- None = 0
- Arthalgia = 1
- Uveitis = 1
- Erythema Nodosum = 1
- Aphtous ulcer = 1
- Pyoderma gangrenosum = 1
- Anal Fissure = 1
- New Fistula = 1
- Abscess = 1

Total Harvey Bradshaw Index score: [sum of all above items]
Remission = <5
Mild Disease = 5-7
Moderate Disease = 8-16
Severe Disease >16

1. General Well-being Descriptors

General well being includes fatigue in the overall rating and how you feel today. Record the worst you have felt today. Compare yourself to someone else of your age, how would they rank their general wellbeing? Below are some descriptors to help you rank your category of general well being.

- **Very Well:** General health is not generally a problem. You're feeling very good or great and under control.
- **Slightly Below Par:** You're getting through things but feeling below par and not normal. Something overall is preventing you from saying " I feel wonderful ". You're feeling good but not great. You can work, socialize, and function on a day to day basis.
- **Poor:** Your symptoms bother you. You occasionally miss work, school, Or social activities. You have some embarrassing moments with fecal incontinence. You have diarrhea, abdominal pain, fatigue, and basically just feeling unwell, but you are still able to function. You're getting through the day, doing all your normal stuff but it is a struggle.
- **Very Poor:** Your getting through a part of the day, but can't do you're your normal stuff. You can't attend social events in evening. You sometime leave home from work early. You feel pretty bad and are not doing much activity – only those absolutely
- **Mild:** You're aware that the abdominal pain is there but it does not interfere with your life and you continue with activities such as work and pleasure. You feel and hear rumbles, gurgles and cramps.
- **Moderate:** You're aware of your abdominal pain and must alter your activities to manage the pain (ie. lie down to rest, postpone shopping trips until later, and take Tylenol). The pain interferes with your life and daily activities. You may have to miss work or pleasure activities on occasion.

Appendix 3.5 Partial Mayo Score

Patient, please enter number of daily bowel motions you would have when in remission or before your diagnosis or symptoms of ulcerative colitis began. T**his number will be Your Normal:** ☐

Patients, please complete Questions number 1 and 2.

1. Stool Frequency (based on the past 3 days)

- ☐ Normal number of stools = 0
- ☐ 1-2 stools more than normal = 1
- ☐ 3-4 stools more than normal = 2
- ☐ 5 or more stools more than normal = 3

☐

2. Rectal Bleeding (based on the past 3 days)

- ☐ No blood seen = 0
- ☐ Streaks of blood with stool less than half the time = 1
- ☐ Obvious blood with stool most of the time = 2
- ☐ Blood alone passed = 3

☐

Physician, please complete Questions number 3.

3**. Physician's Global Assessment (to be completed by Physician)**

- ☐ Normal (sub scores are mostly 0) = 0
- ☐ Mild Disease (sub scores are mostly 1) = 1
- ☐ Moderate Disease (sub scores are mostly 1 to 2) = 2
- ☐ Severe disease (sub scores are mostly 2 to 3) = 3

☐

The physician's Global Assessment acknowledges the Sub scores, the daily record of abdominal discomfort and functional assessment and other observations such as physical findings, and the patient's performance status

Total Partial Mayo Index Score [sum of all above items] ☐

Remission = 0-1
Mild Disease = 2-4
Moderate Disease = 5-6
Severe Disease =7-9
Version June 2009