

**Investigating Fibroblast Growth Factor 19
expression in primary and secondary bile acid
diarrhoea**

**Dr Jonathan David Nolan
Clinical Research Fellow
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Abstract:

Introduction: Bile acid diarrhoea (BAD) results from excess bile acids (BA) in the colon. Most BAs are reabsorbed at the terminal ileum. Increased colonic BA occur secondary to ileal disease or resection (common in Crohn's disease [CD]) leading to BA malabsorption and secondary BAD (SBAD). Primary BAD (PBAD) occurs in the absence of overt ileal disease or malabsorption. The peptide FGF19 is produced from enterocytes in response to BA absorption and is a negative regulator of BA synthesis. Defects in FGF19 production may underlie the excess faecal BA loss observed in PBAD.

Aim: This thesis reports studies testing the following hypotheses: (1) Ileal FGF19 expression is altered in both CD and PBAD; (2) Serum FGF19 is a diagnostic marker of BAD in CD; (3) Stimulating FGF19 with a BA analogue in patients with SBAD will have beneficial symptomatic effects.

Methods: Groups of patients underwent fasting blood sampling or ileal biopsies during colonoscopy. Serum FGF19 was measured and biopsies were studied for expression of FGF19 transcripts and other genes before and after incubation with BA. RNA extraction, cDNA synthesis and RT-PCR were used for relative quantification of gene expression. A genotype study on a candidate SNP in the DIET1 gene was performed on a cohort of PBAD patients.

Results: Serum FGF19 levels were lower in CD patients with ileal resection, or diarrhoea or active disease. FGF19 transcripts were strongly induced by BAs in ileal explants but were lower in CD compared to controls. Baseline and BA-stimulated ileal FGF19 expression were related to measures of faecal BA loss. The DIET1 variant was associated with FGF19 levels. An open-label, pilot trial of the BA analogue, Obeticholic acid (OCA), in patients with SBAD produced variable changes in FGF19 levels and symptomatic improvement.

Conclusion: This work increases awareness of FGF19 and dysregulated BA homeostasis in the pathophysiology of certain chronic diarrhoeal diseases. FGF19 holds promise in guiding therapy for BAD

Statement of originality and copyright

This body of work increases awareness of the role of FGF19 and dysregulated BA homeostasis in conditions of bile acid diarrhoea. The work described in this thesis is my own.

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Dr Jonathan David Nolan

List of abbreviations:

μM / μl	micromolar / microlitre
ASBT	Apical Sodium-linked Bile salt Transporter
BA	Bile Acids
BAD	Bile Acid Diarrhoea
BAM	Bile Acid Malabsorption
BMI	Body Mass Index
BSFS	Bristol Stool Form Scale
BSEP	Bile Salt Export Pump
C4	7α-hydroxy-4-cholesten-3-one
CA	Cholic Acid
CCK	Cholecystokinin
CD	Crohn's Disease
cDNA	complementary Deoxyribonucleic acid
CDCA	Chenodeoxycholic acid
Ct	Cycle Threshold
CYP27A1	sterol 27-hydroxylase
DCA	Deoxycholic acid
DMEM	Dulbecco's Modified Eagle Medium

DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FGF15/19	Fibroblast Growth Factor 15/19
FGFR4	Fibroblast Growth Factor Receptor 4
FXR	Farnesoid X Receptor
g	grams
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCDCa	Glyco-chenodeoxycholic acid
HBI	Harvey Bradshaw Index
HPLC	High Pressure Liquid Chromatography
IBABP	Ileal Bile Acid Binding Protein
IBAM	Idiopathic Bile Acid Malabsorption
IBS	Irritable Bowel Syndrome
IBS-D	Diarrhoea predominant Irritable Bowel Syndrome
ICP	Intrahepatic Cholestasis of Pregnancy
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IQR	Inter-Quartile Range
IR	Ileal Resected
IR-CD	Ileal Resected Crohn's Disease
LCA	Lithocholic acid
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
MAF	Minor Allele Frequency
mRNA	Messenger Ribo-Nucleic Acid
NF-κB	Nuclear Factor Kappa of B cells
NR-CD	Non Resected Crohn's disease
OCA	Obeticholic Acid
OSTa/OSTβ	Organic Solute Transporter alpha / Beta
pg	Picograms
PBAD	Primary bile acid diarrhoea
RT-qPCR	Real time Quantitative polymerase Chain reaction
SeHCAT	23-seleno-25-homotaurocholic acid
SHP	Short Heterodimer partner
SNV	Single Nucleotide Variant
TGR5/ GPBAR1	The G protein-coupled bile acid receptor 1
TNFα	Tumour Necrosis Factor Alpha
UDCA	Ursodeoxycholic acid

Contents page

Acknowledgements	12
Chapter 1 Introduction	14
1.1 Bile acid (BA) homeostasis and the enterohepatic circulation of BA	14
1.2 BA-FXR-FGF19 signalling	15
1.3 Secondary bile acid diarrhoea (SBAD) in Crohn's disease (CD)	17
1.4 Bile acid studies in Crohn's disease	18
1.4.1 Post ileal resected-Crohn's disease (IR-CD)	18
1.4.2 Non resected Crohn's disease (NR-CD)	19
1.4.3 Altered BA- FXR- FGF19 signalling in Crohn's disease	20
1.5 Primary Bile acid diarrhoea (PBAD)	22
1.6 Diarrhoea as altered FGF19 signalling in Crohn's Disease	25
1.7 Bile acids, FXR and intestinal barrier function	26
1.7.1 FXR agonists protects against chemically induced animal models of colitis	27
1.7.2 The vicious cycle: FXR activation is repressed by inflammatory cytokines, and inflammatory cytokine expression is repressed by FXR	29
1.8 FXR agonists and a therapeutic role in SBAD of Crohn's Disease	31
1.9 Genotype phenotype relationships in bile acid homeostasis and colonic function	33
1.9.1 DIET1 single nucleotide variant: rs12256835	34
Hypothesis	36

Chapter 2: General Methods	37
2.1 Ethical considerations, patient recruitment and consent	37
2.2 Serum FGF19 studies in Crohn's disease	37
2.2.1 FGF19 assay	37
2.2.2 C4 and Bile acid assays	39
2.3 Ileal FGF19 expression studies	39
2.3.1 Sampling during routine colonoscopy	39
2.3.2 Recruitment and selection of healthy controls and patients	38
2.3.3 Samples from retrospective cohort of patients with chronic diarrhoea	40
2.3.4 Samples for basal ileal FGF19 transcript expression studies	40
2.3.5 Explant incubations and bile acid preparations:	41
2.3.6 Tissue disruption, lysis and RNA extraction	43
2.3.7 cDNA synthesis	44
2.4 Quantitative real-time reverse-transcription PCR (RT-PCR)	45
2.4.1 Primer design	45
2.4.2 RT-PCR protocol	45
2.5 Statistical analysis and interassay variability	47
2.6 Culture fluid ELISA for FGF19	48
2.7 <i>DIET1</i> genotyping study: Allelic discrimination assay	49
2.8 Clinical trial: Obeticholic Acid in Bile Acid Diarrhoea: OBADIAH1:	51
2.8.1 Study design	51
2.8.2 Subjects	52
2.8.3 Statistical analysis	53

Chapter 3: Serum FGF19 levels in Crohn's disease	54
Abstract	54
3.1 Background	55
3.2 Aims	56
3.3 Specific methods	57
3.4 Results	58
3.4.1 Patient demographics	58
3.4.2 FGF19 levels in Ileal resected (IR)-CD patients	58
3.4.3 FGF19 levels in Non-Resected CD patients	59
3.4.4 Serial FGF19 levels during the day	63
3.4.5 Serial FGF19 in patients on treatment	64
3.5 Discussion	65

Chapter 4: Basal expression of FGF19 and other BA regulated genes in human ileum

Abstract	68
4.1 Background	70
4.2 Aims	72
4.3 Specific methods	72
4.3.1 Prospective study of ileal and colonic FGF19 expression	72
4.3.2 Analysis of retrospective cohort of patients with chronic diarrhoea + PBAD	73
4.3.3 Sample processing and RT-PCR	73
4.3.4 Using RT-PCR relative quantification to compare basal expression levels of FGF19 between patient groups	74
4.4 Results:	77

4.4.1 RNA concentrations and purity	77
4.4.2 Validation experiments and primer efficiencies	78
4.4.3 FGF19 mRNA is basally expressed in human ileal but not in colonic biopsies obtained at colonoscopy	80
4.4.4 Ileal transcript expression levels of FGF19 and ASBT are related to SeHCAT retention levels in a cohort of patients with chronic diarrhoea	83
4.4.5 Reduced coexpression of ileal transcript levels in BA regulated genes in PBAD	85
4.4.6 FGF19 ileal transcript levels in patients with Crohn's disease	87
4.5 Discussion:	88

Chapter 5: Stimulation of fibroblast growth factor 19 expression in the human

ileum by bile acids	91
Abstract	91
5.1 Background	92
5.2 Aims	96
5.3 Specific methods:	96
5.3.1 Patient selection: Healthy controls	96
5.3.2 RT-qPCR: Comparative Ct method	96
5.3.3 Patient demographics	99
5.4 Results	99
5.4.1(a) Six-hour 'control' incubation of explants does not alter the basal expression of FGF19 mRNA	99
5.4.1(b) GAPDH expression is stable despite incubation with BA	101
5.4.2 Potent stimulation of FGF19 transcript expression by CDCA and GCDCA in the human ileum	101

5.4.3 Bile acid dose response analysis	102
5.4.4 Time course studies:	103
5.4.5 Relationship between CDCA stimulated FGF19 fold changes and relative quantities of FGF19 expression levels in the unstimulated control explants	105
5.4.6 BA stimulated FGF19 expression is not demonstrated in human colon	105
5.4.7 The induction of FGF19 mRNA in human ileum by different BA correlates with their relative potencies as FXR agonists.	106
5.4.8 BA stimulated FGF19 expression by BA analogues: INT 747 vs 767 vs 777	107
5.4.9 FGF19 protein in culture fluid from ileal explants correlates with fold change in FGF19 mRNA	109
5.5. Potential sources of error in experimental design and suggested solutions	110
5.5.1 Primer efficiency	111
5.5.2 FGF19 protein concentrations in culture supernatant	112
5.5.3 Variability of fold change in BA stimulated FGF19 transcript expression	113
5.6 Discussion	115
 Chapter 6: Reduced bile acid stimulated expression of FGF19 is associated with conditions of bile acid	 121
Abstract	121
6.1 Background	122
6.2 Aims	123
6.3 Specific methods	124
6.2.1 Patients with Crohn's disease	124
6.2.2 Patients assessed by SeHCAT with chronic diarrhoea	124
6.4 Results: Crohn's disease	125

6.4.1 Patient demographics	125
6.4.2 Ileal BA stimulated FGF19 transcript levels in CD	126
6.4.3 Median CDCA stimulated FGF19 transcript levels are lower in CD ileum compared to healthy controls	127
6.4.4 Post-hoc analysis of BA stimulated FGF19 transcript levels in IR-CD, neo-terminal ileum	129
6.4.5 BA stimulated Ileal SHP and IBABP transcript levels in patients with NR-CD	130
6.4.6 Post-hoc analysis of Ileitis and BA stimulated expression of FGF19 transcripts in non ileal resected patients	131
6.5 Results: PBAD: Positive correlation between BA stimulated FGF19 transcript levels and SEHCAT retention levels	133
6.6 Pilot data on the effects of TNF α on BA stimulated FGF19 transcript 6.3 BA stimulated FGF19 expression in neo-terminal ileum	135
6.7 Discussion	135
Chapter 7: The response of patients with secondary bile acid diarrhea to the farnesoid X receptor agonist obeticholic acid	141
Abstract	141
7.1 Background	143
7.2. Results	143
7.2.1 Study patients	143
7.2.2 FGF19	144
7.2.3 7 α -hydroxy-4-cholesten-3-one (C4)	147
7.2.4 Total bile acid values	147
	10

7.2.5 Changes in clinical symptoms	147
7.2.6 Ileal resection length	148
7.2.7 Idiopathic diarrhoea controls	149
7.2.8 Tolerability, liver enzymes and lipid analyses	149
7.3 Discussion	150
 Chapter 8: Variation in <i>DIET1</i> may modify the PBAD phenotype by influencing serum FGF19 levels	 154
Abstract	154
8.1 Background	155
8.2 Specific methods genotyping	156
8.3 Results: The minor allele may attenuate the reduced FGF19 levels associated with PBAD	 156
8.4 Discussion	161
 Chapter 9: Conclusion and future work	 163
References	169

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1 Introduction

1.1 Bile acid (BA) homeostasis and the enterohepatic circulation of BA

Bile acids are the end product of cholesterol metabolism and their secretion is the main determinant of hepatic bile flow. Bile is released into the duodenum after a meal in response to cholecystokinin (CCK) which induces contraction of the gallbladder and relaxation of the sphincter of Oddi. The amphipathic nature of bile acids (BA) enables them to emulsify the lipid component of a meal and form mixed micelles with fatty acids and monoglycerides within the small bowel. This greatly enhances the passive diffusion and absorption of fatty acids and monoglycerides at the brush border membrane.

The majority of BAs are conjugated and ionized before secretion from the liver, which ensures they stay in the intestinal lumen until they meet the BA transporter proteins of the terminal ileum. The specific BA transporter protein is the apical sodium-linked bile acid transporter (ASBT) which mediates a Na-dependent reabsorption of BAs from the intestinal lumen, against a concentration gradient of BA into the enterocytes of the terminal ileum[1, 2]. Passive diffusion of unconjugated BAs throughout the distal small bowel also occurs [3]. The ileal reabsorption of conjugated BA is highly efficient, with less than 5 % of BA passing the ileum into the colon at each pass. The reabsorption of this specific component of biliary secretions in the distal small bowel leads to a pool of bile acids that cycles several times (5-12 cycles) between liver and ileum after each meal. The bile acid pool is approximately 2-3g. The liver secretes around 12g per day but only needs to synthesise an average of 400mg per day to keep up with the small amounts lost from the body . The homeostasis of this enterohepatic circulation of bile acids is complex. BA production in the liver matches the amount of BA lost in the faeces.

1.2 BA-FXR-FGF19 signalling

In animal studies, diverting bile acids away from this enterohepatic circulation (such as a biliary fistula) will lead to increased hepatic BA biosynthesis[4]. Yet animal models involving bile duct ligation, which increase hepatic concentrations of BA are also found to have an increase in hepatic synthesis of BA[5]. In this situation duodenal administration of bile acids (chenodeoxycholic acid, CDCA) reduces hepatic BA synthesis but intra-venous administration does not[6]. An intestinal negative feedback signal from the gut to the liver was proposed. In 2005, such a signal was found to be an atypical Fibroblast growth factor, FGF15 in the mouse [7]. FGF15 inhibits *de novo* BA synthesis by inhibiting the transcription of cholesterol 7 alpha hydroxylase CYP7A1, the rate limiting enzyme in the production of primary BA, through the hepatic FGF receptor 4 (FGFR4) and the c-Jun NH2-terminal kinase (JNK) pathway [8]. FGF15 is under the transcriptional control of the Farnesoid X Receptor (FXR), a nuclear receptor that is widely regarded as an intracellular BA receptor for BAs. BA are taken up by the ileal enterocyte where they bind and activate the transcription factor FXR. CDCA is the most potent natural ligand for FXR [9]. FXR upregulates the transcription of many BA-responsive genes but the most responsive in the mouse ileum is FGF15 [10]. This mechanism ensures that reduced ileal uptake of BA is met with counter regulatory increases in hepatic bile acid synthesis.

BA can also signal via a G-protein coupled plasma membrane receptor, TGR5 leading to activation of adenylyl cyclase and increasing the production of cAMP[11]. TGR5 is expressed on neuroendocrine cells secreting GLP1. Interest is now also mounting on systemic effects of BAs on energy consumption, glucose homeostasis and lipid metabolism via TGR5 receptors on non- enterohepatic tissues[12].

There are some intriguing differences between FGF15 and other distal peptide gut hormones. FGF15 is produced in enterocytes rather than neuroendocrine cells, and it does not appear to be released from preformed vesicles. The expression of FGF15 in the mouse is largely restricted to the ileum [13]

unlike PYY and GLP1 whose expression is fairly widespread throughout and ileum and colon [14].

FGF15 is secreted from ileum (by unknown mechanisms), then travels to the liver via the portal circulation and binds to the cell surface receptor FGFR4.

FXR is expressed in liver and intestine. BA can also down-regulate their own synthesis within the liver directly through FXR-mediated down-regulation of CYP7A1 transcription[15]. Gut and liver specific FXR knockout mice have shown that liver FXR $-/-$ still retain the capacity to inhibit hepatic biosynthesis in response to CDCA whereas ileal FXR $-/-$ cannot [16]. BA FXR activation in the liver is likely to be important in responding to increasing intracellular concentrations of BA, particularly in situations of intra-hepatic cholestasis. Liver specific FXR activation transactivates BA transporters BSEP, MDR3 and OATP1B3, which promote BA efflux from the hepatocyte, limiting BA-induced intracellular toxicity[17, 18]. Mutations in such genes are associated with conditions of intra-hepatic cholestasis [19]. Ileal BA FXR activation is likely to be important in responding to changes in intraluminal BA concentrations leading to a protein signal FGF15/19 is sent to the liver.

FGF19 is the human orthologue of FGF15, a 21kDa protein, which is able to send feedback signals to more proximal parts of the GI tract, similar to other distal gut hormones (PYY, enteroglucagon)[20, 21]. Serum FGF19 levels [22] increase in the blood a few hours after meals, when ileal BA absorption is maximal. BA synthesis is asynchronous with FGF19. Both BA synthesis and FGF19 levels are affected by fasting. FGF19 is not usually expressed in the liver, but in cholestasis, expression can be demonstrated, suggesting an additional role perhaps in helping to protect the liver from the toxic effects of BAs [23]. Some authors have also demonstrated in the mouse that FGF15 represents a post-prandial signal that antagonizes CCK induced gallbladder contraction and promotes gallbladder re-filling[24]. Another group conducting human studies have shown that administration of oral CDCA increases FGF19 levels along with gallbladder volume up to 6 hours after administration[25].

Patients suffering from intestinal failure on long term parenteral nutrition can develop a liver disease characterised by steatosis and cholestasis. Such patients often have an anatomically short small intestine. The liver disease is therefore thought to relate to a disruption of the normal enterohepatic circulation of BA. These patients often lack an intact distal ileum and it is possible that such patients lose a protective influence from ileal -BA -FXR related FGF19 production [26].

With regards to BA homeostasis , it appears that more is known about how overexpansion of the BA pool size is prevented by inhibiting the addition of new BAs at the liver rather than increasing excretion into the faeces at the ileum. BA are present in all classes of vertebrates including primitive forms such as the lamprey [27]. Even the selective BA transporter, ASBT is present in the everted gut sac of the lamprey [28] , but it is not clear how far down the evolutionary tree the FGF15/19 pathway is found. The FGF15/19 pathway may have evolved in higher vertebrates due to deleterious consequences of increasing concentrations of BA in the colon. Increased aqueous concentrations of BA in the colon promote water and chloride secretion, which stimulates colonic motility and watery diarrhoea. In human population studies there is evidence to suggest a possible association between increased secondary faecal bile acids with the incidence of colorectal cancer [29, 30].

1.3 Secondary Bile Acid Diarrhoea (SBAD) in Crohn's disease (CD)

Diarrhoea is a major symptom in Crohn's disease and causes significant concern in patients with inflammatory activity and also in surgically-treated patients. It is a key component of the Crohn's Disease Activity Index used in most studies of drug efficacy. There are multiple mechanisms that lead to diarrhoea, including impaired absorption and secretion of electrolytes and water in the inflamed parts of the intestinal tract. Bile acids which are normally absorbed in the ileum as part of an entero-hepatic circulation, may be implicated as they will produce a secretory diarrhea in the colon if ileal function is abnormal, as is usually the case in Crohn's. Many studies have demonstrated secondary bile acid malabsorption in most Crohn's patients with diarrhoea, whether or not they

have undergone ileal resection. Corticosteroids increase expression of bile acid transporters and improve diarrhoea. Therapy with bile acid sequestrants can be beneficial.

Another potential mechanism for bile acid diarrhoea has become apparent recently. It has been shown that the ileum produces a secreted hormone, fibroblast growth factor 19 (FGF19), which inhibits hepatic bile acid synthesis. In our pilot data, we have measured the expression of FGF19 in human ileum and in blood, and have found very low levels in some patients who have undergone ileal resection for Crohn's disease. This is proposed to result in increased hepatic bile acid synthesis, which in the presence of sub-optimal ileal reabsorption of bile acids, will further worsen the secretory effects of bile acids in the colon, producing diarrhea.

We hypothesize that FGF19 production is altered in patients with Crohn's disease and so may serve as a marker for functioning ileal tissue and for disease activity. Reduced FGF19 may contribute as a novel, unappreciated cause of the diarrheal symptoms

1.4 Bile acid studies in Crohn's disease

1.4.1 Post ileal resected-Crohn's disease (IR-CD)

The length of the small intestine is highly variable. Autopsy studies reveal a range from 2.5 – 6 metres. The transition from intestinal secretion to active absorption of sodium and water is a gradual one from jejunum to ileum throughout the mid-small bowel. Active uptake of BA is more confined to the distal ileum [3, 31]. This is likely to relate to increasing expression of ASBT towards the terminal ileum[32] . Ileal resections as small as 30cm are associated with significant faecal BA loss [33].

The increased faecal BA concentrations after ileal resection often lead to post operative diarrhoea. This situation was termed 'cholerrheic diarrhoea' by Alan Hofmann [34] and also type I, or secondary bile acid malabsorption (BAM). The cholerrhesis refers to the loss of bile acids, from the entero-hepatic circulation between liver and small intestine, into the colon. Only a small proportion

of BA can be reabsorbed from the colon as secondary BA. BA kinetic studies reveal that endogenous BA synthesis can increase dramatically to match the faecal BA losses in this situation[35].

Diagnosis of BA malabsorption is usually made by the SeHCAT test in countries where this is available. The SeHCAT test determines the 7-day retention of a ^{75}Se tagged bile acid using simple nuclear medicine gamma scanning. An important study using SeHCAT scans revealed that 90% of Crohn's patients suffering from chronic watery diarrhoea after ileal resection have evidence of BAM[36, 37]. Usually the lower the SeHCAT retention level, the more likely the patient will respond to BA sequestrants such as cholestyramine, colestipol or colesevelam, which bind BAs in the gut and prevent their secretory actions in the colon. A therapeutic trial with one of these agents is often used to indicate diarrhoea is due to BA malabsorption.

With larger ileal resections, hepatic BA synthesis is not sufficient to keep up with faecal BA loss. One particular study elegantly showed faecal fat excretion was found to increase in direct proportion to the length of small bowel resected greater than 100cm [38]. This observation was thought to be due to reduced BA pool sizes, with consequent decreased small bowel luminal BA concentrations and fat maldigestion. Previously mentioned *in vitro* studies on human ileal tissue has demonstrated greatly increased BA uptake in the distal 100cm of ileum compared to more proximal ileal tissue [31].

1.4.2 Bile acid studies in non-resected Crohn's disease (NR-CD)

Similar observations are present in patients with NR-CD and regional ileitis. SeHCAT testing reveals that the majority of patients with active ileitis will have a decreased SeHCAT retention [39, 40]. Older studies using radio-isotope dilution studies have observed reduced BA pool sizes in such patients with ileal lesions that had been thought to be much less than 100cm[41]. In another study such observations have been attributed to concomitant colitis interfering with the colonic absorption of secondary BAs[42]. In others, it has been speculated that the ileal BA malabsorptive defects were more extensive than that revealed by the radiological studies. One particular animal model of

chemically induced terminal ileal inflammation revealed evidence of reduced BA absorption in ileal tissue some distance from the area of regional ileitis[43].

The idea that patients with NR- CD have a defect in ileal BA uptake in the absence of overt ileal inflammation has been further elaborated. Two groups have found decreased uptake of BAs in non-inflamed human ileal tissue from patients with colonic CD[44, 45]. Smith et al observed that up to 50 % of non-resected patients thought to be in remission but suffering from chronic diarrhoea have evidence of reduced SeHCAT retention. Further recent work has identified that patients with NR-CD in remission have a reduced expression of ASBT mRNA and protein in terminal ileum. The same group also demonstrated that the expression of ASBT can be increased by the administration of the drug budesonide[46].

Budesonide is an oral medication which is particularly useful for patients with ileal CD for a number of reasons. Firstly it is a glucocorticoid, and members of this family of drugs have long been a mainstay of treatment for active CD due to their anti-inflammatory properties. Secondly the drug has a very high first-pass metabolism limiting any systemic glucocorticoid effects and localizing its effects to the intestine. Thirdly current capsule formulations are available, which lead to drug release gradually within the small intestine rather than the stomach.

One of the most commonly associated symptoms of active ileal CD leading to a prescription of budesonide is diarrhoea [47]. Some authors have suggested in addition to its anti-inflammatory properties, some of the beneficial symptomatic effects of budesonide may be attributable to an enhancement of BA absorption[48]. This idea is supported by the observation in a prospective study of patients (diagnosed with microscopic colitis) treated with budesonide that SeHCAT retention improves [49].

1.4.3 Altered BA-FXR-FGF19 signalling in Crohn's disease

Rodent gene knockout studies on any part of the BA- FXR –FGF15 signalling pathway from ileum to liver reveal the same phenotypic changes in BA homeostasis, which include: 1. Increased CYP7A1 mRNA expression, 2. Increased BA pool sizes and 3.increased faecal BAs. The gene knockout studies that have been performed include intestine specific FXR $-/-$, aswell as FGF15 $-/-$, FGFR4 $-/-$ and SHP $-/-$ [7, 16, 50]. The intestine specific FXR knockout phenotype was generated by using a 'floxed' FXR transgenic mouse model. To gain intestine specific knockdown of FXR, homozygous floxed FXR mice were crossed with those harbouring cre-recombinase under the the control of the villin promoter to inactivate FXR specifically in intestine but not the liver [51].

Similar changes in BA homeostasis associated with severe watery diarrhoea, were also shown in a primate study of anti-FGF19 antibodies [52]. With the new knowledge of the FXR-FGF15 signalling pathway in the mouse, more recent studies have examined how this pathway may be disturbed by BAM in humans, particularly in Crohn's disease.

The first potential defect in this signalling pathway in CD starts with the decrease in BA uptake into the enterocyte within the terminal ileum. Inflammatory ulceration is likely to limit passive diffusion of unconjugated BA in addition to the active transport of conjugated BA. Not only do patients with CD in remission demonstrate a reduced basal expression of ASBT, but human, rat and mouse ASBT expression and affinity is inhibited by inflammatory cytokines via c-fos interactions with the ASBT promoter [53].

The reduced uptake of BA from the ileum in CD will interfere with FXR binding and activation and ultimately transcription of FGF19 within the ileal enterocyte. The baseline expression of transcripts from another FXR-responsive gene, SHP, has been shown to be reduced in ileal biopsies from patients, in remission, with Crohn's colitis [54]. Again this was attributed to reduced BA uptake within the enterocyte. However another mechanism has been recently discovered. *In vitro* work,

with intestinal cell lines and *ex vivo* mouse ileum has shown that FXR-target gene transcription (FGF15, SHP and IBABP) is repressed by inflammatory cytokines probably through NF-KB[55]. The authors have therefore suggested that the reduced expression of SHP in Crohn's ileum may also be due cytokine mediated inhibition of FXR activation. FXR expression itself is also reduced in biopsies from inflamed areas of colon in patients with CD [56]. As one might expect, fasting circulating levels of FGF19 are significantly lower in patients with ileitis and resections compared with healthy controls [57, 58]. In these patients, levels of 7-hydroxy-4-cholesten-3-one (C4, a metabolic marker of bile acid synthesis) are significantly increased, consistent with a compensatory increase in hepatic *de novo* BA synthesis.

Another explanation for reduced ileal FXR activation may be related to changes in BA composition. With ileal malabsorption of BA, there will be an increase in colonic BAs. Many of the BA entering the colon are dehydroxylated to secondary BA (deoxycholic acid, DCA, and lithocholic acid, LCA) which are known to be weaker FXR agonists. Some of these BA that are still in solution in the colon may enter the BA pool by passive diffusion from the right colon. If the BA pool is enriched with secondary BA then it is possible that there will be a decrease in ileal FXR activation. Although some studies have shown that non-resected CD patients with localised ileal disease appear to have an increase in ursodeoxycholic acid (UDCA, the epimer of CDCA), the most common observation in resected patients is actually a paradoxical decrease in secondary BA[59]. This observation has been attributed to a possible change in the composition of commensal bacterial flora. This could involve a reduction in 7-dehydroxylating bacteria after ileal resection.

1.5 Primary Bile acid diarrhoea (PBAD)

After the first description of cholerheic diarrhoea in post ileal resection patients [60] it became clear that a subgroup of patients without CD or resection with chronic diarrhoea also have increased faecal BA levels and other markers of excess faecal BA loss such as decreased SeHCAT retention levels. A mutation in the *SCL10A2* gene encoding the ASBT protein was found in a family of patients

with PBAD which led to initial theories of ileal BA malabsorption in the absence of any overt ileal inflammation[61] . However, this mutation was found to represent only rare cases of congenital BAD and further case-control studies failed to show any significant associations of common polymorphisms in the ASBT gene in this condition [62].

A number of studies have documented BAM in one-third to one-half of patients with chronic diarrhea or IBS-D, summarized in a recent systematic review [63]. Overall, the systematic review found that BAM was reported in 32% of patients with symptoms consistent with IBS-D, and there was a dose-response relationship to treatment with BA binders, based on severity of BAM assessed by SeHCAT values. More recent studies have confirmed similar results [64, 65]. It has been suggested that BAD could have a population prevalence as high as 1% in western countries [66].

The PBAD phenotype is remarkably similar to animal models of FGF15/19 deficiency. Increased BA synthesis, Increased BA pool size and increased faecal BA losses. It was therefore proposed that defective FGF19 signalling could be the cause of the BAD in these patients [58]. Impaired FGF19 feedback inhibition therefore leads to uncontrolled BA synthesis that exceeds the normal capacity for ileal reabsorption, producing diarrhoea.

Increased faecal BA or seHCAT losses could result from other mechanisms other than ileal malabsorption. Rapid transit would result in a reduced time for reabsorption of bile acids in the terminal ileum. This would lead to increased BA enterohepatic recycling frequency with increased faecal SeHCAT loss[67]. Transit studies in PBAD have failed to demonstrate an association with increased small bowel transit [68, 69]. Increased faecal seHCAT loss could also result from an increased BA pool size. BA recycling frequency could remain unchanged and the quantity of BA absorbed at the ileum could actually increase, but the fractional absorption at each pass would be decreased leading to increased SeHCAT loss over 7 days. This would be consistent with increased BA pool size observed in one study of PBAD patients [70]. Hence,

impaired FGF19 feedback inhibition leads to uncontrolled BA synthesis that exceeds the normal capacity for ileal reabsorption, producing diarrhoea..

In support of this hypothesis, low median FGF19 levels have been demonstrated in these patients compared to idiopathic diarrhoea controls patients [71]. This implies that FGF19 levels are not simply decreased due to diarrhoea per se. FGF19 levels in this group of patients were also found to have an inverse relationship to 7α – hydroxyl-4-cholesten-3-one (C4) levels [72], a metabolic byproduct of bile acid synthesis reflects the rate of new bile acid synthesis [73].

Other potential mechanisms involve the colonic response to BAs. Some patients may be more predisposed to the pro-secretory or motility related actions of BAs. Genetic variation in *GPBAR1* gene predisposes to quantitative changes in colonic transit and BA excretion. GPBAR1 encodes the TGR5 receptor and is a member of the G protein-coupled receptor superfamily that functions as a cell surface receptor for BA [74]. They are abundantly expressed on colonic enterocytes and regulate basal and cholinergic-induced secretion in rat colon and influence colonic transit [75, 76]. In addition to this genetic variation in the proteins involved in FGF19 mediated feedback regulation of bile acid synthesis, specifically *KlothoB* gene and *fibroblast growth factor 4* gene have been shown to associate with radiological assessments of colonic transit [77-79].

1.6 Diarrhoea resulting from altered FGF19 signalling in Crohn's Disease

Recent in vitro studies demonstrating that certain inflammatory cytokines (TNF- α , IL-1 β) profoundly inhibit the FXR mediated transcription of FGF15/19 lead us to an interesting potential defect in ileal CD[55].Cytokine suppression of FXR activation and FGF19 production may be reduced disproportionately to the reduction in FXR activation relating to BAM in early ileal CD. Hypothetically speaking , the result would be impaired FGF19 feedback inhibition leading to excess

BA synthesis, which would exceed the already reduced capacity for ileal reabsorption, producing BA diarrhoea [58] **figure 1**. A recent study of antibodies directed against FGF-19 in cynomolgus monkeys was associated with the development of severe diarrhoea (and high faecal BA concentrations) after a single dose[52]. Conversely, in a mouse model of BAM (Involving knockout of the BA transporter ASBT) both exogenous FGF15 and FXR agonists greatly reduced faecal bile acids [80]. In human studies it would be difficult to separate (almost impossible) the increase in faecal BA resulting from impaired FGF19 and BA overproduction from the increase resulting from BAM clinically, but the relationship between circulating FGF19 levels and conditions of bile acid diarrhoea is of particular interest [25].

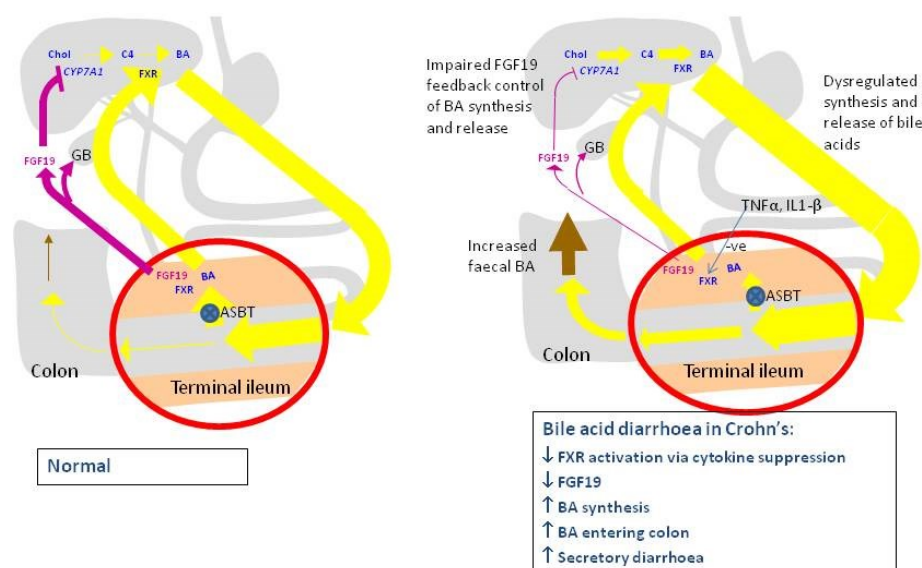


Figure 1. Enterohepatic circulation and BA-FXR-FGF19 signalling. In addition to BAM, faecal bile acids may be increased in CD as a result of cytokine mediated suppression of FXR activation. FXR's transcription of FGF15 in mouse ileum has been shown to be impaired by inflammatory cytokines. If FGF19 production is impaired by cytokines in CD then dysregulated synthesis and release of BA from the liver may occur, further overwhelming the capacity for ileal reabsorption. (Chol = cholesterol, CYP7A1 =hepatic cholesterol 7 α hydroxylase, C4 = 7 α -hydroxy-4-cholesten-3-one, BA=Bile acids, ASBT

= apical sodium-linked bile acid transporter, FXR = Farnesoid X Receptor, FGF19 = Fibroblast Growth Factor 19, GB = Gallbladder).

1.7 Bile acids, FXR and intestinal barrier function

The commensal microbiota of the intestine is several orders of magnitude lower in the small intestine compared to the large intestine. There are a number of reasons for this, including small intestinal peristalsis, defensin secretions and the ileo-caecal valve. Bile acids are also thought to contribute by virtue of direct anti-bacterial effects within the lumen of the small intestine. *In vitro* studies have demonstrated bacterocidal functions via their effects on pH [81]. Animal models involving bile duct ligation display evidence of small intestinal bacterial proliferation. They also display evidence of intestinal mucosal injury, inflammation and bacterial translocation to the mesenteric lymph nodes and systemic circulation [82]. Oral administration of CDCA can limit these effects in animal models [83]. Bacteraemia and sepsis from gut derived organisms is also a common problem in patients with obstructive jaundice [84].

More recent mouse models of BDL have revealed that the increased bacterial proliferation, translocation and mucosal injury are prevented by the oral administration of a specific non-steroidal FXR agonist GW4064 [10]. In addition to this, these protective effects were lost in FXR knockout mice. Microarray analysis of mRNA levels in the ileum reveals the expected upregulation of bile acid responsive genes but also showed an upregulation of many other genes, some of which are thought to have roles in epithelial barrier function. As CDCA is the most potent FXR agonist *in vitro* it was suggested that BA convey anti-bacterial defence through more complex means than simply pH effects within the lumen of the small intestine. Intracellular enteroprotective signalling via FXR activation has become a target of further investigation.

Another FXR agonist currently attracting some attention is the semi-synthetic bile acid 6-ECDCA or INT-747, now known as obeticholic acid. This is chemically derived from CDCA and is also similar in structure to UDCA which has been in therapeutic use for over 30 years. The addition of the 6 ethyl

group greatly increases potency for FXR binding and activation. In vitro work shows it to be 100 times more potent than CDCA [85].

1.7.1 FXR agonists protect against chemically induced animal models of colitis

The importance of FXR activation in the maintenance of intestinal barrier function was first demonstrated by Piero Vavassori et al, by carefully observing the intestinal phenotype in FXR knockout mice. They demonstrated the following important changes after 14-16 weeks, in comparison to FXR wild type mice: 1 Increased colonic mucosal lamina propria (LP) cellular infiltration and collagen deposition. 2 A 60 fold increased expression of mRNA from proinflammatory and pro-fibrotic genes ($\text{TNF}\alpha$, $\text{IL-1}\beta$, $\text{IFN } \gamma$, $\text{TGF}\beta 1$, $\alpha 1$ collagen) using RT-qPCR. 3. Increased basal and stimulated inflammatory cytokines from FXR^{-/-} LP-derived monocytes [56].

The FXR knockout mice developed a more severe phenotype of chemically induced colitis (intrarectal administration of trinitrobenzenesulfonic acid (TNBS)) than FXR wild type mice. Most interesting of all however, was that FXR wild type mice fed concomitantly with INT-747 alongside TNBS administration were protected from the development of colitis. The protective effects of INT-747 were absent in FXR knockout mice. In vitro, INT-747 also attenuates Lamina propria (LP) derived mouse mononuclear cell (MC) responsiveness and inhibits pro-inflammatory gene responses to lipopolysaccharide (LPS). Again, these responses are not attenuated by INT-747 in FXR knockout mice derived MCs. Some evidence was presented demonstrating that in addition to synthetic FXR agonists, endogenous BA can also alter certain innate immune responses. Using THP-1 cells (a leukaemic cell line with upregulated $\text{IL-1}\beta$ expression) LPS stimulated induction of $\text{IL-1}\beta$ mRNA was inhibited by pretreatment with CDCA but not by the weaker endogenous FXR ligand LCA[56].

Gadaleta et al have also demonstrated the protective effects of INT-747 in chemically induced mouse models of colitis. Mice fed with INT747 during intestinal dextran sodium sulphate (DSS) and TNBS administration are protected against chemically-induced colitis[86]. Again using FXR-knockout

mice the protection was shown to be FXR dependent. They also demonstrated INT-747 limited chemically induced increases in intestinal permeability, and limited the induction of several key pro-inflammatory genes in intestinal tissue (IL-1 β , mcp-1, IL-6). They went further to note an induction of certain genes which may be important in mucosal protection (iNOS + cathelicidin in colon and ANG1 in ileum). *In vitro* studies from this group revealed that culturing human stimulated peripheral blood mononuclear cells (PBMC) and gut-derived lamina propria mononuclear cells (LPMC, from 4 patients with IBD) with INT-747 significantly inhibited the release of TNF α (measured by ELISA). With PBMC, 10 μ M of endogenous BA CDCA showed some evidence of attenuating cytokine release but in comparison to 1 – 10 μ M of INT-747, this did not achieve significance. This is likely to relate to the relative potency of INT-747 compared to CDCA with regards to FXR binding and activation[86].

1.7.2 The vicious cycle: FXR activation is repressed by inflammatory cytokines, and inflammatory cytokine expression is repressed by FXR

Many of the pro-inflammatory genes shown to be downregulated by FXR activation by INT747 (TNF- α , IL-1 β) have their expression upregulated by another key transcription factor, Nuclear factor kappa – B (NF-kB). Mutual antagonism between these two pathways has been demonstrated. FXR can inhibit the transcriptional activity of NF-kB and, conversely, NF-kB can inhibit the transcriptional activity of FXR. Fiorrucci's group have highlighted, *in vitro*, possible mechanisms underlying FXR induced inhibition of NF-kB. In THP-1 cell cultures FXR INT-747 activation was shown to promote the transcriptional silencing of IL-1 β by stabilising nuclear receptor corepressors (NCoR)[56]. Conversely, Van Mil's group have explored possible mechanisms underlying NF-kB induced inhibition of FXR. In cultured *ex vivo* mouse ileal tissue FXR BA responsive target genes IBAP, SHP and FGF15 revealed significantly reduced transcription upon stimulation with GW4064 in the presence of TNF α or IL-1 β . Reporter assays overexpressing the NF-kB subunits p50 and p65 also completely abolished GW4046 mediated stimulation of target FXR target genes SHP and IBABP. [55].

If BA have anti-inflammatory properties within the intestinal cells, one hypothesis worth exploring in the future is whether reduced BA absorption into intestinal cells contributes to the upregulated inflammatory signals associated with Crohn's disease. A vicious cycle may exist where reduced BA uptake into cells leads to impaired FXR activation leading to disinhibition of inflammatory cytokine production. Increased inflammatory cytokines may repress FXR activation further. Reduced FXR target gene transcription would also lead to reduced expression of FGF19, **Figure 3**.

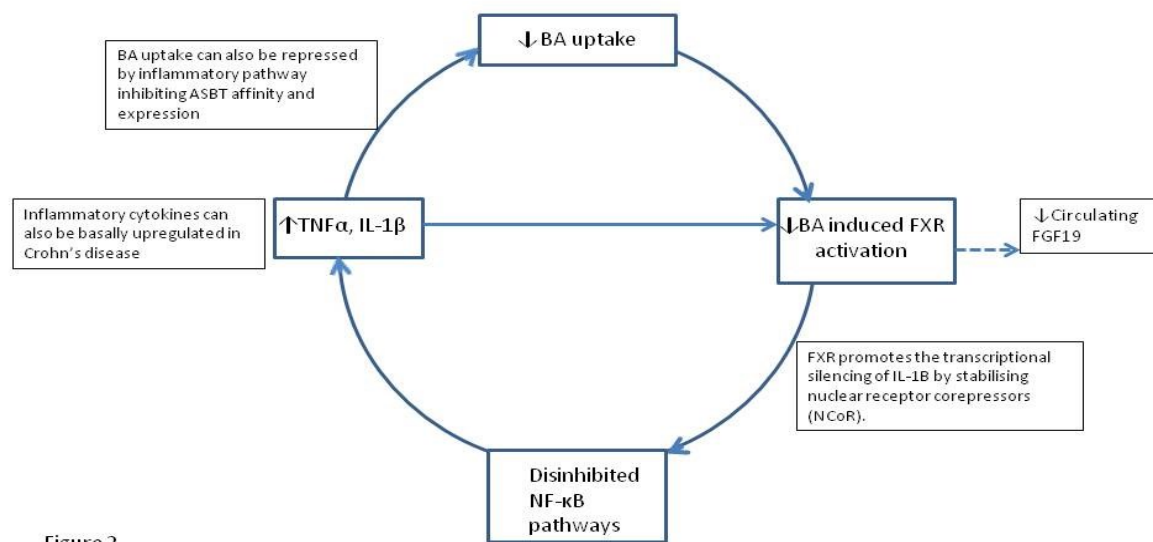


Figure 1.2. Vicious cycle of repressed FXR activation within epithelial enterocytes: Reduced uptake of BA will lead to reduced FXR activation. FXR activation is further restrained by basally upregulated inflammatory cytokines in Crohn's disease. NF-κB pathways will be further disinhibited by impaired FXR activation allowing for further unrestrained transactivation of inflammatory cytokines. Inflammatory cytokines have also been shown to impair the activity of the bile acid uptake transporter ASBT. Hence a self perpetuating cycle of impaired FXR activation and upregulation of the NF-κB pathway ensues (BA=Bile acids, ASBT = apical sodium dependent bile acid transporter, NF-κB = Nuclear factor Kappa B).

1.8 FXR agonists and a therapeutic role in SBAD of Crohn's Disease

We hypothesized that, if potent FXR agonists such as OCA were able to stimulate FGF19 in patients with BAD, this would reverse the impaired regulation of BA synthesis and, by reducing BA production, would lead to clinical improvement. The effect may be most marked in primary BAD and impaired BA absorption could limit the effect in secondary BAD but the potency as an FXR agonist may overcome this in SBAD. The addition to the CDCA by the addition of the 6 ethyl group dramatically increases the bile acids binding and activation of FXR. The importance of FXR signaling in BA homeostasis and colonic function is already hinted at from the observation that constipation was a side effect in prior human studies of obeticholic acid [87] whereas CDCA has been used to treat constipation due to its secretory effects in the colon in others [78] .

Additional benefits may arise from the demonstrated anti-inflammatory effects of FXR agonists observed in animal studies and cell lines [56, 86, 88]. In vitro, the most potent natural ligand for FXR is CDCA [9]. There is some evidence to suggest that FXR activation is impaired in human ileum in CD [54]. The mechanism underlying this finding may be due to malabsorption of BAs (specifically CDCA) or directly resulting from cytokine suppression of FXR. If FXR activation maintains intestinal barrier function it is theoretically possible that impaired FXR activation may contribute to the aetiopathogenesis of ileal Crohn's disease. Initiating events in the early pathogenesis of IBD are thought to be related to inappropriate activation of (Pathogen Pattern Recognition)PPR receptors in response to translocating bacterial antigens with activation of NF-kB pathways. It is possible that in the normal ileum, BA-induced FXR activation contributes to the restraining of this pathway via its antagonistic effects on NF-kB . Impaired ileal BA transport and FXR activation may contribute to the Crohn's disease predilection for localised ileal inflammation. Again it is interesting to speculate whether budesonide and other corticosteroids may have anti-inflammatory effects in part attributable to enhanced BA uptake and possibly increased FXR activation.

FXR agonists have shown protective effects in chemically induced colitis but perhaps their most relevant application in human inflammatory bowel disease will be in ileal Crohn's disease, as this is physiologically where the most significant defect in intestinal FXR activation is likely to occur. In the normal intestine, the protective effects of FXR activation in the colon are probably less important as most of the BA in the colon are either secondary bile acids (which are only weak FXR agonists, in vitro) or not in the aqueous phase. Also reduced fasting circulating FGF19 levels may act as a biomarker of patients in whom ileal FXR activation is most deficient. Fasting FGF19 levels have been shown to increase significantly in patients taking FXR agonists in clinical trials where it is being used in liver disease [89]. This observation confirms that ileal FXR activation is certainly enhanced in this group of patients. Enhanced ileal expression of BA-responsive target genes has also been demonstrated recently in patients with Crohn's colitis after the oral administration of CDCA[25]. This confirms that ileal FXR activation is possible in CD despite the known defects in ileal BA absorption. The next step is to test whether the FXR agonists produce symptomatic improvement in SBAD, followed by further studies in the future regarding the potential anti-inflammatory effects specifically in ileal CD.

1.9 Genotype phenotype relationships in bile acid homeostasis, colonic function and PBAD

Candidate gene studies in patients with PBAD have centred on genes with established roles in bile acid transport and metabolism and the FXR-FGF19 pathway. As previously described the FXR-FGF19 pathway exerts a negative feedback signal to hepatic BA synthesis in response to ileal absorption of BA. After uptake of BA into the ileal enterocyte BAs act as the ligand to bind and activate the nuclear receptor FXR. Ligand bound FXR then upregulates the transcription of FGF19. Receptors specific for FGF19 protein, referred to as FGFR4 are expressed predominantly on hepatocytes in association with a co-receptor klothoB. FGFR4 and klothoB activation downregulates CYP7A1 expression.

A study of 435 IBS patients and 279 healthy subjects testing 15 common SNPs from 7 genes involved in BA homeostasis and associated a Klotho β variant (SNV rs17618244) with accelerated colonic transit in IBS predominant diarrhoea patients [79]. Previous work by this group examined the use of chenodeoxycholate in females with IBS and constipation on colonic transit and ascending colon emptying which were significantly increased compared with placebo [121]. Stool frequency increased and stool form became looser. There was a significant association between fasting C4 levels and bowel transit, although no association with FGF19 was found. It was found that a SNP in the FGFR4 gene and the same SNP in klotho B gene rs17618244 was associated with increased transit with the 2 different doses of CDCA administered. The authors concluded that patients with this genotype may be more susceptible to the effects of exogenously administered BAs [78].

Recently described exploratory studies of certain functional genetic variants including those which have been identified in the FXR gene (*NR1H4*) [90, 91] which might affect FGF19 production have also been described. This research group has also looked at variants in the *FGF19* gene in the 3'-untranslated region which could affect transcript stability. Other candidate genes were explored where variation could affect intracellular bile acids and FGF19 expression include the ileal bile acid

transporters ASBT (*SLC10A2*) and OST α (*SLC51A*). No associations of variants in these key genes were identified to associate with PBAD patients compared to healthy control patients.

1.9.1 DIET1 single nucleotide variant: rs12256835

For the purposes of this thesis we became interested in another gene known as DIET1. The Diet1 gene locus was first localised in a substrain of C57BL/6 (C57Bl/6ByJ (B6By)) mice with low cholesterol levels and resistance to atherosclerosis when fed a high fat diet [92]. The investigators had hypothesized that the atherosclerosis resistant phenotype of this mouse substrain was due to a mutation in an uncharacterized gene diet1 and was associated with increased BA synthesis [93]. The Diet1 gene locus was localised to chromosome 2 and genes in this area were known to affect plasma lipid levels and atherogenesis.

FGF19 secretion is poorly understood but a novel protein, DIET1, has been identified as a key regulator. Diet1 in the mouse is a 236kD protein and promotes FGF19 secretion. The responsible mutation in the mouse diet1 gene was associated with differences in BA metabolism analogous to PBAD with reduced FGF15 expression, increased BA synthesis and pool size and increased faecal BA levels. Diet 1 is localised to intestinal epithelial cells in the mouse small intestine with increasing expression towards the ileum. *Diet1* expression levels in mouse intestine, and *DIET1* expression levels in cultured human enterocytes, correlate with FGF15/19 protein production. Diet1-deficient mice have reduced FGF15/FGF19 levels in the ileum, impaired feedback regulation of hepatic bile acid synthesis, and increased bile acid synthesis, an increased BA pool and increased BA excretion [94]. In the same diet1 deficient mice, transgene expression of diet1 normalized hepatic bile acid synthesis and *Fgf15* expression, confirming that a key function of Diet1 is to promote FGF15 production. HT-29 cells transfected with a *Diet1* expression vector increased FGF19 mRNA levels slightly (~30%) and increased FGF19 protein secretion into the culture medium by 3-fold. Diet1 therefore enhances FGF15/FGF19 expression and secretion. Karen Reue et al have since performed

pilot studies on DNA samples from previously recruited patients with PBAD and healthy controls to sequence the DIET1 gene for variation which may be associated with PBAD (unpublished work) and is further described in chapter 8.

Hypothesis:

The general hypothesis of this thesis is that ileal FGF19 production is suboptimal and causes diarrhoea due to dysregulated BA production leading to increased faecal BA levels and secretory diarrhoea, in patients otherwise described as suffering from 'bile acid diarrhoea'. This hypothesis can be further subdivided into 3 main propositions for investigation in this thesis:

- (1) Ileal FGF19 expression is altered in both CD and PBAD;
- (2) Serum FGF19 is related to ileal resection length and symptoms of diarrhoea in CD and therefore could have utility as a biomarker of BAD in CD;
- (3) Stimulating FGF19 with a BA analogue in patients with CD and SBAD will have beneficial symptomatic effects

2. Materials and Methods:

2.1 Ethical considerations, patient recruitment and consent

The FGF19 expression studies (Chapter 3-6, 8) were approved by the local Institutional Review Board, the Hammersmith, Queen Charlotte's, and Chelsea Hospitals' Research Ethics Committee. REC reference number 2000/5795, UKCRN ID number 7024. The clinical trial in Chapter 7, OBADIAH1 was sponsored by Imperial College London and Imperial College Healthcare NHS Trust and registered with EudraCT (2011-003777-28) and ClinicalTrials.gov (NCT01585025). The drug was provided by Intercept Pharmaceuticals, Inc. (San Diego, CA). Ethical approval was obtained from National Research Ethics Service Committee London Brent (REC reference 12/LO/0123).

2.2 Serum FGF19 studies.

Described in chapter 3, fifty-eight patients with a diagnosis of Crohn's disease were recruited prospectively from the gastroenterology outpatient clinics, inflammatory bowel nurse specialist clinics and gastroenterology wards from 3 sites at Imperial College Healthcare trust. This included Charing Cross, Hammersmith and St Mary's hospital. Written informed consent was obtained and venous blood samples were collected in serum separator tubes. These were then centrifuged at 3000rpm for 10mins and aliquoted for storage at -80°C for subsequent FGF19 assays. Some patients also had 5mls of whole blood collected in an EDTA tube for storage at -20 and later DNA extraction.

The diagnosis of Crohn's had been histologically confirmed in all patients. Twenty-three patients had undergone previous ileal resection (IR-CD) and 35 had no previous intestinal resections (non-resected CD, 'NR-CD'). In 18 of the 23 IR-CD patients the precise length of ileum resected was available according to macroscopic surgical specimen descriptions.

Stool frequency, and Bristol Stool Form Scale (BSFS) were recorded for all patients. Disease activity was assessed using the Harvey Bradshaw Index (HBI)[95] and CRP in those with CD. Some patients also gave stool samples for storage at -80 for later analysis of faecal calprotectin. For comparisons

between patient groups, diarrhea was arbitrarily defined as a stool frequency of ≥ 3 , with stool form of 6 or 7 as in previous studies[71]. A Stool Index (SI) was calculated as (daily stool frequency x BSFS) + loperamide use (mg*3).[96] None of the NR-CD patients were taking loperamide during this study. Distribution of CD was assessed using standard endoscopic and radiological techniques. Of the NR-CD patients, 21 had evidence of ileitis (10 ileal, 11 ileo-colonic CD) and 14 had disease affecting the colon only (colonic CD). Details of the patients are shown in Table 1.

Eleven patients (6 with ileitis and 5 colonic) with active disease (HBI $\geq$ 5) requiring treatment (6 corticosteroids and 4 biologics) to induce remission were part of a longitudinal study. Repeat fasting samples were taken following resolution of diarrhea during a period of medically induced remission (HBI $<$ 5). Where possible, samples were taken before the start of treatments used for inducing remission in patients with active disease. This was possible in all patients starting biologics and three of the six starting corticosteroids. The three patients who were already commenced on corticosteroids during active disease had been on these for no longer than 48h.

Three patients with CD and ileal resection or inflammation (one NR-CD with ileal involvement, and two IR-CD) underwent serial blood sampling for FGF19 pre and post standardized breakfast and lunch.

2.2.1 FGF19 and C4 assays.

Including samples from chapter 3 and 7, blood samples were separated, aliquoted and stored at -80deg C. Assays were performed by operators who were unaware of the clinical conditions of the patient providing the sample. FGF19 was measured by ELISA sandwich assay (FGF19 Quantikine ELISA kit; R&D Systems, Minneapolis) according to the manufacturer's protocol. 100 μ l samples of serum were assayed in duplicate. The assay is linear between 16 and 1000pg/ml. The intra-assay and inter-assay coefficients of variance are about 4-6% (manufacturer's data, confirmed in the

laboratory). All assays were run with mid pool samples (previously collected with known FGF19 levels) to ensure consistency and observe the inter-assay variation.

2.2.2 C4 and BA assays.

Serum 7 α -hydroxy-4-cholesten-3-one (C4) levels from the clinical trial described in chapter 7 were determined by the previously documented procedure in the Clinical Chemistry Department, Western General Hospital, Edinburgh [73]. Following solid phase extraction, samples were analyzed by high performance liquid chromatography. Total serum BA were measured by an enzymatic colorimetric method using 3 α -hydroxysteroid dehydrogenase.

2.3 Ileal FGF19 expression studies:

2.3.1. Sampling during routine colonoscopy

Endoscopically obtained intestinal biopsies were used to study the expression of FGF19 transcripts. Biopsies were to be taken at colonoscopy. Colonoscopy was performed after bowel preparation and fasting for at least 6h. The bowel preparation used is Senna granules and magnesium citrate. The bowel purgative includes magnesium carbonate and citric acid. This reacts within the intestinal lumen to form magnesium citrate which exerts an osmotic effect on cells within the intestine. This leads to secretion of water and stimulation of GI motility. This clears the colon of any stool prior to colonoscopy and biopsies.

During colonoscopy, endoscopic mucosal biopsies were taken by an endoscopist using standard endoscopic radial jaw biopsy forceps (Boston Scientific, M005133300). The first two ileal and/or colonic biopsies were placed in formalin for histological examination. Then 2 – 9 biopsies were taken for research purposes to be used for basal expression studies or ileal explant incubations.

2.3.2 General recruitment and selection of healthy controls and patients

Patients for this part of the study were screened and recruited prospectively whilst attending for routine outpatient colonoscopy. Patients aged 18-80 were included. Patients were excluded if they

were taking anti-coagulants, dual anti-platelet therapy or any other bleeding risks including the need for polypectomy of a polyp ≥ 1 cm. They were given a patient information sheet whilst they were being pre-assessed for colonoscopy. If interested in taking part, written, informed consent was obtained to undergo 2-9 research biopsies in addition to clinical biopsies.

In all patients symptoms of diarrhoea were recorded including the presence or absence of liquid stool and average stool frequency in 24hrs. Drug history and where possible wt, height and BMI were also recorded. Each of the patient groups studied are described in the specific methods of chapters 4,5 and 6. Colesevelam was stopped 48hrs prior to samples in BAD patients.

2.3.3 Samples from retrospective cohort of patients with chronic diarrhoea

Historical stored ileal biopsy samples from a previously recruited cohort of 15 patients was also included in baseline intestinal FGF19 expression studies [71]. These patients had been investigated for chronic diarrhoea (an average of 3 loose, type 6 or 7 stools per day for greater than 3 months) with colonoscopy and SeHCAT scanning. 3-6 ileal biopsies had been taken at colonoscopy and stored in RNA later and immediately frozen and stored at -80 for later processing.

2.3.4 Samples for basal ileal FGF19 transcript expression studies

For this part of the study, groups of 3 mucosal biopsies were taken from the following anatomical regions; terminal ileum, proximal and distal colon. From each region the 3 mucosal biopsies were pooled and placed together in 400ul of RNAlater in separately labelled sterile RNase free, 1.5ml microcentrifuge tubes. Distal colonic biopsies were taken from the sigmoid colon just above the rectosigmoid junction. For each patient, separately labelled ileal, proximal and distal colonic biopsies were then transferred to a -80 freezer for storage and subsequent processing for the analysis of baseline gene expression. Intestinal samples taken from prospectively recruited patients were thawed and processed within 6 months of sampling. The 15 historical ileal samples were thawed and processed as part of this thesis 2 years after the samples were taken at colonoscopy.

2.3.5 Explant incubations and bile acid preparations:

Patients were screened and consented as described above 2.3(a). Two to eight ileal mucosal biopsies were placed in pre-oxygenated Dulbecco Modified Eagle's Medium (DMEM, PAA systems E15-843) on ice for immediate transfer (5-10 mins) to culture media in an incubator at 37°C 5% CO₂ in a primary cell culture lab for 6 hour incubations.

Culture wells were pre-filled with 'complete culture media' from 6 well culture plates (NuncloTM Δ Surface multidishes, ThermoScientific 140685). Complete culture media was prepared by supplementing DMEM with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml leupeptin, 50 µg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The media was also aliquoted to be stored at -20°C for future use. On the day of an incubation frozen culture media was thawed and brought to 37°C (in a water bath) prior to obtaining biopsies. All culture media had high concentration oxygen (95%O₂ and 5%CO₂) bubbled through it with sterile tubing for 5 seconds.

The culture well in the first position of the 6 well dish was always designated a substrate free well (negative control), containing culture media only +/- vehicle. The other wells contained the substrate of choice (a BA or BA analogue). For incubations six or eight terminal ileal biopsies were usually obtained from an individual patient. Groups of 2 or 3 explants (from the pool of 6-9 ileal biopsies from an individual patient) were then placed into each well depending on how many conditions were being studied in parallel (**figure 4**).

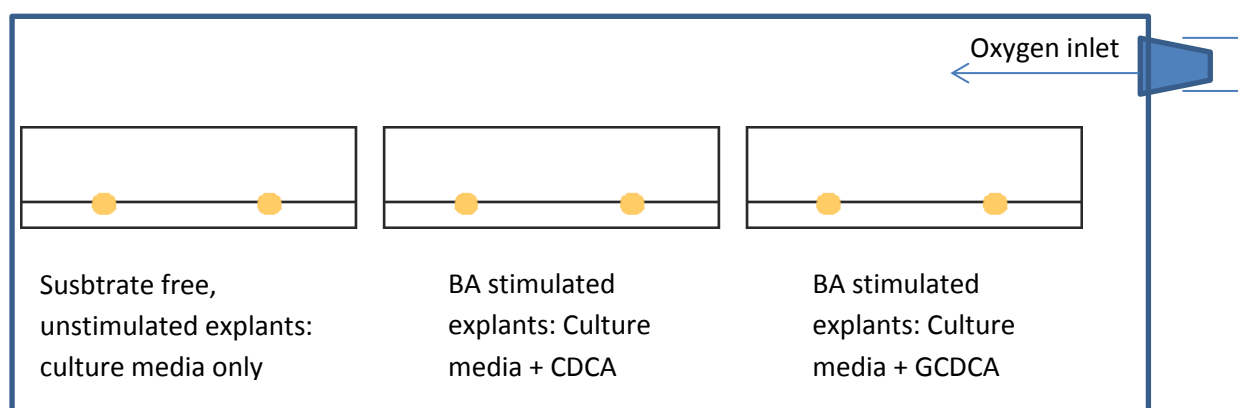


Figure 2.0. An example of an incubation of 6 terminal ileal biopsies (explants) from one patient. Groups of 2 biopsies maintained in 3 parallel culture wells in 3 separate conditions. Each culture dish contained 2mls of complete media. Bile acids (CDCA = Chenodeoxycholic acid, GCDCA = Glyco-chenodeoxycholic acid) were usually added to stimulate BA regulatory genes. Transcriptional gene expression in stimulated explants was then compared to control ‘unstimulated’ explant expression. Stock solutions of bile acids were made to 10mM and the different BA and concentrations used are shown in **table 2.1**. Small aliquots were frozen at -20 for single use aliquots to prevent repeated freeze thaw cycles. The conjugated BA, GCDCA was dissolved directly into DMEM and the unconjugated BAs studied were dissolved in a solvent vehicle, dimethyl sulfoxide DMSO (Sigma UK, D8418) or ethanol. The addition of 10µl of BA to the total volume of 2mls culture media was then required to obtain a concentration of 50µM. This concentration was used most often as it was also used in cell and non cell based FXR activation assays[9]. BA aliquots amounted to no more than 0.5% of the total volume of culture fluid.

Bile acids / BA analogue	Concentration in µM used in explant incubations	Manufacturer
Glyco-chenodeoxycholic acid (GCDCA)	1-100	Sigma UK 739715
Chenodeoxycholic acid (CDCA)	1-100	VWR A1690
Cholic acid (CA)	50	Sigma Uk C1129
Deoxycholic acid (DCA)	50	Sigma Uk D2510
Lithocolic acid (LA)	50	Sigma Uk L6250
6-ethyl-3,7-dihydroxy-5-cholan-24-oic acid, Obeticholic acid, INT 747 (OCA)	1-20	Gift from Intercept pharmaceuticals, San Diego, USA
6-ethyl-3,7,23-trihydroxy-24-nor-5-cholan-23-sulfate sodium salt, INT 767	1	Gift from Intercept pharmaceuticals, San Diego, USA

Table 2.1. (BA) Bile acids and BA analogues and the concentration ranges studied in explant incubations with abbreviations and/or synonyms and manufacturers.

The culture plates were then placed in a sealed plastic container with a small inlet valve allowing for oxygen tubing to be attached allowing for oxygenation intermittently. The container was placed in the incubator at 37°C and 5%CO₂ and oxygenated for 30 seconds at the start and at 2 hourly intervals during each 6 hour incubation. At the end of the incubation, biopsies were placed in RNAlater and stored at 4 °C overnight, then transferred the next day to be stored at -80 °C prior to RNA extraction. Culture fluid from the 6 well plates was also collected and immediately frozen in 500ul aliquots at -80 for later analysis. This experimental set-up is similar to a previous successful set-up described by Balesaria et al for human duodenal transcriptional responses to stimulation by Vitamin D[97].

2.3.6 Tissue disruption, lysis and RNA extraction

Stored biopsies from explant incubations or basal expression samples were thawed suspended in RNAlater to preserve RNA from environmental RNAses. Upon thawing of samples they were immediately transferred to eppendorfs containing lysis buffer including dithiothreitol (DTT) at 40mM concentration. Physical tissue disruption of biopsies was performed using a mechinal rotostator or hand held (RNase free) pestle and mortars (VWR) for each group of explants. When hand hand held pestle and mortar was used for tissue disruption, an additional step was used to enhance tissue disruption and minimise residual tissue debris. This involved centrifuging the lysate through a biopolymer-shredding system in a microcentrifuge spin-column format (QIAshredder, QIAGEN, 70565).

The lysate was then processed using silica membrane based spin columns using the RNeasy Mini kit (Qiagen 74106) according to the manufacturer's protocol to isolate total RNA. To minimise genomic DNA contamination, further RNA purification was performed on-column using a RNase-free DNase set (Qiagen 79254). At the end of the protocol total RNA was eluted in 50µl nuclease free sterile water. RNA concentration of the 'eluate' and purity obtained was then assessed using a

ThermoScientific NanoDrop 1000 Spectrophotometer, and then stored at -80°C. Nanodrop RNA concentrations were expressed as ng/ul.

2.3.7 cDNA synthesis

Synthesis of cDNA from RNA was carried out using a SuperScript kit (Invitrogen 11904-018). To control for any differences in RNA yield or amount of tissue between specimens, all RNA was diluted to obtain 1ug. Reaction volumes of 20 µl were used. The first step was to combine 1 µl dNTP mix (10 mM) and 1 µl random hexamers (50 ng/µl), with the RNA eluate and DEPC treated water to obtain 1 µg RNA to an initial volume of 10ul.

This would mean that RNA eluate of concentrations of 125ng/ul would require the addition of 8ul RNA eluate without dilution + 1ul dNTP mix and 1ul random hexamers. Concentrations above 125ng/ul were diluted with DEPC to achieve a final RNA concentration of 1ug (eg 331.18ng/ml required 3.02uls of RNA eluate and 4.98 uls of DEPC treated water within the 8ul reaction volume) .

This 10ul initial reaction volume was followed by the addition of 2 µl 10X RT buffer, 4 µl MgCl₂ (25 mM), 2 µl DTT (0.1 M), 1 µl RNaseOUT™ (40 U/µl), 1 µl SuperScript II RT (50 U/µl). Reverse transcription was performed at 42 °C for 50 minutes according to the manufacturer's instructions. The reaction was terminated at 70°C for 15 minutes. Minus reverse-transcriptase controls were included, where SuperScript II RT was replaced with 1 µl DEPC-treated water. The RNA template from the RNA:cDNA hybrids were removed by treatment with 1 µl *E.coli* RNase H (2 U/µl) for 20 minutes at 37 °C .

2.4 Quantitative real-time reverse-transcription PCR (RT-PCR)

2.4.1 Primer design

Primer sequences and characteristics are summarised in **Table 2.2**. FGF19 primers were designed using the following criteria: primer length of 18-22 base pairs (generally accepted as optimal), similar melting temperatures (T_m), similar G-C content, no more than 3 consecutive identical nucleotides, presence of G-C clamping and amplicon size between 85 and 150 base pairs. For the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used, as described previously [98], and although amplicon size is >150 base pairs, the primers had amplified successfully in this previous study. For both primer pairs, BLAT (<http://genome.ucsc.edu/>) was used to verify primer specificity and to check that amplicons span at least 2 exons (to prevent genomic DNA amplification). In silico PCR methods were also used to obtain melting temperatures.

Primer	Sequence (5'-3')	Position	Primer melting temperature (T_m , °C)	G-C content (%)	Amplicon size (base pairs)
Human FGF19, forward	CGG TAC CTC TGC ATG GGC	chr11: 69,518,053-69,518,070	62.6	67	95
Human FGF19, reverse	CCA TCT GGG CGG ATC TCC	chr11: 69,514,292-69,514,309	64.0	67	
Human GAPDH, forward	CGA CCA CTT TGT CAA GCT CA	chr12: 6,647,136-6,647,155	60.0	50	203
Human GAPDH, reverse	AGG GGA GAT TCA GTG TGG TG	chr12: 6,647,423-6,647,442	60.0	55	

Table 2.2. FGF19 and GAPDH primer sequences.

2.4.2 RT-PCR protocol

Amplification of cDNA was performed using SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich, UK) in an Applied Biosystems 7900HT Fast cycler. Singleplex RT-PCR assays were performed in triplicate in 25 µl reaction volumes, using MicroAmp® Fast Optical 96-well reaction plates (Applied

Biosystems, UK) or in 8 µl reaction volumes using MicroAmp® Optical 384-Well reaction plates (Applied Biosystems, UK). Final cDNA dilution in each well was 1 in 25 and final primer concentration was 0.4 µM.

Each plate was run with minus reverse-transcriptase controls, and PCR controls (1 in 25 volume of DEPC-treated water added to a triplicate of reaction wells instead of 1 in 25 volume cDNA). The thermal cycling programme was as follows: 10 minutes of 95 °C (to activate Taq polymerase), then 40 cycles of 15 seconds at 95 °C, 1 minute at 60 °C, and 1 minute at 72 °C, ramping at 1.4 °C / sec between each step of the cycle. Dissociation-curve analysis to determine melting temperatures (T_m) of nucleic acids was performed to check for product specificity [99] using the same Applied Biosystems 7900HT Fast cycler, with a temperature range of between 60 °C and 95 °C, ramping at a rate of 0.3 °C / 10 sec.

Results were excluded according to the following exclusion criteria:

1. Any samples where amplification revealed a $C_t > 35$ cycles.
2. Where the standard deviation of the mean delta C_t (ΔC_t) from triplicate samples was >0.3
3. If amplification curves of triplicate samples revealed more than one outlier (>0.5 cycle)
4. Dissociation curve analysis showed more than one specific product.

Results were analyzed using relative quantification methods. Basal expression of target gene transcript levels were calculated by converting delta C_t values to the linear form using the calculation $2^{-\Delta C_t}$ (See specific methods, **chapter 4**). For stimulated explants studies, the comparative C_t method was used to give fold changes of FGF19 expression relative to the 'calibrator' control sample in each patient (see specific methods **Chapter 5**).

Primer sequences and characteristics of other genes are summarized in **table 2**. Other primer sequences were taken from other previously published human studies using SYBR green assays

Primer	Sequence (5'-3')
Human FGF19, forward	CGG TAC CTC TGC ATG GGC
Human FGF19, reverse	CCA TCT GGG CGG ATC TCC
Human GAPDH, forward	CGA CCA CTT TGT CAA GCT CA
Human GAPDH, reverse	AGG GGA GAT TCA GTG TGG TG
Human ASBT, forward	GCC CCA AAA AGC AAA GAT CA
Human ASBT, reverse	GCT ATG AGC ACA ATG AGG ATG
Human IBABP, forward	TCA GAG ATCGTG GGT GAC AA
Human IBABP, reverse	TCA CGC GCT CAT AGG TCA
Human SHP, forward	AGG GAC CAT CCT CTT CAA CC
Human SHP, reverse	TTC ACA CAG CAC CCA GTG AG
Human FXR, forward	AGG ATT TCA GAC TTT GGA CCA TGA
Human FXR, reverse	TGC CCA GAC GGA AGT TTC TTA TT
Human OST α , forward	AGA TTG CTT GTT CGC CTC C
Human OST α , reverse	ATT CGT GTC AGC ACA GTC ATT
Human OST β , forward	CAG GAG CTG CTG GAA GAG AT
Human OST β , reverse	GAC CAT GCT TAT AAT GAC CAC

Table 2.3. Primer sequences used in chapters 4-6.

2.5 Statistical analysis and interassay variability

In the serum studies of chapter 3 results will be analyzed by comparing groups of patients with active and inactive Crohn's. 17-18 patients in each group will have 80% power to detect a difference equal to the SD of the sample at the 0.05 level. Sequential samples will be compared in patients before and after response to treatment with corticosteroids and anti-TNF. In these paired comparisons, 10 patients will give similar power. Non parametric Mann Whitney tests will be used to compare cross sectional comparisons of patients from the serum studies and paired non-parametric Wilcoxon paired test for the 10 patients before and after treatments. Spearman's rank correlation coefficients will be used to assess relationships between FGF19 levels and clinical variables.

In the explant studies of Chapters 4,5 and 6. Non-parametric Mann-Whitney tests and Spearman's rank correlations were also used. Corrections for multiple correlations were not applied in Chapter 4 as this is a relatively small group of patients and the comparisons in Chapter 4.4.4 and 4.4.5 are exploratory in nature.

For PCR assays, baseline expression studies all samples were assayed on the same plate. For Stimulated expression studies a positive control was included in each assay to ensure that the GAPDH Ct was within one cycle of previous measurements. All ELISA assays were run with mid pool samples (previously collected with known FGF19 levels) to ensure consistency and observe the inter-assay variation.

2.6 Culture fluid ELISA for FGF19

Culture fluid collected at the end of each incubation for both BA free explants and BA stimulated explants were analysed and compared. Frozen aliquots of culture fluid were thawed at room temperature. The Quantikine Elisa Assay was used to measure FGF19 in culture fluid using the same ELISA as described previously. Standards are prepared in the same medium as culture fluid to exclude any binding differences. The measured concentrations (in pg/ml) were normalized according

to whether the culture plate well had contained 2 or 3 biopsies and the FGF19 concentration was expressed as pg/explant.

2.7 *DIET1* genotyping study: Allelic discrimination assay.

Previously extracted genomic DNA was studied for the *rs12256835* SNP in a cohort of patients who have been previously described [71]. This cohort consisted of 78 prospectively recruited patients with chronic diarrhoea who underwent SeHCAT testing and fasting FGF19 serum sampling. Other intestinal diseases such as IBD or coeliac disease had been excluded from this cohort and the ethnic origin was predominantly caucasian. The 1000 genome project had revealed a *rs12256835* minor allele frequency (MAF) of 0.28 [100]. We assumed a similar MAF of 0.28 in the diarrhoea control patients from this study. On the basis of recent findings of lower prevalence of the minor allele in PBAD patients, power calculations were therefore designed to detect a low frequency of 0.05. For 90% probability at detecting a significant difference between a prevalence of 0.28 and 0.05 we would need 64 alleles (32 patients) in each group (diarrhoea controls vs PBAD). 50 alleles (25 patients) in each group would have an 80% probability.

Genotyping for the *rs12256835* variant was performed using an end point TaqMan allelic discrimination assay (Life Sciences, CA, USA) on each of the 78 patient DNA samples. PCR was performed on 96 well reaction plates and analyzed using a StepOnePlus RT-PCR machine (Life Sciences, CA, USA).

Reaction volume was 15µl per well including 7.5µl TaqMan genotyping Master Mix (2X), 0.375µl SNP Assay mix (40X), 6.125 nuclease free water and 1µl genomic DNA. Standard PCR conditions were: 95°C for 10 minutes, followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute, followed by 60°C for 30 seconds.

The SNP assay consisted of a custom Taqman *rs12256835* genotype assay involving primer/ probe pairs using Minor Groove Binders (MGB) as the Non –Fluorescent Quencher (NFQ). The probe for allele T was labelled with VIC dye and the probe for allele G was labelled with the FAM dye (Life Sciences, CA, USA, assay ID C__32112379_10). The TaqMan genotyping Master Mix contained AmpliTaq Gold® DNA Polymerase with 5' exonuclease activity (Life Sciences, CA, USA Catalog Number 4371353). Allele 1: VIC/MGB-NFQ, T major allele, Histidine residue position 1721. Allele 2: FAM/MGB-NFQ, G minor allele, Glutamine residue position 1721.

2.8 Clinical trial: Obeticholic Acid in Bile Acid Diarrhoea: Obadiah1:

2.8.1 Study design

This was an open-label, single-center observational study of mechanisms, safety and symptom response of obeticholic acid treatment in patients with BAD. Use of OCA was approved by the Medicines and Healthcare Regulatory authority (MHRA, London, UK). Written informed consent was obtained from all subjects who were fully briefed that they were free to withdraw at any point in the study. The study was conducted according to Good Clinical Practice guidelines. All authors had access to the study data and approved the final manuscript.

The design of the trial is shown in **Figure 6**. This **lasted** six weeks, consisting of a two-week run-in period (weeks 1 and 2), two weeks of treatment with oral OCA 25mg daily (weeks 3 and 4) and a two week follow-up period off OCA (weeks 5 and 6). Treatment with bile acid sequestrants was discontinued at the start of the trial; loperamide (up to 16mg/d) was allowed as rescue therapy. Daily symptom diaries were used to record stool frequency, stool form (type) using the 7-point Bristol Stool Form Scale (BSFS)[101], loperamide usage, and abdominal pain, urgency and bloating scores on a 10-point scale, ranging from none to moderate to severe. Hours of pain were also recorded. Patients were asked to record any adverse events in their symptom diaries. Week 5 and 6 diaries were not returned by several patients.

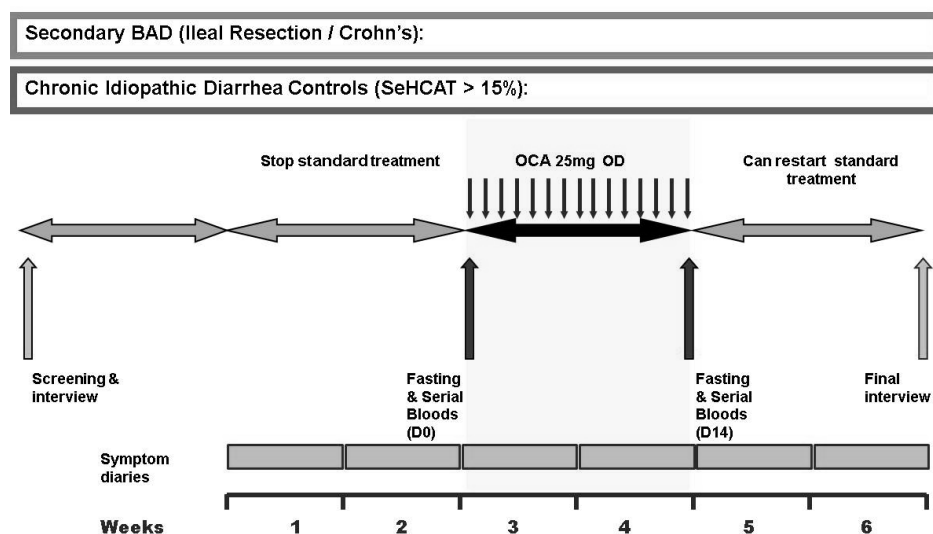


Figure 2.2. Design of the OBADIAH1 study. Three groups of patients were studied over 6 weeks. OCA was given in weeks 3 and 5 with fasting and serial blood sampling at the beginning and end of this period on D0 and D14

Subjects attended on the first and last days of the two week treatment period (D0 and D14), having been asked to fast from 9pm the night before. Blood was taken in the fasting state and then OCA was given on those days. Subjects were then given a standard mixed content breakfast. Further serial postprandial/OCA blood samples were taken at 1-hourly intervals for 6h; a standard mixed content lunch was consumed after 3h.

2.8.2 Subjects

Twenty-three patients were initially recruited from gastrointestinal clinics at the hospitals of Imperial College Healthcare NHS Trust. They were aged 18 – 80y and all had described symptoms of passing an average more than 3 soft or watery stools per day for at least the past three months. Patients recruited into the idiopathic diarrhea control group had received standard investigation for diarrheal symptoms prior to enrolment; other causes of diarrhoea such as colorectal neoplasia, ulcerative colitis, coeliac disease, chronic pancreatitis, drug-induced diarrhoea, active infection or lactose intolerance had been excluded. The patients in the secondary BAD group had Crohn's disease and/or ileal resection. Patients were excluded if they were receiving concomitant

corticosteroids. Patients with clinically significant other disorders including liver disease were excluded.

Work undertaken by others, not included in this thesis also involved recruiting 10 patients with a diagnosis of PBAD. The SeHCAT test was used for a diagnosis of BAD. Patients with primary BAD all had SeHCAT 7d retention $\leq 10\%$. In the secondary BAD group, SeHCAT was not required, as previous studies have shown that over 90% of patients with Crohn's disease, ileal resection and chronic diarrhoea have a SeHCAT $\leq 10\%$ [36, 37]. Two secondary BAD patients with Crohn's disease did not have resections but had SeHCAT tests with retention values of 10% and 4%. The median ileal resection length was 33.5cm, range 0 – 71cm. Several patients also had short colonic resections. Patients in the control group with idiopathic chronic diarrhoea all had normal SeHCAT 7d retention of greater than 15%.

2.8.3 Statistical analysis

The primary endpoint was the change in fasting FGF19 in each group between the samples taken before the first dose of OCA (D0) and after 2 weeks (D14) before the last dose. Secondary endpoints included changes in fasting C4 and BA, liver enzymes, lipids and in clinical symptoms. Peak values during the serial sampling (usually at 5 or 6h) were identified and 0-6h integrated responses (area-under-curve; AUC) values for FGF19 and BA were compared on D0 and D14.

Symptoms recorded in the patient diaries were analyzed as total weekly stool frequency and mean weekly BSFS. Total weekly loperamide use (in mg) was calculated. As patients responded in different ways, to detect a global improvement involving changes in any of these parameters, a weekly diarrhea index (stool index) was calculated using the formula $([\text{weekly stool frequency} * \text{mean BSFS}] + \text{loperamide use} [\text{weekly mg} * 3])$. This equates one 2mg dose of loperamide to one type 6 stool. Comparisons were made between data for W2, the second week of the run-in period off specific treatment, with data for W4, the second week of OCA treatment.

Data are usually reported as medians and interquartile ranges (IQR). Nonparametric tests were used to look for treatment effects between the two groups, Mann-Whitney U unpaired or Wilcoxon paired rank tests and Spearman rank correlations. Statistical analyses were performed using GraphPad Prism 5, Ca, USA. *P* values <0.05 were considered significant.

Chapter 3: Diarrhoea in Crohn's disease: investigating the potential role of the ileal hormone fibroblast growth factor 19

Abstract

Background: Bile acids (BA) are usually reabsorbed by the terminal ileum, but this is frequently abnormal in Crohn's disease (CD). BA malabsorption occurs and excess colonic BA cause secretory diarrhea. Furthermore, the hormone Fibroblast Growth Factor 19 (FGF19) is synthesized in the ileum in response to BA absorption and regulates BA synthesis. We hypothesized that reduced serum FGF19 levels will be associated with diarrheal symptoms and disease activity in both ileal-resected (IR-CD) and non-resected CD (NR-CD) patients.

Methods: Fasting serum FGF19 levels were measured in 58 patients (23 IR-CD patients and 35 NR-CD patients). Disease activity was assessed using the Harvey Bradshaw Index and CRP. Stool frequency, Bristol stool form scale and length of previous ileal resection were recorded. FGF19 levels were also compared to healthy and diarrhea control patients.

Results: FGF19 levels were inversely correlated with ileal resection length in IR-CD patients ($r=-0.54$, $p=0.02$, $n=18$). In NR-CD patients, median FGF19 levels were significantly lower in patients with active disease compared to inactive disease (103 vs 158 pg/ml, $p=0.04$) and in those with symptoms of diarrhea compared to those without (86 vs 145 pg/ml, $p=0.035$). FGF19 levels were inversely correlated ($r=-0.52$, $P=0.02$) with stool frequency, Bristol stool form and CRP in NR-CD patients with ileitis.

Conclusions: Reduced FGF19 levels are associated with diarrhea and disease activity. FGF19 may have utility as a biomarker for functioning ileum in CD. This study supports a potential role of FGF19 in guiding treatments for diarrhoea in Crohn's disease.

3.1 Background

Diarrhoea is a major feature of active disease in Crohn's disease (CD) and a major influence on the ill-health experienced by patients. Clinical disease activity scores are heavily influenced by the frequency of liquid stools.[95] Stool frequency and form are among the least subjective components of the symptomatic scores but do not necessarily reflect active inflammatory disease.

The pathophysiology of diarrhoea in CD is multifactorial but there are two key factors. Colonic water and electrolyte absorption can be impaired directly by colonic inflammation,[102] or indirectly by increased concentrations of bile acids (BA) having secretory effects, [103, 104] referred to as BA-induced diarrhoea (BAD). In healthy subjects, approximately 95% of luminal BA within the small intestine are reabsorbed at the terminal ileum, with only small amounts entering the colon. In CD patients, increased colonic BAs result from impaired reabsorption of BAs at the terminal ileum. This can be either due to ileal inflammation or resection, and may also relate to reduced expression of the ileal apical sodium dependent bile acid transporter (ASBT).[41, 46, 53] This bile acid malabsorption (BAM) can be regarded of as part of the usual phenotype of CD.

Options for diagnosing bile acid malabsorption have recently been reviewed[105]. The gold standard test for diagnosing BAM is the measurement of increased concentrations of fecal BA. This is not commonly done as it is unpopular for patients and lab staff. Other more indirect markers for BAM are measures of BA retention (SeHCAT scan).[106] This involves the oral administration of a radiolabelled BA (SeHCAT) and measurement of its retention within the enterohepatic circulation. Seven day retention is usually reported and reduced retention levels reflect increased losses of BA into the feces. Reduced SeHCAT retention levels are associated with chronic diarrhoea in quiescent CD in the vast majority (90%) of patients with previous ileal resection and as many as 50% of those patients without previous ileal resections[36] [37]. As many as 30% of those previously regarded as having IBS-D also have reduced SeHCAT retention [63] . The majority of these patients gained

symptomatic benefit from BA sequestrants, such as cholestyramine. More recent studies using a newer BA sequestrant, colesevelam, has also demonstrated positive effects on symptoms of diarrhoea in CD.[107]

Another indirect marker of BAM is the BA precursor 7 α -hydroxy-4-cholesten-3-one (C4). Serum levels of C4 are a measure of new bile acid synthesis and increase in response to BA losses from the entero-hepatic circulation.[73] Measuring C4 involves HPLC or tandem mass spectrometry which requires specialized techniques. Increased levels of C4 have been shown to be associated with chronic diarrhoea in pediatric CD.[108] Using older methods markers of BAM were found to relate to disease activity in previous studies

FGF19 is a hormone produced in the enterocytes of the ileum in response to absorbed BAs.[22, 109, 110] BAs bind to the farnesoid X receptor (FXR) in enterocytes and increase transcription of FGF19. Serum FGF19 levels are thus a direct marker of BA absorption and we have shown they are reduced in BA malabsorption.[58] Reduced levels of FGF19 have been demonstrated in patients with both ileal resected and non-resected CD.[72] [57, 111] FGF19 is easily measured using commercially available ELISA. FGF19 levels and C4 are inversely correlated in several groups including CD. ROC curve analysis has revealed that the optimal level of FGF19 for predicting severe BAM (defined by C4>50ng/L)was <60pg/ml with a 80% sensitivity and 68% specificity in CD.[57] We have previously shown an optimal level of FGF19 at <145pg/ml has a sensitivity of 58% and a specificity of 79% to detect C4 levels of >28ng/ml in a cohort of BAD patients including CD.[72]

3.2 Aims

(1) We aimed to investigate the relationship between serum FGF19 levels and CD disease activity scores, and in particular symptoms of diarrhoea (the main symptomatic consequence of BAM) in patients with CD.

(2) We aimed to study the relationship between serum FGF19 levels and length of previous ileal resection in patients.

(3) To study how FGF19 levels are influenced by anti-inflammatory treatments used to treat patients with diarrhoea and active disease.

3.3 Specific methods

Described in serum FGF19 studies Chapter 2.2

3.4 Results:

3.4.1 Patient demographics

	NR-CD		IR-CD	Idiopathic* diarrhea	Healthy* controls
	Ileal disease	Colonic			
Number	21	14	23	68	19
Median Age	35 (25-40)	38 (29-55)	50 (39-67)	50 (33-61)	47 (23-61)
Gender					
Female	10 (48%)	10 (71%)	12 (52%)	46 (68%)	9 (47%)
Male	11 (52%)	4 (29%)	9 (48%)	22 (32%)	10 (53%)
Clinical indices					
CRP mg/dL	10 (2-24)	5 (2-27)	3 (1-9)	-	-
HBI	6 (3-7)	4 (0-6)	4 (2-6)	N/A	N/A
Stool Freq	3 (1-3)	3 (1-4)	4 (2-5)	5 (4-8)	1 (1-1.5)
BSFI	5 (2-6)	5 (3-7)	6 (5-6)	6.5 (6-7)	-
Median Resection length(cm)			40		
Resection length range (cm)			10-96		
Current medications					
5-ASAs	4 (20%)	3 (20%)	3 (13%)	-	-
Immunomodulators	7 (33%)	4 (29%)	8 (34%)	-	-
Biologics	4 (20%)	2 (14%)	0	-	-
Corticosteroids	5 (24%)	2 (14%)	1 (5%)	-	-
No Medication	7 (33%)	5 (36%)	11 (48%)	-	-

Table 3.1 *These results are from previously studied patients for comparison only and were not recruited as part of this PhD. Medians and Interquartile ranges are shown. Percentages are shown where indicated. For comparisons, additional results are included from sixty-eight previously studied [71] patients with idiopathic chronic diarrhea (without evidence of inflammatory bowel disease, previous resections or bile acid diarrhea and SeHCAT >15%) as an 'idiopathic diarrhea control' group. Nineteen previously studied healthy control subjects were also included.[58]

3.4.2 FGF19 levels in Ileal resected (IR)-CD patients:

FGF19 levels were lower in IR-CD patients (median 68pg/ml) than in the previously described healthy controls (231 pg/ml; $p=0.0002$) [Walters CGH] confirming previous findings.[57, 72] We found a significant inverse correlation between the recorded length of ileum resected and the fasting serum FGF19 in IR-CD patients (Spearman $r = -0.55$, $P = 0.02$, **Figure 3.1**). Over half of the IR-CD patients, 14 / 23 (60%) were suffering from symptoms of diarrhea. Five of these patients were taking regular BA

sequestrants and 4 others were unable to tolerate them. There was a trend towards lower FGF19 levels in those patients suffering from chronic watery diarrhea compared to those without ($P = 0.09$).

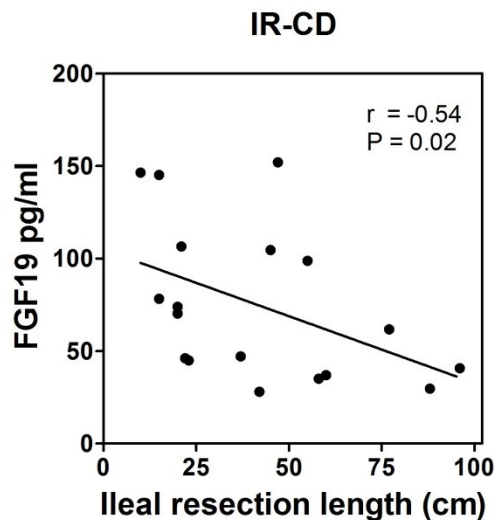


Figure 3.1. Significant inverse correlation between fasting FGF19 vs previous ileal resection length obtained from surgical records. Spearman's rank correlation coefficient (r) is shown. NR-CD = Non resected Crohn's disease.

3.4.3 FGF19 levels in Non-Resected CD patients

The median FGF19 levels in NR-CD (112 pg/ml) were 52% lower than in previously described healthy controls ($p < 0.0001$). Comparisons with previously described diarrhea controls were also highly significantly different. Median FGF19 levels were similar in healthy control patients and diarrhea control patients (231 and 237 pg/ml). Within the cohort of NR-CD patients, those with diarrhea had median FGF19 levels 41% lower than those without diarrhea (86 vs 145 pg/ml, $p = 0.035$, **Figure 3.2**). Median FGF19 levels were 35% lower in patients with NR-CD and active disease (as shown by the HBI) compared to those with inactive disease (103 vs 158 pg/ml, $P = 0.04$, **Figure 3.3**)

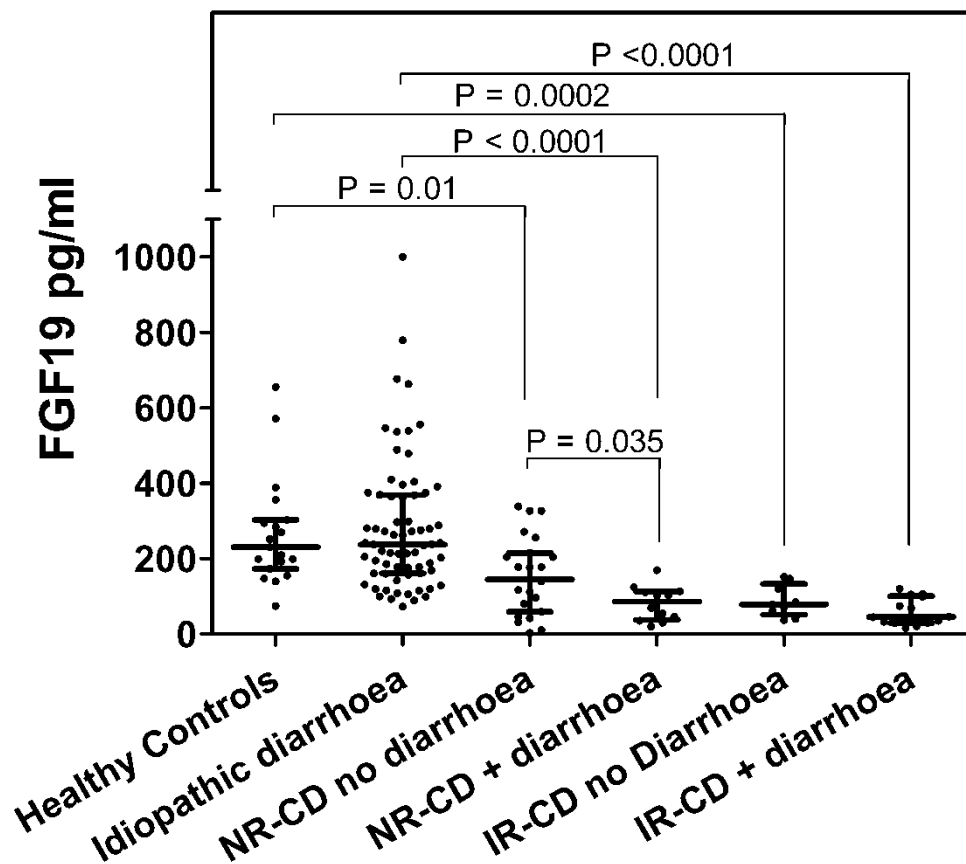


Figure 3.2. Fasting levels of FGF19 in groups of patients with and without diarrhea (stool frequency ≥ 3 , BSFS > 5). NR-CD = Non-resected Crohn's disease, IR-CD = Ileal resected Crohn's disease. Median and Inter Quartile Ranges are shown; Mann Whitney U test was used for comparisons.

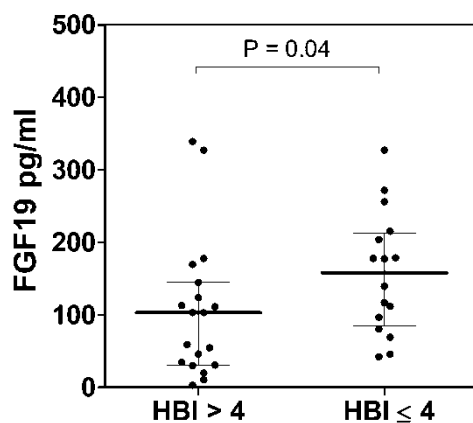


Figure 3.3. Fasting FGF19 levels are significantly lower in NR-CD patients with disease activity (HBI >4) than those in clinical remission (HBI ≤ 4). HBI = Harvey Bradshaw Index. Mann Whitney U test, Medians and Interquartile ranges shown.

Of the 19 NR-CD patients with active disease at the time of sampling, 11 of these patients were suffering from diarrhoea. Symptoms of diarrhoea were present in both colonic CD (5 /6 patients) and in those with ileal involvement (6/13 patients). In the patients with ileal involvement, median FGF19 levels were 71% lower in patients with diarrhoea than those without (40 vs 177 pg/ml respectively). The similar trend in the patients with colonic CD was non-significant (**Figure 3.4**). Patients with ileal involvement and diarrhoea had significantly lower median FGF19 levels than those with colonic CD and diarrhea ($P=0.004$). Significant inverse correlations between FGF19 levels and stool frequency, BSFS, CRP and stool index were found; these inverse correlations were strongest in those patients with ileitis (**Figure 3.5, table 3.2**).

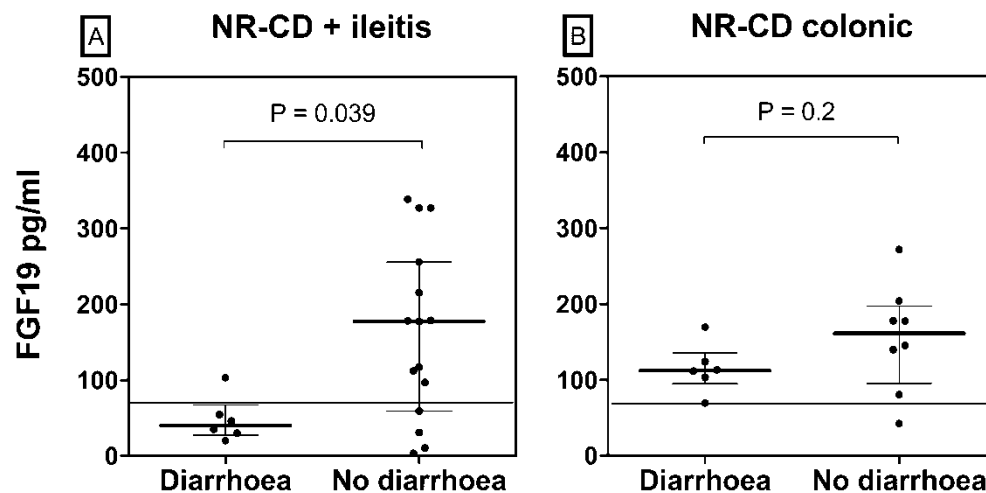


Figure 3.4 Fasting FGF19 levels in NR-CD (Non-resected CD) patients in those with (a) ileal involvement and (b) colonic involvement, respectively, according to the presence or absence of diarrhoea (Stool frequency ≥ 3 , BSFS > 5). Line represents an FGF19 level of 60pg/ml. Levels below 60pg/ml have been shown to be associated with severe BAM (C4 levels > 50) in a cohort of CD patients from another study.[57] Mann-Whitney U test, median levels and Interquartile ranges are shown.

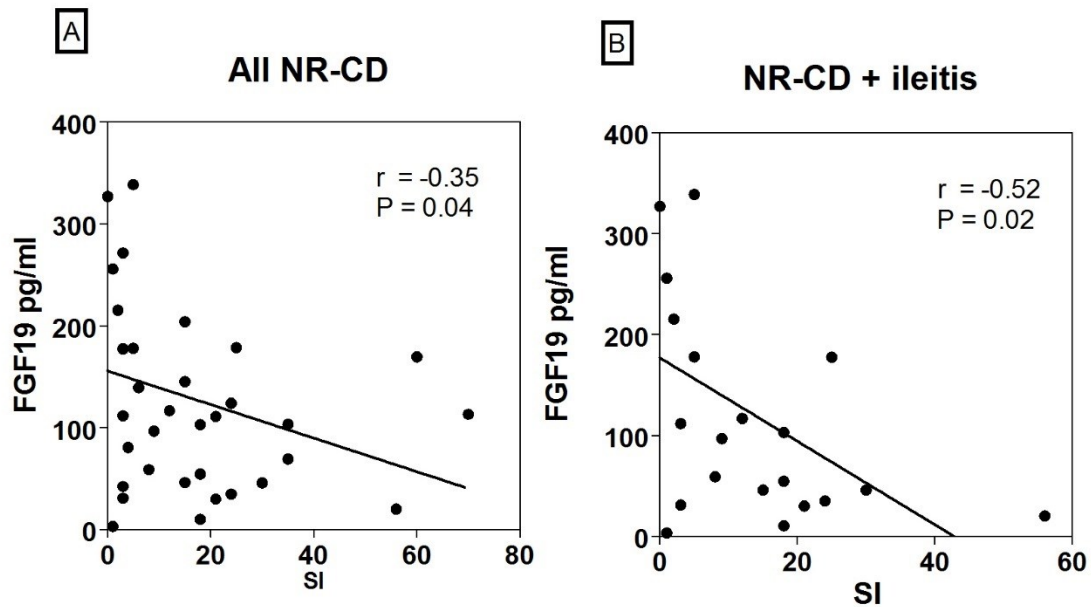


Figure 3.5. Significant inverse correlations between FGF19 levels and a calculated SI (stool index) in (A) all patients with NR-CD and (B) in those with ileitis (ileal or ileo-colonic disease). Spearman's rank correlation coefficient (r) shown and regression lines.

	Stool freq/ day	BSFS	SI	HBI	CRP (mg/dL)
NR-CD all (n=35)					
Spearman r	-0.34	-0.36	-0.35	-0.24	-0.37
P value	0.04	0.03	0.04	0.15	0.03
NR-CD ileitis (n=21)					
Spearman r	-0.49	-0.45	-0.52	-0.39	-0.58
P value	0.02	0.04	0.02	0.08	0.006
Diarrhea controls (n=68)					
Spearman r	0.07	0.04	0.24		
P value	0.56	0.80	0.07		

Table 3.2 FGF19 correlations with clinical indices. Spearman's rank correlation coefficients shown.

BSFS = Bristol Form Stool Index. SI = Stool index, HBI = Harvey Bradshaw Index

Using a cut-off value of 145pg/ml (previously shown to have a 80% sensitivity and 60% specificity as a marker of excess BA losses assessed by SeHCAT retention levels in PBAD[71]), levels below 145pg/ml were significantly commoner in those with symptoms of diarrhea in all 35 NR-CD patients studied ($P=0.028$, Fisher's exact test). Using a lower cut-off value of 60pg/ml (previously shown to have a 80% sensitivity and 68% specificity as a marker of more severe BA losses assessed by C4 levels in IBD[57]) revealed that levels below 60pg/ml were significantly associated with symptoms of diarrhea in the 21 NR-CD patients with ileitis ($P=0.046$, Fisher's exact test).

3.4.4 Serial FGF19 levels during the day

Repeated FGF19 sampling pre and post meals were compared between patients with CD and diarrhea control patients. Serial FGF19 levels remained low with no evidence of diurnal variation pre and post meals in the 3 CD patients compared to the 4 diarrhea control patients (**figure 3.6**).

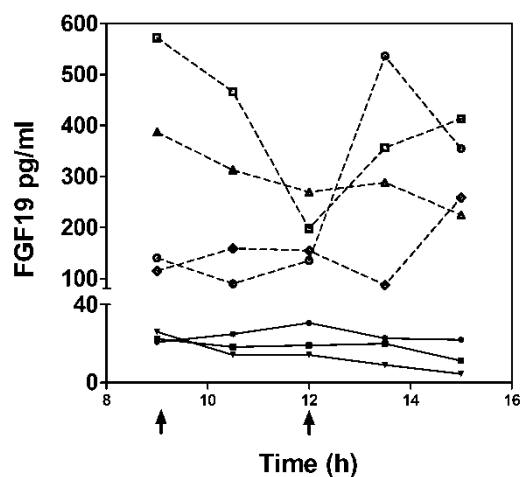


Figure 3.6. Diurnal variation in FGF19 levels in CD patients vs non inflammatory diarrhea control patients. Closed shapes and solid lines = 3 patients with CD and diarrhea (one with ileitis and the other 2 with previous ileal resections). Open shapes and dotted lines = 4 idiopathic diarrhea control patients (without evidence of Primary BA Diarrhea, SeHCAT retention >10%). Arrows indicate standardized meals (9am and 12 midday).

3.4.5 Serial FGF19 in NR-CD patients on treatment

In the longitudinal studies, initial serum FGF19 levels were measured in patients in clinical remission on different days to look for intra-individual variability. There was no significant difference between the paired levels ($n = 6$, Wilcoxon $P = 0.68$). Median FGF19 levels were significantly lower in the patients with ileitis while their disease was active (median 40 pg/ml, range 3 – 92) compared to paired levels taken after medically induced clinical remission and resolution of diarrhea (median 140 pg/ml, range 106 – 184, $P=0.03$, paired Wilcoxon). Median FGF19 levels were not statistically different in the 5 patients with colonic CD during active disease (median 113 pg/ml, range 50 – 120) compared to paired levels taken during medically induced clinical remission and resolution of diarrhea (median 70 pg/ml, range 40 - 120, $p=0.5$ Wilcoxon, **Figure 3.7**). The median time interval between samples taken during disease activity and medically induced remission was not significantly different between these groups (median interval: colonic 98 days, range 28 – 168 vs ileitis 83 days range 40 -159, $p = 0.66$ Mann Whitney U test).

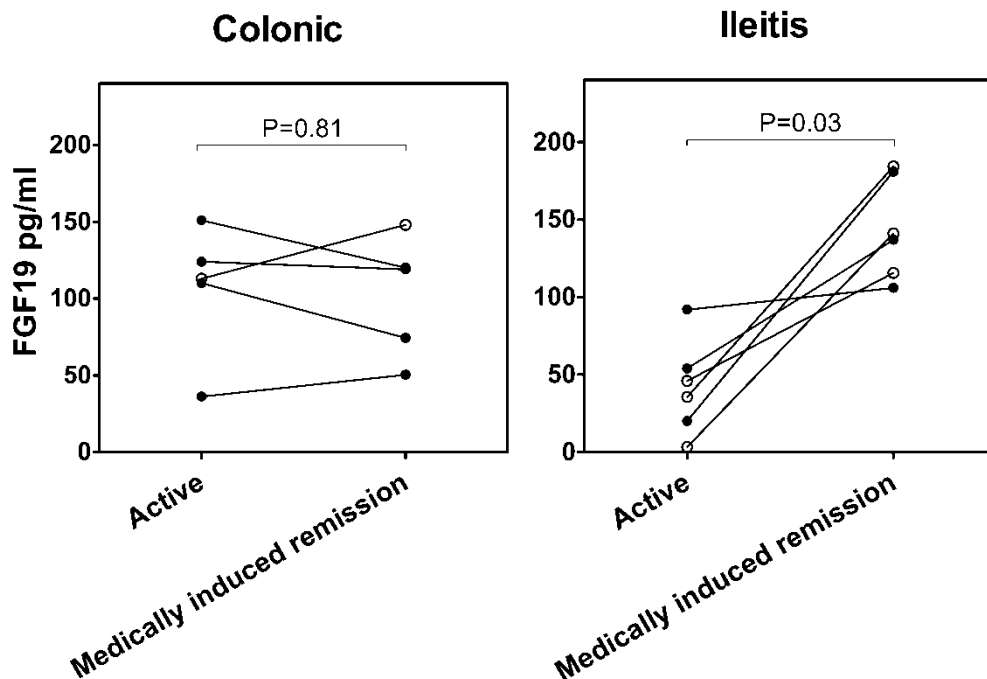


Figure 3.7. FGF19 levels during a period of disease activity followed by a repeat sample during clinical remission after anti-inflammatory treatments used to induce remission (biologics or corticosteroids).

Two out of five patients with colonic CD were treated with corticosteroids and five out of six patients with ileitis were treated with corticosteroids. The remaining patients were treated with biologics (infliximab or adalimumab) only. The open circles represent patients that were still receiving corticosteroids when the repeat FGF19 sample was taken (Paired values were compared using the Wilcoxon signed rank test).

3.5 Discussion

Overall, significantly reduced median FGF19 levels were associated with active disease and more specifically with symptoms of diarrhea in NR-CD. Median FGF19 levels were found to be significantly

lower in those patients with ileitis and diarrhea than those with disease restricted to the colon and diarrhea. In addition to this, FGF19 levels increased in NR-CD patients who received anti-inflammatory treatments leading to clinical remission and resolution of diarrhea in those patients with ileitis but not colonic CD. As excess fecal BA are more likely to contribute to the diarrhea of patients with ileitis than those with pure colonic disease, these differences support the role of FGF19 as a marker of both BAD and ileitis in NR-CD.

The association between ileal inflammation and markers of BA malabsorption have previously been demonstrated in NR-CD patients using older markers of BA loss techniques.[41] Similar to our findings with FGF19, inverse relationships between symptoms of disease activity and SeHCA retention values have also been demonstrated previously in NR-CD.[41, 42]

A significant inverse correlation was found between fasting FGF19 and length of terminal ileal resection despite the possible confounding variables that may affect this (such as total small bowel length, type of anastomosis, and gut microbial metabolism of bile acids). We also observed a complete loss of normal post prandial diurnal variation in FGF19 levels throughout the day in the patients with ileal resection or inflammation. The pattern is similar to those observed in healthy controls that remain fasted throughout the day.[22] These findings suggest that the terminal ileum is critical to the basal production of serum FGF19.

The higher FGF19 levels post treatment in patients with ileitis may also have been influenced by the use of corticosteroids. Budesonide treatment has been shown to increase the expression of ASBT protein in the terminal ileum of healthy volunteers.[46] In vitro reporter assays have revealed a Glucocorticoid Receptor (GR) response element within the promoter region of ASBT and various animal models have demonstrated an increase in ASBT expression and function after corticosteroid

treatments.[46, 112] Hence, the higher post treatment FGF19 levels seen in some patients in this study may have been influenced by corticosteroid induced increases in ASBT expression and BA absorption.

It is important to note that FGF19 synthesis occurs after absorbed BAs bind and activate the intracellular BA receptor Farnesoid X Receptor (FXR).[113] The most FXR responsive BA regulatory gene in human ileum is FGF19. [109] However, FXR activation may also enhance anti-inflammatory genes and down regulate pro-inflammatory genes. [56] [86] Obeticholic acid, a potent, semisynthetic FXR agonist has demonstrated anti-inflammatory and anti-fibrotic properties within the liver,[114, 115] [87] and possibly within the intestine. [116, 117] Perhaps the most relevant application in human IBD will be in ileal CD, as this phenotype is most likely to be associated with a deficit in endogenous intestinal FXR activation.[54] Hence reduced fasting circulating FGF19 levels may also serve as a biomarker of impaired FXR activation.

FXR agonists may therefore have dual functional effects in ileal CD. Stimulating FGF19 may have beneficial symptomatic effects by the inhibitory actions on BA synthesis reducing excess colonic BAs. The FXR agonist Obeticholic acid has shown positive effects on diarrhea symptoms in an open label phase II study of primary and secondary BAD patients.[96] Additional positive effects on ileal FXR mediated barrier function may also result and requires further study.

It has already been established that the majority of patients with chronic diarrhea and previous ileal resection are likely to be suffering from BAD and will have symptomatic benefit following treatment BA sequestrant therapy.[36] However, using reduced FGF19 as a marker of BAD in this study has revealed that the majority of NR-CD patients with ileitis, disease activity and symptoms of diarrhea are also likely to gain symptomatic benefit from treatments for BAD. FGF19 levels may have utility in

the work up of a NR-CD patient suffering from an acute flare of disease activity and diarrhea.

Diarrhea associated with higher levels of FGF19 is more likely to be associated with colonic inflammation and less likely to respond to such treatments. Levels less than 60pg/ml are likely to be associated with severe BAM, ileal inflammation and BAD. These patients are likely to benefit from BA sequestrants, budesonide or potential future treatments to restore FGF19 levels and reduce excess colonic BA such as FXR agonists.

Chapter 4: Basal expression of FGF19 and other BA regulated genes in human ileum

Abstract

Background: Animal models of FGF15/19 deficiency develop a phenotype similar to PBAD characterized by increased BA synthesis, BA pool size and excess faecal BAs. Primary bile acid diarrhoea (PBAD) is a chronic diarrhoeal disease characterized by excess faecal BA loss and reduced SeHCAT 7d retention (<15%). The condition has previously been shown to be associated with an increased BA pool size and increased BA synthesis. PBAD could result from FGF19 deficiency or ileal BA malabsorption. We set out to investigate the normal and PBAD related basal expression of FGF19 alongside other BA regulated genes (ASBT, IBABP, OSTa/b, SHP, FXR) in human ileum.

Aim: To determine ileal and colonic transcript expression of FGF19 and genes related to BA absorption in healthy controls, CD and patients with chronic diarrhoea and known SeHCAT values.

Methods: 7 healthy controls, 6 CD patients and 15 patients with undiagnosed chronic watery diarrhea (stool >3/d, Bristol stool form scale >5, duration >3 month) who had undergone SeHCAT tests were recruited and ileal or colonic biopsies were obtained at routine ileo-colonoscopy after standard bowel preparation and > 6h fasting. Biopsies were stored in RNAlater. After RNA extraction and cDNA synthesis, transcript levels for FGF19 and GAPDH were measured in all samples and ASBT, IBABP, OST α , OST β , SHP, FXR and GAPDH, in the chronic diarrhoea cohort were determined by qRT-PCR. Data were analysed by Mann-Whitney U and Spearman Rank correlation tests.

Results: FGF19 transcripts were detected in all ileal biopsies in all patients studied. FGF19 was not detected in any of the colonic biopsies. From the chronic undiagnosed diarrhoea cohort, 8 patients had SeHCAT values <15% (range 3-13%), diagnostic of PBAD, and 7 had values >15% ("diarrhea-controls"). Two patients (SeHCAT 5% and 13%) were excluded from further analysis as ASBT, SHP and FGF19 values were below detection limits, suggesting a non-ileal source. No significant

differences in median gene expression were detected between the groups. Overall, SeHCAAT values were significantly correlated with FGF19 ($r=0.59$, $p=0.027$) and with ASBT ($r=0.49$, $p=0.043$) but with no other transcripts. FGF19 and ASBT were strongly correlated ($r=0.88$, $p=0.0002$), as was IBABP with OST α ($r=0.87$, $p<0.0001$) and with ASBT ($r=0.63$, $p=0.01$). FXR and SHP were associated with many genes (FGF19, ASBT, IBABP, OST α and each other) but OST β did not correlate with any other transcript. All associations were stronger in the diarrhoea-controls. In the PBAD group, the associations with SeHCAAT were absent, and, although the correlation of FGF19 and ASBT remained strong, those between FXR and OST α or IBABP were lost.

Conclusions: FGF19 is expressed in human ileum but not colon. In patients with undiagnosed chronic diarrhoea, SeHCAAT values are related to fasting levels of transcripts for both ASBT and FGF19. Although expression of many BA-associated genes are closely related, these associations are less strong in PBAD, implying impaired basal regulation. Their response to BA absorption may also differ.

4.1 Background

FGF15 mRNA has been detected in relative abundance in mouse ileum but not in other tissues using RT-PCR [13]. In-situ hybridization techniques have revealed that transcripts are largely confined to the epithelium [7]. The localised ileal expression is a feature unique to FGF15 compared to other distal gut peptides such as PYY and GLP-1 which tend to be more widely expressed throughout ileum and colon.

We aimed to study FGF19 transcriptional expression within human intestine. As transcripts have been demonstrated in mouse intestinal epithelium we would expect mucosal biopsies obtained at ileo-colonoscopy to be sufficient to detect human expression. Balesaria et al had previously confirmed the basal expression of FGF19 in human ileum and duodenum and demonstrated that the expression is higher in the ileum than duodenum [118]. However FGF19 expression was detected using FGF19 taqman probes and reagents. We aimed to use SYBR green technology and this would require designing new primers as at the time of starting this study there were no published FGF19 primers for use in SYBRgreen assays.

The previous study had also investigated BA regulated gene expression in patients with chronic diarrhoea. However only a minority of these patients had BA losses quantified with SeHCAT scanning. We aimed to investigate the relationship between BA regulated gene expression levels to SeHCAT retention levels.

SeHCAT, selenium homo tauro cholate, is a radiolabelled BA that becomes incorporated into the enteroheptic circulation after oral administration and has BA kinetics similar to cholic acid. SeHCAT remains in the enterohepatic circulation and is excreted exclusively in the faeces (see chapter 3).

Patients with PBAD (seHCAT retention levels <15%) have diarrhoea due to the secretory effects of increased faecal BA concentrations within the colon. SeHCAT retention levels have been shown to be inversely related to faecal BA concentrations

SeHCAT retention levels are dramatically reduced in patients with secondary BAD, such as in CD, due to terminal ileal resection or inflammation reflecting increased faecal BA losses in these patients. It is usually inferred from such studies that SeHCAT retention is largely determined by the re-absorption of BA at the terminal ileum.

However patients with PBAD have an intact terminal ileum and the active uptake of BA into terminal ileal mucosa has been found to be normal or increased. Reduced SeHCAT retention may also arise as a result of increase in BA synthesis and pool size or enterohepatic recycling frequency. Defective production of FGF19, the negative feedback signal to BA synthesis may produce these effects as reflected in various animal models.

The initial aim of this study was to establish an efficient protocol to examine human intestinal FGF19 transcript expression. This would require successful extraction of enough RNA from small bowel endoscopically obtained mucosal biopsies. Then to develop a primer for FGF19 using RT-PCR and SYBR green chemistries (which had not been done prior to this work) to enable detection of transcripts from these samples. SYBR green chemistries are more cost effective than Taqman based primers which have been used to detect FGF19 in human intestine previously [118]. It was important to establish the SYBR green technique as we aimed to assess multiple samples between patients groups.

4.2 Aims:

- (1) To successfully extract RNA from endoscopic mucosal biopsies to detect FGF19 transcripts.
- (2) Establish if FGF19 transcripts are detectable within human ileal mucosal biopsies using RT-PCR and SYBRgreen chemistries.
- (3) Establish whether FGF19 transcripts basally expressed in human colonic mucosal biopsies?
- (4) To perform exploratory studies on basal expression of FGF19 and other BA regulated gene transcripts in ileal mucosal biopsies from patients with SeHCAT diagnosed PBAD and CD.

4.3 Specific methods:

4.3.1 Prospective study of ileal and colonic FGF19 expression

In total, 27 subjects were recruited prior to undergoing routine ileo-colonoscopy. 13 of these patients were recruited prospectively and gave informed, written consent. All subjects had fasted overnight in preparation for colonoscopy and had received bowel preparation, usually with oral Senna and magnesium citrate. Exclusion criteria were as follows: patients under 18 yr old, patients on anticoagulant or dual-antiplatelet therapy. Intestinal mucosal biopsies were taken with the same type of disposable biopsy forceps (radial jaw 3; Boston Scientific, Hemel Hempstead, UK), giving standardized mucosal samples. The study was approved by the local Institutional Review Board, the Hammersmith, Queen Charlotte's, and Chelsea Hospitals' Research Ethics Committee.

For this part of the study, groups of 3 mucosal biopsies were taken from the following anatomical regions; terminal ileum, proximal and distal colon. From each region the 3 mucosal biopsies were pooled together in 400ul of RNAlater in separately labelled sterile RNase free, 1.5ml microcentrifuge tubes. Distal colonic biopsies were taken from the sigmoid colon just above the rectosigmoid junction. For each patient, separately labelled ileal, proximal and distal colonic biopsies were then

transferred to a -80 freezer for storage and subsequent processing for the analysis of baseline gene expression. Intestinal

4.3.2 Analysis of previously recruited patients with chronic diarrhoea + PBAD

15 of these 27 patients had been previously recruited and samples taken prior to this PhD and were in storage at -80. These ileal mucosal biopsy samples came from patients who have been previously described in studies of serum FGF19 levels in PBAD [71]. These patients had been investigated for chronic diarrhoea (an average of 3 loose, type 6 or 7 stools per day for greater than 3 months) with SeHCAT scanning and serum samples obtained for fasting FGF19. Ileal biopsies (n=3-6 biopsies) had been taken at colonoscopy and pooled together in 400ul of RNeasy lysis buffer in individually labelled RNase free 1.5ml microcentrifuge tubes. The samples had been stored at -80 for 2 years prior to the processing described below.

4.3.3 Sample processing and RT-PCR

RNA extraction and cDNA synthesis was performed according to the manufacturer's protocols and as described in **chapter 2.4 – 2.5**. Primers were designed for FGF19 (**Chapter 2.6**) and otherwise included previously published human primer SYBR green sequences for the other genes (GAPDH, ASBT, OSTa+b, IBABP, FXR, SHP). RT-PCR was then performed on the cDNA using specific primers at 400nM in singleplex reactions using SYBR green chemistry according to the manufacturer's protocol (**chapter 2.6.2**).

To assess the relative efficiencies of the target amplification and the reference (endogenous control) amplification, standard curves were run for each amplicon utilizing the same sample. We aimed to cover 5 logs of input cDNA amount to assess the dynamic range of the assay for the FGF19 and GAPDH primers. We therefore performed serial 3 fold serial dilutions of the sample template.

4.3.4 Using RT-PCR relative quantification to compare basal expression levels of FGF19 between patient groups:

To explore for possible difference in FGF19 expression in ileum from patients with CD and PBAD we used RT-qPCR with relative quantification methods. This method quantifies target gene transcript levels relative to a control / reference gene. Uniformity of expression levels in the control gene, GAPDH across the samples or patients is assumed using this method.

Using RT-qPCR 40 thermal cycles are run to achieve standard cycles of denaturing, annealing and extension. The sample cDNA is amplified using a solution containing DNA polymerase, nucleotides, SYBR green probes and primers that are complementary to the target DNA sequence. When this solution is heated the taq polymerase (DNA polymerase) is activated. As the solution cools, the primers anneal to the target sequences on the first strand cDNA. The DNA polymerase then forms a new strand by extending the primers with nucleotides, creating a complimentary copy of the target cDNA sequence and dsDNA. Repeated thermal cycling leads to denaturation of dsDNA, annealing and extension. This leads to an exponential increase in the number of target DNA sequences referred to as amplicons. During this cycling, SYBR green probes bind to the minor groove of the dsDNA. Binding to the minor groove of dsDNA increases the SYBR green fluorescence. The TR-qPCR 7900 ABS system detects this increasing fluorescence representing increasing PCR product.

Cycle thresholds (Cts) are calculated as the point at which the fluorescence for a sample becomes significantly greater than the background fluorescence. A qPCR curve has typically an exponential phase followed by a plateau phase. The Ct measure needs to be taken in the exponential phase, where the curve is linear. The horizontal blue line shows the cycle threshold, which can be automatically or manually set to any value that is: (1) above background fluorescence, (2) below plateau regions, (3) within the geometric phase of amplification. Cycle time (Ct) values can then be determined by reading off the cycle threshold, as shown by the dotted black lines (**figure 4.1**).

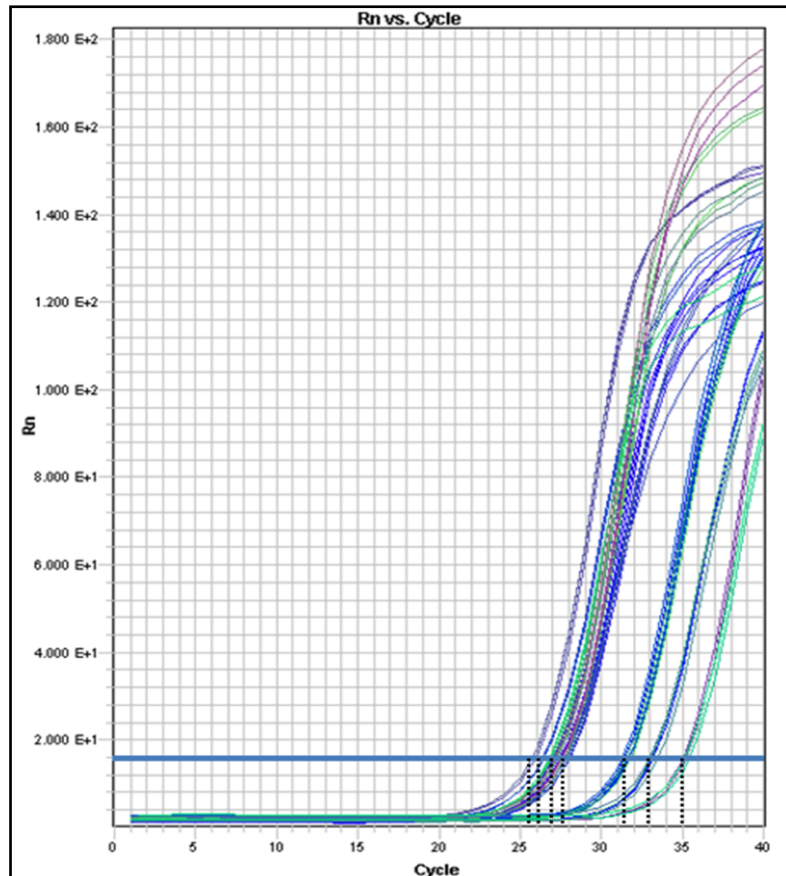


Figure 4.1 PCR amplification curves with Ct's indicated by dashed vertical lines.

The later the PCR cycle threshold is reached (ie the higher the Ct, 30-35) the less the amount of starting template within the sample as you need more cycles of amplification for the fluorescence to be detected. If the Ct has a small value (10-15), the gene is highly expressed. The Ct is therefore inversely proportional to the quantity of starting template.

FGF19 transcript levels are therefore expressed relative to GAPDH as ΔCt values which is calculated as:

Ct target gene – Ct endogenous control

Target gene Ct's values were generally higher than control gene Ct's. The mean Ct for FGF19 was 29.9 and 24 for GAPDH. This means that FGF19 amplification occurs 6 cycles after GAPDH. This means

that there is a greater relative abundance of GAPDH than FGF19. Due to the exponential nature of PCR this difference represents a mean of 64 times (2^6) the abundance of GAPDH template in these samples than FGF19 template.

This equation leads to ΔCt values that are inversely related to expression levels of target gene transcripts. High ΔCt levels would reflect low relative FGF19 transcript levels. To convert the ΔCt to a linear form the equation $2^{-(\Delta Ct)}$ is used and expressed as arbitrary units (AU) for comparisons of basal FGF19 expression levels between patients. These arbitrary units are then directly proportionate to expression levels, with higher values (AU) reflecting a greater abundance of FGF19 transcript levels.

4.4 Results:

4.4.1 RNA concentrations and purity

RNA concentrations and purity were within acceptable limits following RNA extraction (**table 1**). The total RNA preparations were diluted to obtain a starting amount of 1ug for each sample. This is well within the range of starting RNA amount required for the reverse transcriptase (SuperScript™ First-Strand Synthesis System for RT-PCR, Cat. No: 11904-018) according to the manufacturer's protocol (range - between 1ng and 5ug total RNA).

Minus reverse transcriptase controls after cDNA synthesis always revealed flat amplification plots after RT-PCR consistent with the fact that the FGF19 primer crosses 2 exons. The GAPDH Minus reverse transcriptase controls did reveal some amplification with Cts of 33 or later which was always > 10 cycles later than the respective RT +ve sample. This implied approximately 0.1% of the amplification in the RT+ve samples was attributable to genomic DNA. This was considered an acceptably low level of genomic DNA.

PCR assays were examined and results were excluded according to the criteria described in Chapter 3. All PCR assays revealed a specific product for each primer pair (**figure 4.2**). Interassay variability between multiple 96 well plates was considered acceptable if there was no more than 1 cycle difference between Cts from 2 separate PCR plates using a pooled positive control sample. Analysis of multiple genes on the samples from the 15 previously recruited patients was performed on 2 x 384 well PCR plates with 2 positive controls on each plate to minimise interassay variability.

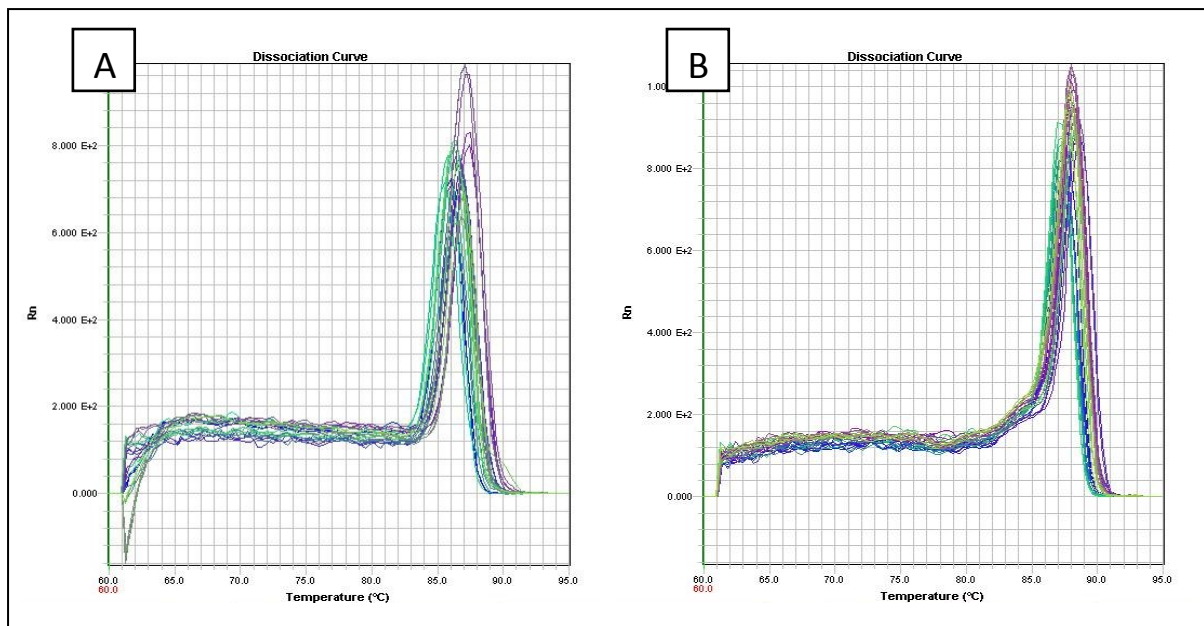


Figure 4.2 Dissociation-curves show that both the FGF19 primer pair **(A)** and GAPDH primer pair **(B)** dissociate with only one amplification peak, indicating a pure product.

4.4.2 Validation experiments and primer efficiencies

For the comparative Ct method to be valid the efficiencies of the target amplification and endogenous control needed to be similar. A seven-fold serial dilution of a positive control sample was performed in singleplex triplicate reactions for target gene and endogenous control gene. The $\text{Log}_{10}(\text{template concentration})$ was plotted against the Ct. The gradient of the resulting trend line represents a function of the amplification efficiency. A gradient (S) of -3.32 is consistent with an efficiency of 100%. Amplification efficiency for each primer was then calculated from the slope with the equation: $\text{Efficiency} = 10^{(1/-S)}$. Primer efficiencies shown in **figure 4.3**.

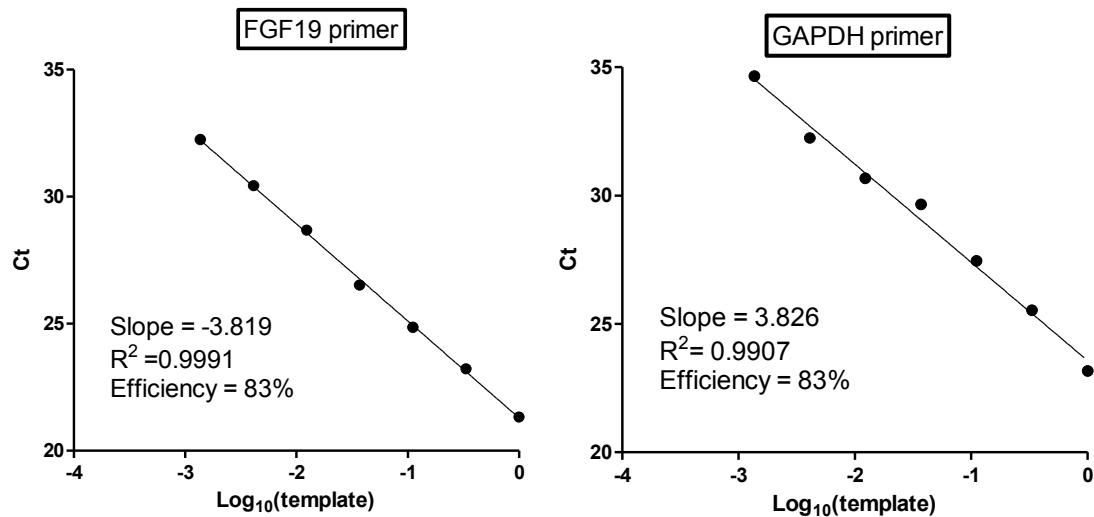


Figure 4.3. Analysis of primer efficiencies. Initial input amount of RNA for cDNA synthesis is 1µg; this initial amount is then serially diluted using 3-fold dilutions. RT-PCR is performed to give Ct values for the serially diluted samples. Primer efficiencies are calculated using the following equation: $\text{Efficiency} = 10^{(-1/\text{gradient})} - 1$, where the gradient is calculated from plotting Ct against log₁₀ (input amount of RNA). Efficiencies are 83% for both FGF19 primer pairs **(A)** and GAPDH primer pairs **(B)**. R² values are > 0.99 in both graphs, indicating that the estimated values determined by the trendline correspond extremely closely with the experimental values.

The variation of ΔCt ($\text{Ct}_{\text{target}} - \text{Ct}_{\text{endogenous control}}$) according to template dilution was plotted against Log₁₀(template concentration). The absolute value of the slope of the ΔCt vs Log₁₀(template concentration) was 0.007. Absolute slopes of the ΔCt vs Log₁₀(template concentration) are considered to have passed a validation experiment if the slope is <0.1. The primer pairs achieved this standard (**figure 4.4**).

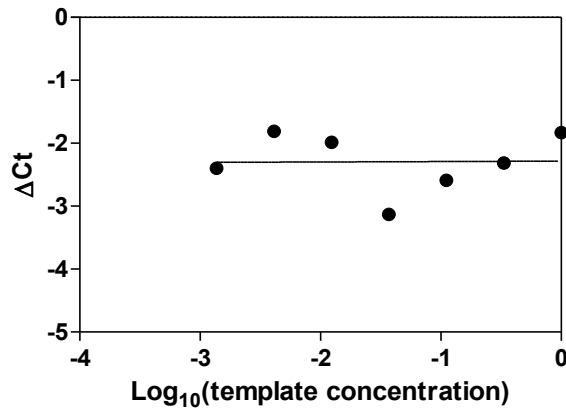


Figure 4.4. Validation of primers for the comparative Ct method. Initial input amount of RNA for cDNA synthesis is 1μg; this initial amount is then serially diluted using 3-fold dilutions. A gradient of <0.1 on a semi-log graph of ΔCt (Ct_{FGF19} minus Ct_{GAPDH}) against $\log_{10}$ of the input amount of RNA indicates matching primer amplification efficiencies.

4.4.3 FGF19 mRNA is basally expressed in human ileal but not in colonic biopsies obtained at colonoscopy

We first set out to compare basal FGF19 transcript levels between terminal ileal and colonic mucosa (including proximal and distal colon). Healthy control patients (n=7) and 2 patients with NR-CD disease in clinical remission were prospectively recruited whilst attending for colonoscopy (see table 2.1 and chapter 2.3a(ii) respectively). 8 patients underwent groups of 2-3 biopsies from ileum and proximal colon, a further patient underwent colonic biopsies only (**table 4.1**).

N=9	Healthy controls (n=7)	NR-CD (n=4)	Previous IR (n=2)
Age	36 (26-46)	26 (23-39)	40 (27-73)
Male	6	2	5
Female	3	2	4
Indications for colonoscopy			
Change in bowel habit (no diarrhoeal)	6	0	4
Assessment of Crohn's disease	1	4	2
PR bleed	1	0	1
Anaemia	0	0	1
Polyp surveillance	1	0	1
Drug History			
No medication			2
Regular medication	7		6
Lactulose	1		1
Aspirin	1		
Immunomodulator	0	4	0
Biologic	0	2	0
Ileal resection length (cm)			51 , 15

Table 4.1 Patient demographics for basal expression studies of FGF19 between ileum and colonic samples. NR-CD, Non resected Crohn's disease; IR, ileal resection.

After, RNA extraction, cDNA synthesis and RT-qPCR, FGF19 transcript levels were either completely undetectable or beyond the limits of reliable quantification (Ct>35 cycles) in the groups of colonic biopsies from each patient. This finding was independent of colonic region sampled including proximal or distal colon (**table 4.2**). The mean Ct for GAPDH was 24.1 with a standard error of the mean of 0.14 suggesting low variance in the expression levels of the control gene, and there was no significant difference between colonic and ileal biopsies.

Patient	Biopsy site	GAPDH mean Ct	FGF19 Mean Ct	(RNA) ng/ul	Purity 260/280
Pt A	Ileum	24.48	29.14	432.52	2.04
Pt B	Ileum	23.53	29.15	300.32	2.07
	Prox colon	24.25	> 40	1057.81	2.07
Pt C	Ileum	23.13	25.43	390.87	2.04
	Dist colon	23.45	36.14	395.32	2.05
Pt D	Ileum	24.39	29.22	189.59	2.09
	Prox colon	23.82	36.86	249.37	2.08
Pt E	Ileum	24.15	32.74	279.52	2.08
	Prox colon	25.21	> 40	848.48	2.06
	Dist colon	24.13	36.87	415.16	2.04
Pt F	Ileum	24.59	32.54	655.71	2.10
	Prox colon	22.96	> 40	681.67	2.10
	Dist colon	23.06	37.25	618.19	2.09
Pt G	Ileum	24.54	30.64	394.48	2.06
	Prox colon	23.54	> 40	939.50	2.08
	Dist colon	24.13	> 40	330.75	2.05
Pt H	Prox colon	24.12	> 40	946.11	2.05
	Dist colon	23.57	38.15	743.15	2.04
Pt I	Ileum	24.91	30.62	591.55	2.07
	Prox colon	24.72	> 40	750.86	2.05
	Dist colon	24.81	> 40	738.73	2.07

Table 4.2. RNA concentrations and Cycle thresholds results for according to different regions sample during colonoscopy. RNA concentrations were adequate for subsequent cDNA synthesis. The higher the Ct, the later the amplification, so the lower the gene expression. Dark blue boxes represent samples that had no amplification after 40 PCR cycles. Light blue boxes highlight samples which amplified at the limits of reliable quantification (>35 cycles). All samples expressed FGF19 at <0.004 times the expression seen in the ileum of the same patient.

FGF19 transcripts were reliably detected in all samples from ileum. Cts for FGF19 were higher than GAPDH with a mean of 29.9 (reflecting a lower relative abundance of FGF19 transcripts relative to GAPDH). The standard error of the mean for FGF19 Δ Ct values was 1.14 again suggesting that the ileal samples from different patients had a low variability of up to twofold difference in basal FGF19 expression.

4.4.4 Ileal transcript expression levels of FGF19 and ASBT are related to SeHCAT retention levels in a cohort of patients with chronic diarrhoea

Expression of FGF19 and other relevant transcripts was studied in ileal biopsies from the cohort of 15 patients (see 2.3(a)) with chronic diarrhoea who also underwent SeHCAT testing. Patient demographics are shown in **table 4.3**. Eight patients had PBAD with SeHCAT values <15% (3 to 13%) and 7 were ID controls (values >15%). Two patients (SeHCAT 5% and 13%) were excluded from further analysis as FGF19 and ASBT transcript values were outside the limits of reliable quantification with no amplification in one sample and a Ct of 36 in another. This could have been due to a non ileal source of biopsy.

	Age		PMH	DHx	bmi	FGF19	seHCAT
BD72	67	f	Hypothyroid	Enalapril, thyroxine	29	263	90
BD13	49	m			25	489	59
BD84	44	m		Statin	32	281	48
BD21	51	f			22	178	39
EX45	38	m					
BD10	56	f			29	288	23
BD87	41	m	Depression	Quetiapine, diazepam, loperamide	24	368	19
BD115	72	f	Cholecystectomy, osteoporosis	allendronate	34	206	13
BD46	57	f	Thyroidectomy	Thyroxine, enalapril, lipitor	26	227	12
BD52	48	f	Hypothyroid, Chol	Thyroxine, simvastatin		50	9
BD25	38	f			22	146	7
BD06	38	f			32	56	5
BD37	29	f			28	68	5
BD51	48	m		aspirin	23	349	4
BD75	55	m			26	129	3

Table 4.3. Patient demographics from a cohort of patients previously recruited and described elsewhere [71]. Bmi, body mass index; PMH, past medical history; DHx, drug history; seHCAT, tauro-selenium cholic [75 selenium] acid retention level at 7 days (% retention shown).

A further 2 samples were also excluded from analysis of FGF19 expression as the ΔCt SD values for FGF19 obtained were >0.3 . On these 2 samples small peaks were also seen on the dissociation curve analysis for FGF19 at 80°C rather than the usual 85°C which implied possible primer dimer formation. Pipetting error may also have affected these results. For these 2 samples however, ΔCt SD and dissociation curves were adequate for all other genes analysed leaving 13 patients for further gene analysis.

Values obtained for GAPDH had low variability across this cohort of patients. RNA concentrations and purity were adequate. Even in this small cohort of patients the median serum FGF19 levels were significantly lower in the PBAD patients compared to the ID controls (117, IQR 55- 197 vs 368, IQR 285-537, $p=0.02$). No significant differences in median target gene expression levels were detected between the two groups.

There were no significant correlations between serum FGF19 levels and transcript expression levels or SeHCAAT levels. However, SeHCAAT values were significantly associated with FGF19 ($r_s = 0.59$, $p = 0.03$, $n = 11$) and with ASBT ($r_s = 0.49$, $p = 0.04$, $n = 13$) but not with the other transcripts (**table 4.4**). Expression of FGF19 and ASBT were strongly correlated ($r_s = 0.88$, $p = 0.0002$). Additionally IBABP correlated with OST α ($r_s = 0.87$, $p < 0.0001$) and IBABP with ASBT ($r_s = 0.63$, $p = 0.01$). FXR and SHP expression were associated with many genes (FGF19, ASBT, IBABP, OST α and each other) but OST β did not correlate with any other transcript.

		All Subjects							
		Serum FGF19	SeHCAT	FGF19	ASBT	IBABP	OST α	OST β	SHP
SeHCAT	r_s	0.56							
	p	0.08							
FGF19	r_s	-0.15	0.59						
	p	0.67	0.03						
ASBT	r_s	-0.05	0.49	0.88					
	p	0.88	0.04	<0.001					
IBABP	r_s	0.20	0.35	0.41	0.63				
	p	0.53	0.12	0.11	0.01				
OST α	r_s	0.42	0.24	0.32	0.37	0.87			
	p	0.17	0.22	0.17	0.10	<0.001			
OST β	r_s	-0.05	-0.08	0.18	0.08	-0.11	-0.02		
	p	0.87	0.40	0.30	0.40	0.36	0.47		
SHP	r_s	-0.14	-0.04	0.61	0.53	0.55	0.51	-0.05	
	p	0.64	0.44	0.02	0.03	0.02	0.04	0.44	
FXR	r_s	-0.06	0.31	0.67	0.79	0.61	0.43	-0.18	0.54
	p	0.84	0.15	0.01	<0.001	0.01	0.07	0.28	0.03

Table 4.4 Correlations between basal expression levels of ileal transcript and SeHCAT levels / serum FGF19 in patients with chronic diarrhoea assessed by SeHCAT retention. Spearman rank correlation coefficients (r_s) and p values are shown. Significant p values (<0.05) are shown in bold with significant correlation coefficients in bold red.

4.4.5 Reduced coexpression of ileal transcript levels in BA regulated genes in PBAD

Associations between ileal transcript expression levels were stronger in the ID controls. In the PBAD group, the positive correlations between FGF19 and ASBT with SeHCAT were absent. Although the correlation of FGF19 and ASBT remained strong, those between FXR and OST α or IBABP were lost (table 4.5 and 4.6).

		PBAD n = 6							
		SeHCAT	FGF19	ASBT	IBABP	OSTa	OSTb	SHP	FXR
FGF19	r_s	-0.20							
	p	0.35							
ASBT	r_s	-0.14	0.94						
	p	0.38	0.00						
IBABP	r_s	0.07	0.26	0.18					
	p	0.44	0.31	0.35					
OSTa	r_s	0.14	-0.09	-0.21	0.75				
	p	0.38	0.44	0.32	0.03				
OST b	r_s	-0.04	0.77	0.50	0.36	0.32			
	p	0.47	0.04	0.13	0.22	0.24			
SHP	r_s	0.18	0.60	0.71	0.61	0.14	0.43		
	p	0.35	0.10	0.04	0.07	0.38	0.17		
FXR	r_s	0.07	0.43	0.61	0.00	-0.21	-0.29	0.39	
	p	0.44	0.20	0.07	0.50	0.32	0.27	0.19	

Table 4.5 Correlation of basal levels of ileal transcript expression in BA regulated genes in patients with PBAD, primary bile acid diarrhoea (SeHCAT >15%). Spearman rank correlation coefficients (r_s) and p values are shown. Significant p values (<0.05) are shown in bold with significant correlation coefficients in bold red.

		ID controls n = 7							
		SeHCAT	FGF19	ASBT	IBABP	OSTa	OSTb	SHP	FXR
FGF19	r_s	0.49							
	p	0.16							
ASBT	r_s	0.29	0.77						
	p	0.27	0.04						
IBABP	r_s	0.11	0.77	0.93					
	p	0.41	0.04	0.00					
OSTa	r_s	-0.07	0.83	0.79	0.93				
	p	0.44	0.02	0.02	0.00				
OST b	r_s	0.32	-0.09	0.07	-0.14	-0.21			
	p	0.24	0.44	0.44	0.38	0.32			
SHP	r_s	-0.54	0.66	0.61	0.71	0.82	-0.14		
	p	0.11	0.08	0.07	0.04	0.01	0.38		
FXR	r_s	0.29	0.77	1.00	0.93	0.79	0.07	0.61	
	p	0.27	0.04	0.00	0.00	0.02	0.44	0.07	

Table 4.6. Correlation of basal levels of ileal transcript expression in BA regulated genes in ID, idiopathic diarrhoea controls (SeHCAT >15%). Spearman rank correlation coefficients (r_s) and p values are shown. Significant p values (<0.05) are shown in bold with significant correlation coefficients in bold red.

4.4.6 FGF19 ileal transcript levels in patients with Crohn's disease

Exploratory studies to investigate basally expressed ileal FGF19 in patients with Crohn's disease and/or ileal resections were also performed. FGF19 was basally expressed in neo-terminal ileal samples from 2 patients with previous ileal resections (one patient had Crohn's disease) and in ileal samples from 4 patients with NR-CD. There was no significant difference in transcript expression levels between these patients and the 6 healthy control patients (figure 5.3).

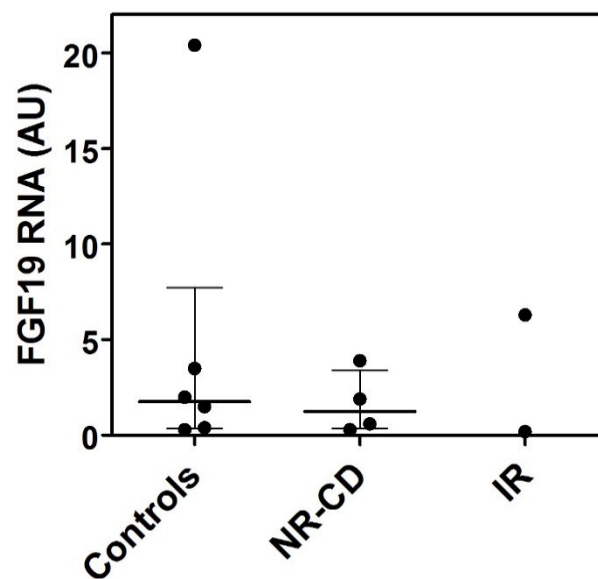


Figure 4.5. Basal expression of FGF19 in healthy control patients compared to NR-CD (non-resected Crohn's disease) and IR- ileal resected patients. The quantity of FGF19 mRNA relative to GAPDH has been calculated using the equation $2^{-(\Delta Ct)}$ and expressed as AU, arbitrary units.

4.5 Discussion:

In this study we have confirmed the presence of FGF19 gene expression within human ileal tissue in healthy control patients. FGF19 transcript levels were lower relative to the expression of the endogenous control gene GAPDH and other BA regulated genes. FGF19 transcripts are not basally expressed in the human colon.

These results suggest that the colon is unlikely to be important in intestine-liver hormonal signaling for the maintenance of BA homeostasis. FXR is expressed in more locations in the gastrointestinal tract than FGF19, including the colon [119], indicating that other factors must be involved in the transcriptional control of FGF19 in ileum. Other transcription factors which may be candidates for regulating the distribution of FGF19 expression include homeobox transcription factors and GATA4 [120-124]

Although FGF19 is not basally expressed in colonic biopsies from patients that are fasted and have received bowel laxatives prior to sampling, it is possible that FGF19 expression may be induced by exposure to increased BA concentrations. Induction of expression of colonic fgf15 expression has been demonstrated in mice that have undergone ileo-cecal resection[125]. This would lead to higher colonic concentrations of BA. BA stimulated induction of FGF19 in the human colon is explored further in **chapter 5**.

The transcript expression studies from ileal mucosal biopsies in the group of patients with chronic diarrhoea who had undergone SeHCAT testing is particularly informative. FGF19 transcripts are significantly lower in basal, unstimulated, fasting samples from patients with lower SeHCAT values. However ASBT transcripts are also lower; if ASBT protein levels are also reduced, it could be that this results in reduced bile acid uptake and consequently reduced bile acid/FXR-dependent stimulation of FGF19 transcription. This finding is not consistent with previous findings of normal or enhanced ileal active uptake of BAs from patients with PBAD diagnosed by low SeHCAT levels [126-128].

There is some evidence to support a potential negative feedback influence of BA on ASBT expression within the terminal ileum [129, 130]. If reduced FGF19 expression is the primary defect in patients with PBAD, with excessive BA synthesis and an enlarged BA pool then the terminal ileum would be exposed to increased levels of BA. This could lead to inhibition of ASBT expression in a homeostatic attempt to re-set the BA pool size by increasing faecal BA losses.

It is also important to observe that the non-significant positive correlation in ASBT and FGF19 to SeHCAT retention is absent in the PBAD patients. This could suggest FGF19 expression is not simply related to ASBT and BA uptake into cells in PBAD but may be influenced by other factors. In addition to this, co-expression between the BA activated transcription factor FXR and known FXR responsive gene transcripts for FGF19, ASBT, IBABP and OSTa was lost in patients with PBAD. This implies impaired basal regulation of BA associated genes via FXR. Such a finding could reflect defects in BA binding and activation of the transcription factor FXR. Regulatory cross talk between nuclear transcription factors and FXR have been described [55, 131, 132] and are discussed further in **Chapter 5 and 6.**

Chapter 5: Stimulation of fibroblast growth factor 19 expression in the human ileum by bile acids

Abstract

Background: Fibroblast growth factor 19 (FGF19) is proposed to be a negative feedback regulator of hepatic bile acid (BA) synthesis. We aimed to investigate human ileal ex vivo bile acid stimulated FGF19 expression in a novel ileal explant system.

Methods: Ileal biopsies were obtained at colonoscopy and ileal explants were incubated with physiological concentrations of various BA for up to 6h, and expression of FGF19 and other genes was determined.

Results: FGF19 mRNA levels remained stable in the control explant incubations. Ileal FGF19 transcript expression was induced 350-fold by 50 μ M chenodeoxycholate (CDCA, n=24, p<0.0001) and 161-fold by 50 μ M glycochenodeoxycholate (GCDCA, n=12, p=0.0005). The response of other genes to CDCA or GCDCA (50 μ M) were smaller: median increases of IBABP, OST α , OST β and SHP were 2.4 to 4.0-fold; ASBT and FXR showed little change. The EC50 for FGF19 transcript induction by CDCA was 20 μ M. FGF19 protein concentrations were significantly higher in the culture fluid from BA-stimulated explants. FGF19 induction with cholate was 81% of that found with CDCA, but deoxycholate (40%) and lithocholate (4%) were significantly less potent. The synthetic FXR agonist obeticholic acid was much more potent with 70-fold FGF19 stimulation at 1 μ M.

Conclusions: FGF19 expression in human ileum is very highly responsive to BA. Changes in ileal FGF19 induction are likely to be important in disorders of BA homeostasis.

5.1 Background

Bile acids (BA) undergo an enterohepatic circulation. They are synthesized in the liver, secreted into the duodenum, reabsorbed in the terminal ileum, and recirculated back to the liver via the portal vein. Enterocytes in the terminal ileum naturally express ASBT in both human and mouse ileum and mediate its functional specialization within the intestine for the process of a highly efficient BA reabsorption into the enterohepatic circulation. This ensures up to 95% of BA are retrieved from the lumen of the terminal ileum leaving only small amounts entering the colon to be lost in the faeces.

BA themselves can exert negative feedback on BA uptake and increase export from hepatocytes via the binding and activation of the 'farnesoid X receptor' FXR. Transcriptional changes in FXR target gene expression has been well studied in human liver and mouse liver and ileum [7, 133] The most responsive gene in mouse ileum was found to be FGF15. FGF15 is a fibroblast growth factor which lacks the usual heparin binding sites that usually mediate tissue binding of the growth factor. This means it can exert effects systemically rather than simply in a paracrine fashion.

In the mouse, FGF15 transcript and protein expression is abundant in ileum [134], and, except for a small amount of FGF15 expression in jejunum, it is undetectable in other tissues. In human intestinal studies, FGF19 the human homologue, expression was reported in ileum, but was not reliably detected in duodenum [118]. Recently, FGF19 expression has also been reported in human gall bladder and biliary tissues [135].

The farnesoid X receptor (FXR, NR1H4) was previously classed as an orphan nuclear receptor. The natural ligand was initially unknown and Farnesoid was shown to activate the receptor. The natural ligand to FXR was found to be CDCA in 1999 [136]. Concentrations of unconjugated chenodeoxycholic acid (CDCA) ranging from 3 – 100 $\mu\text{mol/l}$ have been demonstrated to be effective

in binding, activating FXR and inducing transcription responses in reporter assays in human CV-1 cell lines [9, 136, 137]. However FXR reporter transcription was only observed with taurine- and glycine-conjugated BA when cell CV-1 cell lines were also transfected with ASBT. ASBT is thought to mediate the uptake of these more polar hydrophilic BAS into these cell lines for FXR binding and activation to occur.

Transcriptional changes in FXR target gene expression has now been well studied in mouse ileum with the most responsive gene found to be FGF15. 84 fold increases in FGF15 transcripts after have been shown after 24hrs of feeding FXR agonist GW4046 in mice [10]. Authors investigating FGF15 in the mouse proposed that this gut peptide represented a negative feedback signal via its inhibitory effects on CYP7A1 the rate limiting enzyme of new BA synthesis. It was described as an 'enterohepatic signal regulating BA homeostasis'.

The authors investigating FGF15 have based this hypothesis on observations from FGF15 gene transcription studies. FXR $-/-$ and FGF15 $-/-$ both lead to increased expression of CYP7A1 with an increase in the BA pool size and increased faecal BAs. On the converse, mice fed with the FXR agonist GW4046 have reduced expression of CYP7A1 and reduced pool size and faecal BAs which is dependent on FXR expression. The authors have demonstrated secretion of FGF15 protein from Caco-2 cells transfected with adenovirus expressing a recombinant FGF15 protein. However, in these animal models, confirming this pathway by measuring circulating FGF15 protein in the blood has been difficult and has only recently been confirmed with a combination of assay techniques [138].

In humans, FGF19 can be easily detected and measured and a commercially available ELISA is in common is studies on FGF19. Human studies reveal that serum FGF19 protein levels are probably

dependent on the trans-intestinal flux of BA, based on the serum peaks observed 90 -120 minutes after the postprandial rise in serum BA. In support of this, serum FGF19 was found to decrease after the treatment with an intestinal BA-binding resin and to increase upon oral administration of CDCA [22].

Homeostatic regulation of the enterohepatic circulation of BA occurring at human terminal ileum is ill defined. Some Authors have observed small BA induced transcriptional changes in ASBT, IBABP, OST a/b in rodent studies [53]

We therefore hypothesized that FGF19 is also the most responsive BA responsive target gene in human ileum as it is in mouse. This would support the notion that BA homeostasis is achieved from the ileum by signaling to the liver rather than regulating intestinal BA uptake and transport.

Our ultimate aim from this research was also to set up a system of stimulating FGF19 gene expression with BAs in human ileum to enable us to compare responses in patients with PBAD and CD to healthy controls.

This research group has previous experience in measuring intestinal (duodenal) transcriptional responses and we aimed to use similar techniques to investigate FGF19 transcriptional response [97]. The techniques included incubating duodenal mucosal biopsies in tissue culture for 6 hours. Similar techniques have also been used in animal intestinal samples [139]. In these experiments the intestinal biopsies were stimulated with vitamin D and TRV6 transcripts were measured. We would use the endogenous primary BAs CDCA and GCDCA, to attempt to stimulate FGF19 transcription in human ileal tissue in a similar set up. CDCA and GCDCA are the most potent natural FXR ligands and are therefore most likely to induce a response.

BA can be toxic in high doses. Previous experiments have used BA in concentrations ranging from 1-100 μ M in human cell lines / hepatic tissue. This is within the range of physiologic concentrations of individual BAs within terminal ileal effluent [140]. Using both conjugated and unconjugated BA to stimulate FGF19 is also consistent with normal physiology as small bowel luminal contents have been shown to consist of equal amounts of both conjugated and unconjugated BA at the terminal ileum due to bacterial de-conjugation [130, 140, 141]. These human intestinal catheter perfusion studies have shown that GCDCA absorption displays active, saturable dose response kinetics dependent on the active transporter (ASBT) uptake. Whereas CDCA uptake displays linear kinetics consistent with absorption via passive diffusion. As Terminal ileum tissue will naturally express ASBT, we may observe a different time course and magnitude of response with the conjugated and unconjugated BAs.

FGF15 has been shown to have an N terminal sequence suggesting it is a secreted protein. Animal studies on FGF15 protein have been impaired by problems developing an assay to measure circulating protein. We therefore intended to attempt to measure FGF19 protein in culture supernatants at the beginning and the end of the incubations. We aimed to use the commercially available FGF19 ELISA (R+D systems) to measure protein release in explant supernatants. This ELISA has been used on cell supernatants by other groups [133].

5.2 Aims:

- (1) To establish a short term primary cell culture system using ileal mucosal biopsies explanted into a culture media directly after endoscopic ileal biopsies (ileal explants) to investigate FGF19 transcriptional responses to micromolar concentrations (1-100uM) of CDCA or GCDCA
- (2) To characterise dose response, time course and changes in protein expression for BA stimulated FGF19 expression in this human ileal explant system.
- (3) To compare the time course and magnitude of response in paired stimulation experiments with both GCDCA and CDCA
- (4) To determine if FGF19 expression be induced in colonic explants by micromolar concentrations of BA

5.3 Specific methods:

5.3.1 Patient selection: Healthy controls

Patients were recruited according to criteria described in **chapter 2.3.2**. To study normal human ileum, healthy controls were selected according to pre-defined inclusion and exclusion criteria as in **chapter 4**, by excluding patients with a previous diagnosis of IBD, coeliac disease and ensuring ileal histology was normal on routine biopsies. All patients recruited to this part of the study had clinical samples taken from terminal ileum to ensure normal histology in addition to research samples.

5.3.2 RT-pPCR: Comparative Ct method

In the explant studies in this chapter we aimed to determine BA stimulated target gene transcript expression levels. This involves comparing expression levels in an unstimulated 'calibrator' sample to levels in a stimulated 'target' sample. We refer to these unstimulated 'calibrator' samples as control samples when describing explant studies.

The expression levels in the stimulated 'treated' sample can then be converted to fold change in expression with respect to the unstimulated 'calibrator' sample. Fold changes are calculated using the comparative Ct method to calculate the fold change or 'RQ' (relative quantity). A fold change or RQ of 10 means that the transcript level is 10 times more expressed in the stimulated sample compared to the unstimulated control sample.

$$\Delta Ct = Ct_{\text{FGF19}} - Ct_{\text{GAPDH}}$$

$\Delta\Delta Ct$ values can then be calculated. The $\Delta\Delta Ct$ values represent the normalised FGF19 expression of a cDNA treated sample relative to a calibrator sample (e.g. CDCA-stimulated sample relative to an unstimulated control sample):

$$\Delta\Delta Ct = \Delta Ct_{\text{treated sample}} - \Delta Ct_{\text{calibrator}}$$

$\Delta\Delta Ct$ values can be converted into fold change or RQ values. Fold changes are easier for readers to interpret than $\Delta\Delta Ct$ values. The following equation is used:

$$\text{RQ or fold change} = 2^{-\Delta\Delta Ct}$$

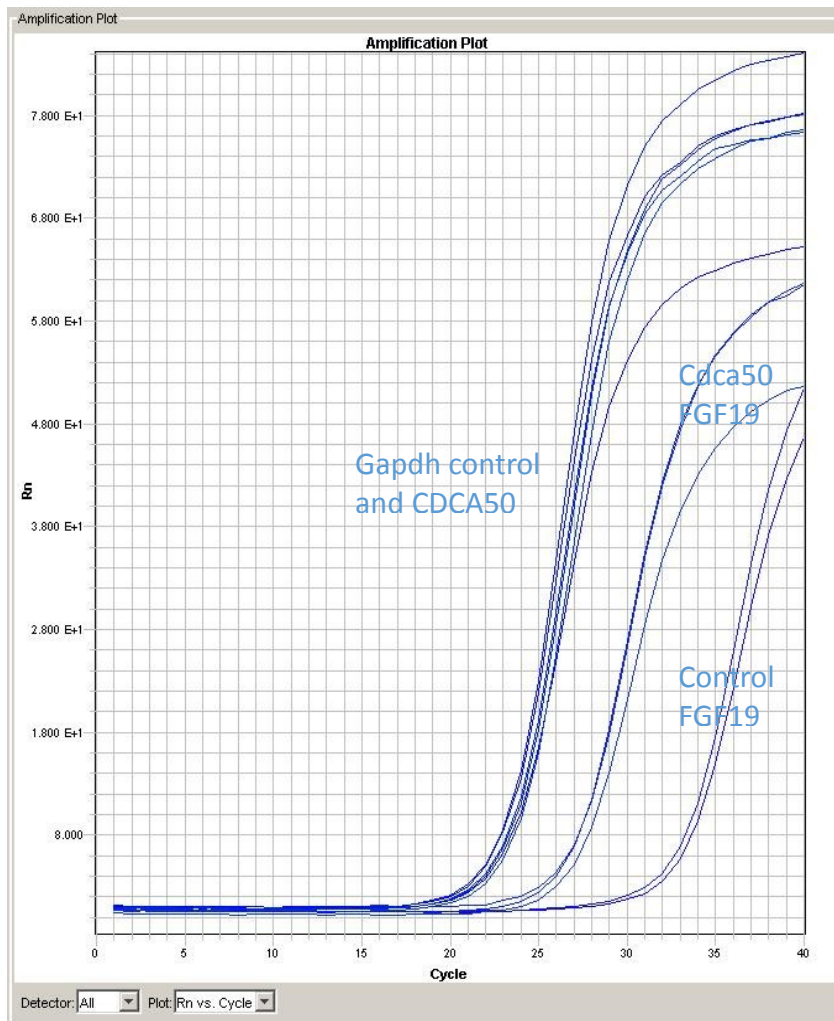


Figure 5.1. Amplification curves from ileal explants from one individual patient. The amplification curves furthest to the right are for FGF19 from the control incubation (BA free media), labelled 'Control FGF19'. This reveals relatively late amplification and therefore less abundant template in these samples. The amplification curves labelled 'Cdca50 FGF19' are from explants incubated with 50uM of Cdca and amplify several cycles earlier than the control FGF19 samples. Due to the exponential nature of PCR this several fold earlier amplification represents several hundred fold greater quantity of template in these BA stimulated samples. The amplification curve furthest to the left are the amplification curves of the GAPDH primers for both control and BA stimulated samples which is more abundant but suggesting similar template quantities regardless of BA stimulation. Using the comparative Ct method (also known as the $\Delta\Delta C_t$ method). Cts are read off the amplification plot from the target and reference gene curves from both of the stimulated and unstimulated samples. Using the Ct values, one can calculate the ΔC_t value of each sample (the expression of a target gene normalised to an endogenous control, which in this case is FGF19 expression relative to GAPDH expression): Cdca = cheno-deoxycholic acid.

5.3.3 Patient demographics

Healthy controls for ileal explants, N=28	
Age	48 (34-58)*
Male	13
Female	15
Indications for colonoscopy	
Diarrhoea (stool freq $\geq 3/24$ hrs and BSFS >5)	3 (11%)
Non-diarrhoeal indications	25 (89%)
Change in stool frequency +/- form	10
PR bleed	4
Anaemia	5
FH of CRC	1
Polyp surveillance	7
PR mucous	1
Drug history	
No medication	20
Regular medication	8
Proton pump inhibitor	3
Statin	2
ACE-i	2
Antibiotic (co-amoxiclav)	1
Oral hypoglycaemics	1
Ca- channel blocker	2
Thiazide	1
Carbimazole	1
Paracetamol	2
Codeine	1
Pro-kinetic	1

Table 5.1 Patient characteristics recruited for explant stimulation experiments. *Median and Interquartile Range, % shown where applicable.

5.4 Results

5.4.1(a) Six-hour 'control' incubation of explants does not alter the basal expression of FGF19 mRNA

Due to the use of Fetal Calf serum in our culture media explant incubations it was important to establish that the expression of FGF19 was not being altered by constituents within the culture media. The relative quantities of FGF19 transcripts in all of the unstimulated ileal explant

experiments incubated in control culture media for 6 hours (n=35) was compared to the expression of FGF19 in ileal biopsies used for basal expression studies (biopsies immediately placed in RNAlater and frozen at -80 for analysis, n=11). No significant difference in expression levels of FGF19 (relative to GAPDH) was found between these incubated control samples vs non incubated samples ($p=0.5$, Mann-Whitney) **Figure 5.2 (A)**. In 4 patients, 4 ileal biopsies were taken with 2 biopsies being placed in control culture media for 6 hours and the other 2 biopsies from the same patient being placed immediately in RNAlater and frozen at -80 for analysis. No significant difference was seen in the expression levels of FGF19 between these incubated vs non incubated samples from the same patients (n=4, $p= 0.2$, Mann Whitney) **Figure 5.2 (B)**.

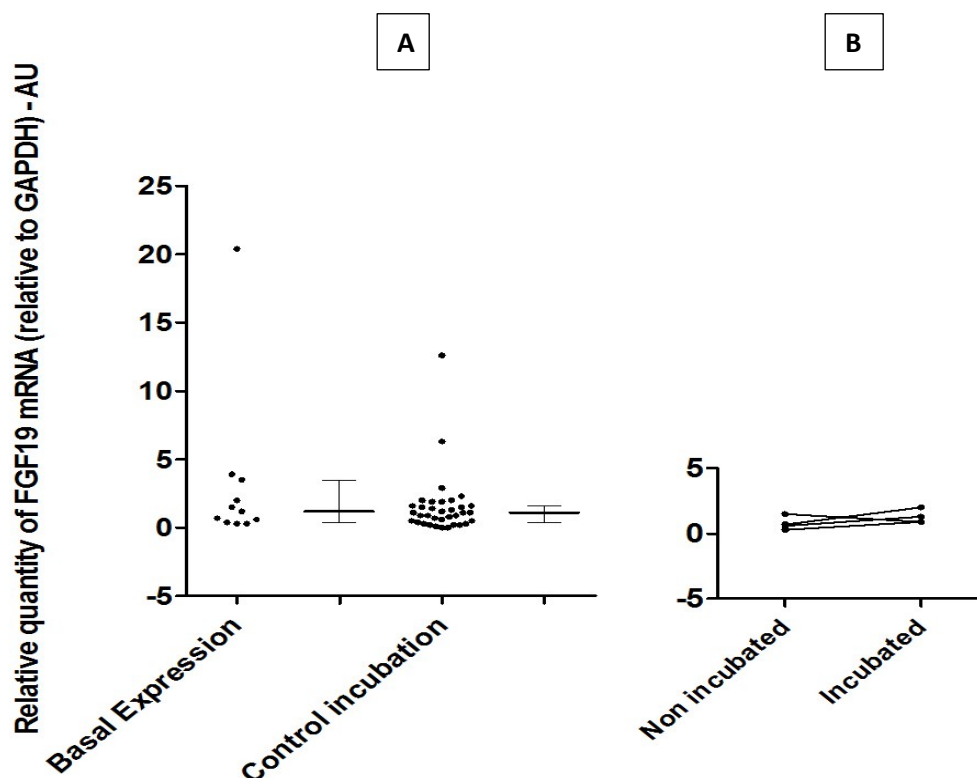


Figure 5.2: FGF19 mRNA expression is unchanged during 6h explant culture. **A:** FGF19mRNA in ileal biopsies stored for immediate processing (basal expression, n=11) and those incubated in control

culture media for 6h (control incubation, n=35) are shown, together with medians and interquartile ranges. There is no statistically significant difference between them, $p = 0.5$, Mann-Whitney test.

B: The relative quantities of FGF19 mRNA are similar in paired samples taken from the same patient Basal expression and that after control incubation are shown (n=4). The quantity of FGF19 mRNA relative to GAPDH has been calculated using the equation $2^{-(\Delta Ct)}$.

5.4.1(b) GAPDH expression is stable despite incubation with BA

There was no significant difference in mean Ct levels observed for GAPDH in ileal biopsies incubated in BA free control culture media compared to those incubated with BAs. This suggests that differences in BA stimulated expression of FGF19 relative to GAPDH occur due to increases in FGF19 rather than decreases in GAPDH expression.

5.4.2 Potent stimulation of FGF19 transcript expression by CDCA and GCDCA in the human ileum

FGF19 transcript levels were significantly higher in BA stimulated explants compared to unstimulated explants after 6hrs. Median levels of FGF19 were 350-fold higher in explants stimulated with **50 μ M** CDCA (n = 24, $p < 0.0001$, two-tailed Wilcoxon test), and 161-fold higher in explants stimulated with **50 μ M** GCDCA (n = 12, $p = 0.0005$). There was no significant difference in FGF19 transcript levels comparing CDCA and GCDCA-stimulated explants ($p = 0.3$, two-tailed Wilcoxon test). Results are expressed graphically in **Figure 5.3**.

The responses of other relevant genes to CDCA and GCDCA at 50 μ M (n = 3 - 4) showed median levels of IBABP, OST α , OST β and SHP between 2.4 and 4.0-fold higher, whereas ASBT and FXR showed little difference (Figure 4 (iii)). The unstimulated relative quantity of FGF19 was lower (with a high deltaCt value) than the other genes, but two other weakly FXR-responsive genes differed greatly, with SHP levels similar to FGF19, but IBABP much higher (with a lower deltaCt value).

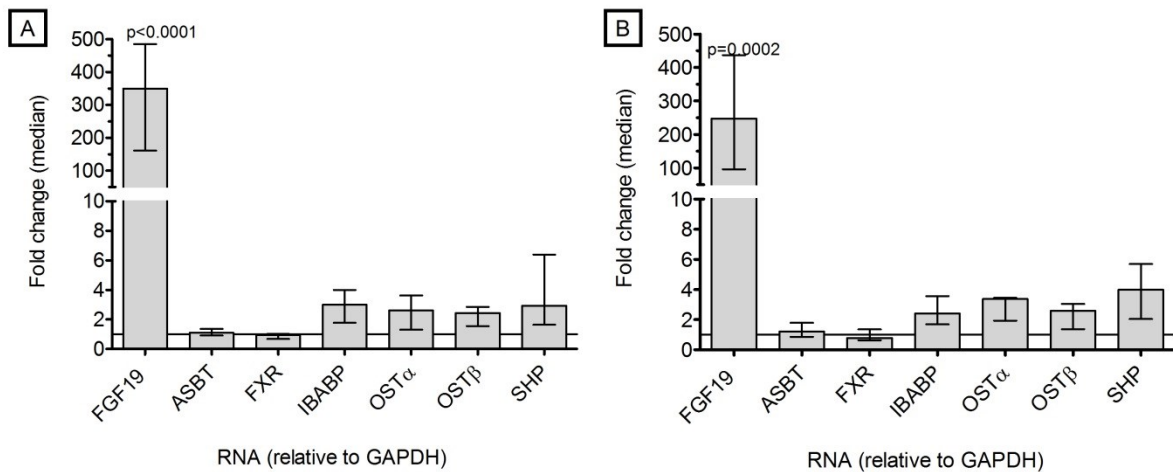


Figure 5.3 A. Transcript expression after 6h incubation with 50 μ M CDCA. Number of subjects(n) and p values were: FGF19 (n=24, $p < 0.0001$); ASBT (n=4, $p = 0.23$); FXR (n=4, $p = 0.30$); IBABP (n=4, $p = 0.02$); OST α (n=3, $p = 0.11$); OST β (n=4, $p = 0.02$); SHP (n=4, $p = 0.03$). **B:** Transcript expression after 6h incubation with 50 μ M GCDCA. n and p values: FGF19 (n=12, $p = 0.0005$); ASBT (n=4, $p = 0.40$); FXR (n=4, $p = 0.52$); IBABP (n=4, $p = 0.06$); OST α (n=3, $p = 0.10$); OST β (n=4, $p = 0.07$); SHP (n=4, $p = 0.06$). Data are expressed as median and interquartile ranges of expression ratio fold-changes, calibrated to matched unstimulated control samples, relative to GAPDH. Control sample mean Ct values for GAPDH, FGF19, ASBT, FXR, IBABP, OST α , OST β and SHP were 22.5, 29.5, 21.9, 24.9, 20.7, 25.7, 23.9 and 27.8 respectively. P values were calculated from dCT values by paired t-tests.

5.4.3 Bile acid dose response analysis

Incubations with a range of 2 - 5 different CDCA concentrations (5- 100 μ M) were performed in parallel in 10 patients. The induction of FGF19 mRNA was similar at 100 μ M and 50 μ M CDCA (**Figure 5.4**). A non-linear, least squares, sigmoidal curve fitting program was used (GraphPad Prism 5) to derive the EC50 for studies which included 4 or 5 different CDCA concentrations (n = 4). The median EC50 was 20 μ M (range 16-52).

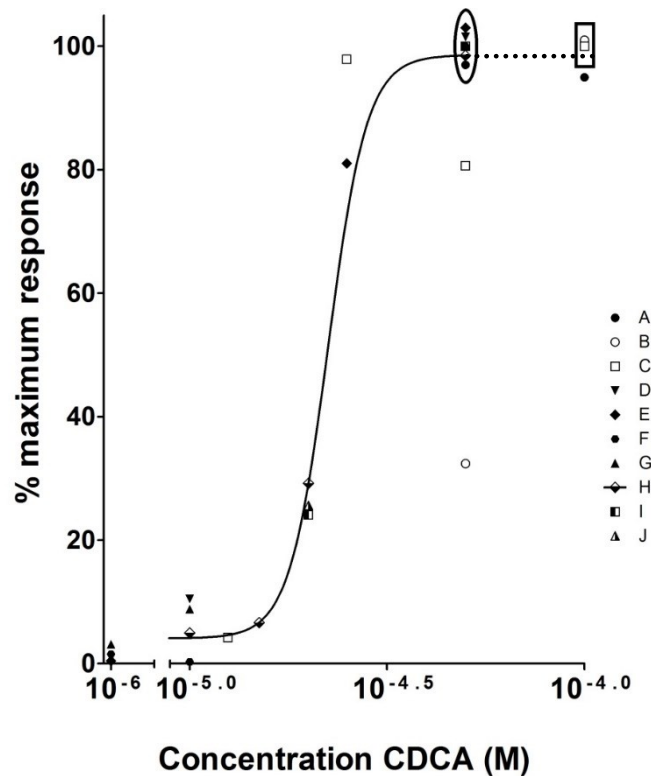


Figure 5.4. Dose ranging studies of CDCA induction of FGF19 mRNA: Graph shows pooled data from 10 explant studies that were incubated with 2 – 5 different concentrations of CDCA in parallel. Concentrations ranged from 1 μ M to 100 μ M. Data points are shown from each individual explant from each individual patient (see data points A-J). Fold changes in mRNA were converted to a percentage of maximum response which was observed at 50 μ M in those studies whose data points are shown in the oval ring and 100 μ M for those shown in the rectangular box.

5.4.4 Time course studies:

Time course experiments showed that FGF19 expression increased over the course of 6h incubation with 50 μ M CDCA (Figure 4). Stimulation of FGF19 expression increased from under 10-fold at 2h to median >40-fold at 3h, to several hundred fold at 6h incubation.

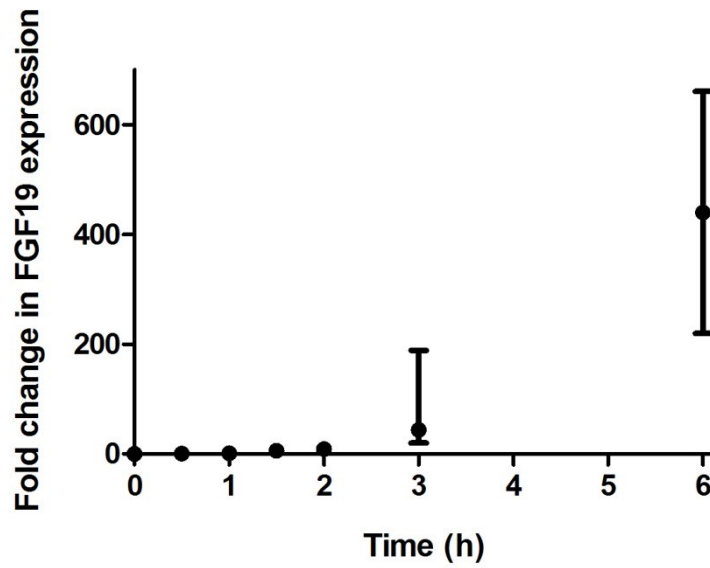


Figure 5.5. Time courses (n=4) showing FGF19 expression increased over the course of 6h incubation with 50 μ M CDCA. Incubations with CDCA were for 0.5h, 1h, 1.5h, 2h, 3h or 6h. Explants were placed into RNAlater immediately after incubation. FGF19 expression at 0.5h and 1h showed no significant change, at 1.5h and 2h was <10-fold, at 3h was >40-fold, and at 6h was dramatically higher at several hundred fold. Data are expressed as median fold-changes, calibrated to matched unstimulated control samples, relative to GAPDH. Interquartile ranges are shown where appropriate.

5.4.5 Relationship between CDCA stimulated FGF19 fold changes and relative quantities of FGF19 expression levels in the unstimulated control explants

An inverse correlation did exist between FGF19 transcript levels in the unstimulated explant to the fold change in the stimulated explants, but this did not achieve statistical significance (**figure 5.6**). Explants with relatively higher levels of basal FGF19 transcripts in the unstimulated explants tended to have lower fold changes in BA stimulated explants.

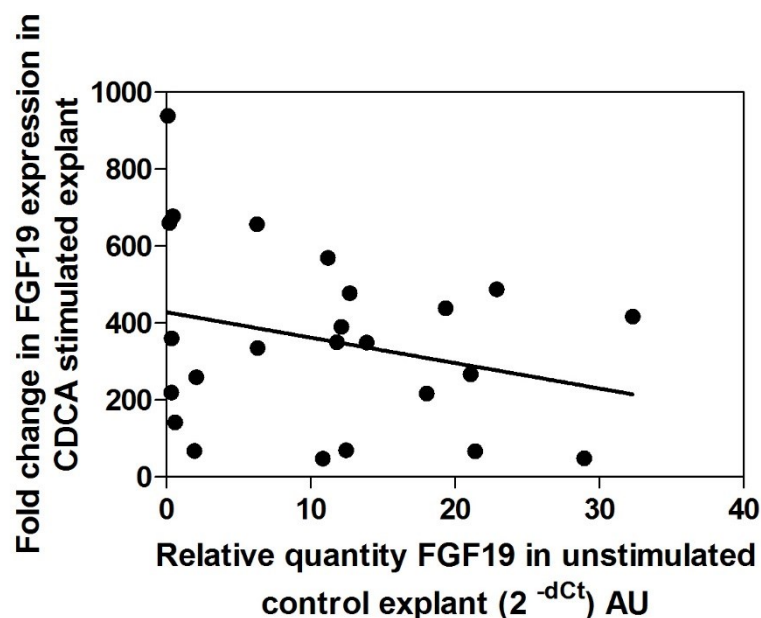


Figure 5.6. Relationship between relative quantities of FGF19 transcripts in unstimulated control explants vs fold change in FGF19 transcript levels in CDCA stimulated explants. AU – Arbitrary units of expression.

5.4.6 BA stimulated FGF19 expression is not demonstrated in human colon

Colonic explant studies were performed on 6 patients. Groups of 2-3 biopsies from the same patient were incubated separately in BA free media (unstimulated control), 50uM CDCA (positive control), 1uM OCA and either 1uM of INT 767 or INT 777. One of the explant results for INT-777 was excluded due to a control gene GAPDH outlier Ct result (see table 3). In 3 of the patients the

unstimulated control explant revealed RT-PCR amplification of FGF19 beyond 35 cycles and was regarded as undetectable. In one of these patients, the CDCA and INT-767 stimulated explant was just within the limits of reliable detection with a Ct of 34.7 and 34.9 respectively. In 3 patients, FGF19 was detectable in the unstimulated control explant but there was no significant difference in the relative quantity (dCt) of FGF19 mRNA in the CDCA (n=3) or OCA (n=3) stimulated explants (p= 1.0, Wilcoxon two tailed).

5.4.7 The induction of FGF19 mRNA in human ileum by different BA correlates with their relative potencies as FXR agonists.

Ileal explants were incubated with CDCA in parallel with other natural BA (CA, DCA, and LCA) all at 50uM or with the semi-synthetic FXR agonist OCA. Median fold changes in BA stimulated expression of FGF19 mRNA were not significantly different with CDCA and CA (n=4). Median fold change in LCA stimulated expression of FGF19 mRNA was significantly lower than CDCA (n=5). The trend in relative potencies of BA stimulation of FGF19 mRNA expression suggested that DCA was intermediate between CA/CDCA and LCA. The mean induction of FGF19 mRNA expression with other BAs at 50uM, normalized to the paired CDCA incubation at 50 μ M, was 81% for CA and significantly lower with DCA and DCA at 40% and 4% respectively (**Figure 5.7**). Mean induction of FGF19 expression with 1uM of OCA normalized to paired 50uM CDCA was 70% (n=3).

After incubations with 1 μ M OCA for 6h, a median fold-change of 70 (range 40 – 258, n = 3) was observed, although no significant changes in FGF19 mRNA expression were observed with 1 μ M CDCA (n = 3). In paired incubations involving both 20 μ M CDCA and 20 μ M OCA, median induction in FGF19 mRNA expression was 5 times greater (range 3 - 45, n = 3) with OCA. The order of potency for stimulating FGF19 expression from this data is OCA>CDCA/CA>DCA>LCA.

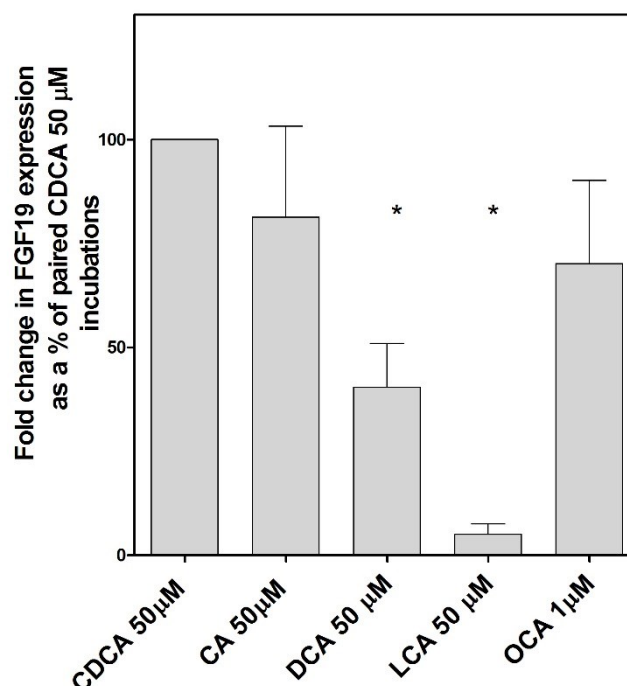


Figure 5.7. Induction of FGF19 mRNA expression with a range of BAs normalised to CDCA stimulated expression. Induction of FGF19 mRNA is expressed as a percentage of the induction observed in explants stimulated with 50 µM CDCA from the same patient. Mean percentages and range are shown. The mean induction of FGF19 mRNA as a percentage of paired CDCA stimulated explants was 81% with cholic acid (CA) ($n = 4$), 40% with deoxycholic acid (DCA) ($n = 3$) and 4% with lithocholic acid (LCA) ($n = 5$). Statistical significance: CA, $P = 0.5$; DCA, $P = 0.03$; LCA, $P < 0.001$; paired t -tests % vs. CDCA 50 µM. With obeticholic acid (OCA), induction of FGF19 at OCA 1 µM was comparable with that found with CDCA 50 µM ($n = 3$).

5.4.8 BA stimulated FGF19 expression by BA analogues: INT 747 vs 767 vs 777

A further cohort of 14 patients was recruited to study the effects of various synthetic BA analogues on FGF19 expression. 8 patients underwent ileal biopsies and 6 underwent colonic biopsies for explant studies. Groups of 2-3 biopsies from the same patient were incubated separately in BA free media (negative control), 50uM CDCA (positive control), 1uM OCA and either 1uM of INT 767 or INT 777. In this cohort of ileal explants ($n=8$), the median fold change in FGF19 RNA expression stimulated by CDCA was 110 (IQR 53–500). FGF19 RNA expression was significantly higher in ileal explants ($n=8$) stimulated with INT-767 compared to those stimulated with OCA ($p = 0.007$, Mann

Whitney **Figure 5.8**). The potency of INT-767 for stimulating FGF19 mRNA expression was approximately 10 fold higher than OCA. No significant induction of FGF19 expression was seen in colonic explants with either OCA or INT 767 ($p=0.25$, Mann Whitney). No significant induction of FGF19 was seen in either ileal or colonic explants with INT-777.

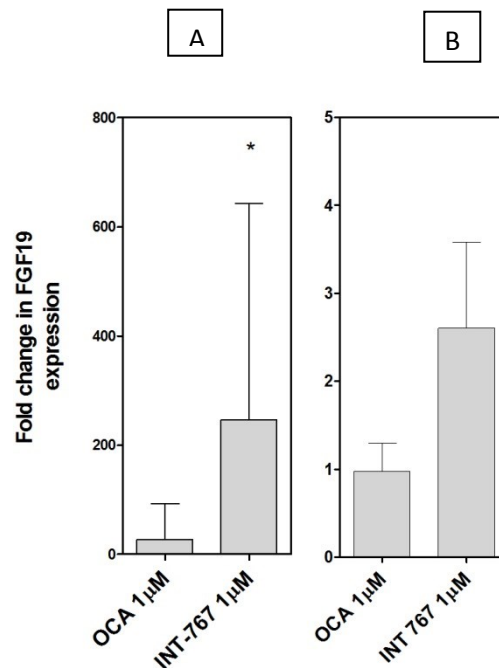


Figure 5.8: Relative potencies of FXR agonists OCA and INT 767 at inducing FGF19 mRNA expression in ileal explant **(A)** and colonic explants **(B)**. Median induction is significantly higher in ileal explants incubated with INT 767 vs OCA (245-fold vs. 27-fold, $p = 0.02$, Mann Whitney). No significant induction of FGF19 occurred in colonic explants with either agonist ($n=5$, OCA, $n=3$, INT 767, Medians and IQR shown).

5.4.9 FGF19 protein in culture fluid from ileal explants correlates with fold change in FGF19 mRNA

Culture fluid supernatant concentrations of FGF19 protein were measured in 16 of the explant studies by a specific ELISA (Quantikine Ref). The measured concentrations (in pg/ml) were normalized according to whether the culture plate well had contained 2 or 3 biopsies and expressed as pg/explant (see methods 2.6). The groups of 2 or 3 biopsies were not weighed before each incubation but a separate pilot study on 2 sets of 3 biopsies gave wet weights of 12 and 20ugs.

Levels of FGF19 protein were undetectable or at the limits of the detectable range of the assay in many of the fluid samples from unstimulated explants. Concentrations of FGF19 were reliably

detectable in all fluid samples from stimulated explants. The median concentration of FGF19 protein was significantly higher in the supernatants from explants incubated with 50 μ M CDCA (56 pg/explant, range 8 – 169) compared to the control explants (7 pg/explant, range 0 – 46, $n = 10$, $p = 0.003$, two-tailed Mann-Whitney). The median level of FGF19 was also significantly higher ($n = 9$, $p = 0.002$, two-tailed Mann-Whitney) in the culture fluid from explants incubated with 50 μ M of GCDCA (median 24 pg/ml/explant, range 3 – 113) compared to the matched control explants (6 pg/ml/explant, range 0 – 13, **Figure 5.9a**). There was also a significant correlation between the culture fluid protein and fold changes in mRNA of FGF19 (CDCA: $n = 10$, $r = 0.70$, $p = 0.005$, and GCDCA: $n = 9$, $r = 0.76$, $p = 0.003$, Spearman's rank test; **Figure 5.9b**).

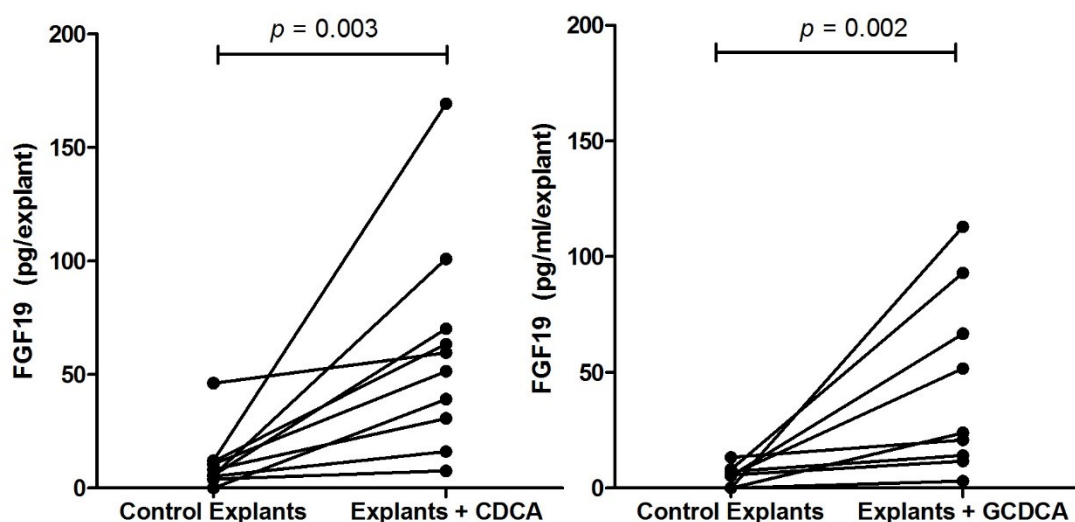


Figure 5.9a Increases in FGF19 protein in culture fluid after BA incubations; CDCA ($n=10$), GCDCA ($n=9$) FGF19 protein levels were measured by specific ELISA (FGF19 Quantikine ELISA kit, R&D Systems, Minneapolis, MN) at the end of 6h incubations. FGF19 protein levels (pg/explant) are higher in the culture fluid from every BA-stimulated explant compared to their respective paired control explant

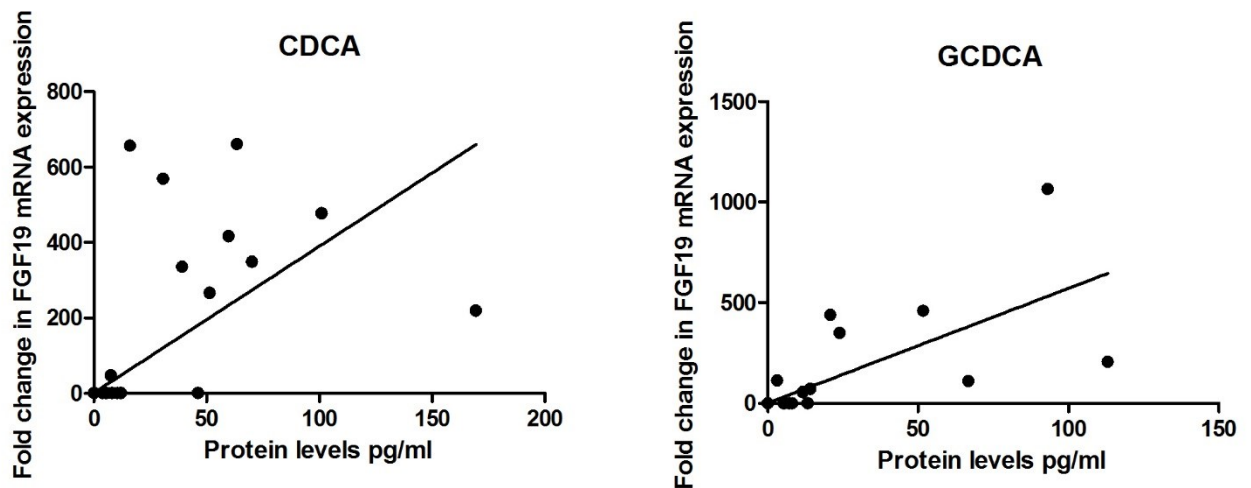


Figure 5.9b Correlation between the culture fluid protein levels and fold changes in mRNA levels of FGF19 (CDCA (n=10) and GCDCA (n=9))

5.5. Potential sources of error in experimental design and suggested solutions:

5.5.1 Primer efficiency

At the time of initiating this research, there were no published primer sequences available for measuring human intestinal FGF19 mRNA using SYBR green assays. The primer was designed by using BLAT software with the in-silico PCR function. The initial optimization experiments revealed a FGF19 primer efficiency of 83%. This is below generally accepted 90% primer efficiencies. This low primer efficiency was considered accepted in these experiments for 3 reasons:

1. The demonstration of similar pcr efficiency in the reference gene primer (from a previously published source) with a similar efficiency of 83%.
2. Pilot experiments revealed our target gene amplification in unstimulated control samples (calibrators) as being generally detectable repeatedly less than 35 cycles and usually with Cts of 29-33.

3. We aimed to compare BA stimulated expression of fold changes in FGF19 mRNA between patient groups. Pilot studies had revealed large fold changes rather than small fold changes (eg below 2-4 fold).

However it is possible that this low primer efficiency could have contributed to the wide variability in stimulated expression in FGF19 mRNA found.

5.5.2 FGF19 protein concentrations in culture supernatant:

Comparisons of FGF19 protein concentrations in culture fluid supernatants between unstimulated and stimulated explants was limited by inaccurate normalization for amount of tissue. Measured concentrations of FGF19 in the supernatant were only normalised according to the number of biopsies contained within each well. This was either 2 or 3 biopsies. However, although the same endoscopic biopsy forcep was used for all samples, the amount of tissue obtained will vary according to a number of factors (eg. Angle of forcep applied to target tissue, degree of pressure applied and consistency of target tissue). A better quantitative expression of protein could have been expressed in pg/mg of tissue. It was considered more important to immediately transfer the endoscopically obtained biopsies from culture fluid into the complete media and into the incubator rather than further manipulating them into separate containers to obtain separate weights for each group of biopsies.

The detection of FGF19 protein in unstimulated 'BA free culture supernatant' has 3 interpretations:

1. There is basal secretion of FGF19 in the absence of additional exogenous BA (this has been shown with longer cell cultures on intestinal cell lines).
2. Cell death and lysis may be occurring releasing preformed protein
3. The ELISA's specific binding may be affected by constituents of the complete culture media

Investigating the possibility of cell death and lysis could have been further evaluated by measuring culture supernatant fluid LDH levels which is a marker of cell lysis. Secondly, a number of explants could have been placed in formalin at the end of the incubations and examined histologically. Finally the third possibility is limited by diluting and the known FGF19 standards in a buffered protein solution, similar to culture fluid (RD5P diluent), for the standard curve. This will aim to limit binding differences arising from interference with the assay from constituents within the media.

5.5.3 Variability of fold change in BA stimulated FGF19 transcript expression

There are a number of experimental factors that may have influenced this observed trend, including luminal BA concentrations and composition at the time of colonoscopy when the primary ileal tissue was sampled. Even in the fasted state BAs are present in the lumen of the TI [141] and variability in the luminal concentrations may influence the basal expression of FGF19 at the time of sampling. This may lead to different baseline levels of expression of FGF19 at the beginning of the 6 hour incubations between individuals. Ileal explants exposed to relatively high concentrations of endogenous luminal BA in vivo may harbour saturated receptors (eg ASBT or FXR) upstream from FGF19 transcription. In this situation then lower induction of FGF19 transcription may be observed with the addition of exogenous BA. For example, active uptake of conjugated BA via ASBT is known to display saturable kinetics [140].

The variability from in vivo endogenous BA concentrations at the time of sampling was controlled for by physical washing of explanted ileal biopsies. This involved washing explants with PBS and transferring them from a transport media to an incubation culture media. A further optimization step could have been leaving the explants for 1-2 hours to reach a steady state in BA free culture media prior to the addition of exogenous BA.

It is unknown what effect the bowel purgative has on ileal FGF19 expression but it is possible that it affects luminal BA concentrations and that patients response to the purgatives in different ways. The purgative that we used includes magnesium carbonate and citric acid. This reacts within the intestinal lumen to form magnesium citrate which exerts an osmotic effect on cells within the intestine. This leads to an osmotic shift of water into the intestinal lumen and stimulates GI motility. The net secretion of water into the intestinal lumen and increased small bowel transit may reduce the physiological concentrations of BA within the ileum to varying degrees. A non-significant trend towards lower serum FGF19 levels after bowel prep has been described in a small group of patients (Sanjeev Pattni, PhD, Imperial College 2012). Further variability in luminal BA concentrations may be influenced by compensatory increases in BA synthesis, biliary secretion and gallbladder function.

To control for variability in luminal BA concentrations at the time of colonoscopy, patients were fasted for a similar time period and have undergone colonoscopy at similar time points after having taken the purgatives. It would be difficult to optimize this variability further. It is possible that PEG based laxatives would lead to less variability as this does not lead to a net secretion of water into the intestinal lumen. Mannitol based bowel preparation has not been shown to affect SeHCAT retention levels in one small cohort [142].

Ileal luminal BA composition may vary between individuals. CDCA, CA and DCA have been shown to be the majority of luminal BA . Differing levels of conjugated BA will depend upon quantity and types of commensal bacteria within the ileum. This will have effects on ASBT. In addition to this, the proportion of luminal secondary BA may affect FGF19 stimulation. Luminal LCA and DCA may have been absorbed and may have bound to FXR at the time of sampling. These secondary BA are weaker FXR agonists than primary BAs for FXR activation [9]. Intracellular DCA and LCA could potentially compete with exogenous CDCA in binding to FXR and may influence the kinetics of FGF19 transcription.

Further variability may be related to the sampling of ileal mucosa. FGF19 is thought to be restricted to expression within enterocytes only from the intestinal epithelium. Ileal mucosa is known to have areas that have interruptions in this enterocyte epithelial lining known as Peyer's Patches. The epithelium in these areas has specialized M-cells at the surface of the mucosa in place of the columnar epithelial enterocytes. Biopsies taken from mucosa harbouring a Peyer's patch is likely to have substantially lower levels of FGF19 expression and therefore lower levels of stimulation. A biopsy from a Peyer's patch would lead to both variability between individuals but may also affect the relative quantification of FGF19 transcript levels between stimulated and unstimulated explants. If the unstimulated explants harbour a sample from a Peyer's patch then the transcript levels would be significantly lower. The stimulated expression is calculated as a quantity (fold change) relative to the unstimulated explant. This would lead to erroneously higher fold change in these stimulated explants. However, Peyer's patches are said to be micrometers in diameter on electron microscopes whereas biopsies specimens tend to be 2-3mm in diameter. Also 2-3 biopsies are taken and grouped for each condition in the incubations to attempt to control for this variability.

5.6 Discussion

After confirming human ileal expression of FGF19 (chapter 4) we have proceeded to show for the first time that ileal FGF19 is greatly stimulated by physiological concentrations of BA. Prior to this study, the effect of BA on FGF19 transcription in human ileum was unknown, because no system existed to investigate this. We have now developed an explant system which is functional and does not result in loss of FGF19 mRNA. The potential applications of this system are numerous, as it enables investigation of FGF19 stimulation in human ileum under controlled conditions. This system is more akin to natural human physiological conditions compared to previous systems which used mouse ileum, hepatocytes or human cell lines. The use of explanted human tissue in this system

also allows ileal FGF19 stimulation to be directly linked to patients, so that ileal FGF19 stimulation may be investigated in different disease states.

We have demonstrated that stimulation of the human ileum with 50 μ M CDCA or 50 μ M GCDCA induces FGF19 transcript expression in all individuals, with a median induction of approximately 300-fold. This is much greater than the approximately 80-fold FGF15 induction previously shown in mouse ileum after oral administration of the synthetic FXR agonist GW4064 [10]. Species differences may have contributed to this much higher FGF19 induction in human ileum compared to FGF15 induction in mice. The large induction in ileal FGF19 expression we have shown is also larger than that previously observed in primary cultures of human hepatocytes [133]. In those experiments, control samples had low expression of FGF19 but a dose dependent increase in FGF19 mRNA levels was seen, of about 80-120 fold after 6 – 24h incubation with CDCA (50 μ M) or with GW4064 (1 μ M).

The large 300-fold changes in FGF19 mRNA expression appear to dwarf the <4-fold changes of the other BA-regulated genes investigated in this study. IBABP, OST α and OST β genes have been shown to have FXR-mediated transcriptional responses which are predicted to lead to an increase BA-ileal fluxes, but their induction in our system was only a fraction of FGF19. SHP, involved in BA-mediated transcription regulation, and previously shown to be highly induced in mouse ileum [143] was also stimulated much less than FGF19. This incongruence may suggest that FGF19 has the dominant role in the negative feedback regulation of the BA enterohepatic circulation.

Due to difficulties in assaying the FGF15 protein in previous mouse studies we are the first to show FGF19 release and possibly secretion from primary human ileal tissue on exposure to BA. FGF19 was detected in a number of the 'unstimulated explant' supernatants but it was measured at higher

levels in all of the BA stimulated explants. Previous groups had used a similar technique to show FGF19 in cell culture fluid from primary human hepatocytes using the same ELISA[133]. One of the main effects of FGF19 is the negative feedback regulation of BA synthesis, and for this to be achieved, FGF19 transcripts must be translated into protein and released into the portal circulation. Our results reveal significant differences in magnitude between the ≈ 300 -fold change in transcript expression and the much lower ≈ 8 -fold-changes in FGF19 protein. This is likely to be due to the extra time needed for translation and export of the protein, but may reflect some additional regulation of those processes.

It is also important to note that the measurements we have made in our explant system are steady-state transcript levels at a particular time, rather than direct measures of FGF19 synthesis or degradation. In order to get a better idea of the dynamics of FGF19 transcript expression, we conducted limited time course experiments, which showed that FGF19 transcript expression were not increased by 1h of incubation, but after then, continued to increase up to 6h. This suggests that as the length of time of BA exposure increases, more FGF19 transcripts are synthesized than degraded, resulting in a net increase in FGF19 transcript levels.

We studied both unconjugated CDCA and conjugated GCDCA in most experiments. GCDCA being a polar, hydrophilic BA is likely only to enter enterocytes expressing the BA transporter ASBT. Expression of ASBT is a key finding in the ileum with and has been shown to be reduced in ileal biopsies from patients with Crohn's colitis in clinical remission [46]. In human cell lines, ASBT transcription may be reduced by c-fos mediated inflammatory pathways. However other studies have refuted a link between ASBT expression and ileal inflammation [144]. In our experiments, stimulation was found in ileal explants in all cases with GCDCA, but this control cohort of patients has not included any subjects with identified ileal disease or histologic microscopic inflammation.

CDCA also induced FGF19 expression in all of the explants in our experiments confirming entry into cells potentially independent of ASBT.

It is widely acknowledged that the most potent physiological FXR agonist is CDCA but there has been some ambiguity regarding the relative potency of CA as an FXR agonist [9, 136, 137, 145]. Minimal FXR activation was found in non-intestinal CV-1 cells with CA, when compared to CDCA and secondary BA [9], and similar findings were reported using human FXR and a reporter gene in HepG2 cells [137]. However, other groups have shown CA is able to stimulate FXR responses [136, 145]. Here we have shown that FGF19 is strongly induced with both CDCA and CA, and, to a lesser extent, with DCA. Our study used human tissue in a physiological system where BA transport occurs, giving confidence that this system generated accurate data regarding human ileum. Nonetheless, putting this data into the context of previous findings does raise the question of whether alternative mechanisms to FXR activation may be responsible for inducing FGF19.

LCA was poor at inducing FGF19 expression in human ileal explants. Our data is in keeping with the reports that LCA binds and antagonizes FXR [146] but does not concur with other findings [9]. A more recent study reported that LCA induced FGF19 expression independently of FXR following Pregnane X Receptor (PXR, NR1I2) activation [147]. However LCA has toxic properties [146] which could have had a negative bearing on the stability of our ileal explant system. Further data are required to fully elucidate the influence of secondary BA on FGF19.

A number of studies indicate possible candidates for FXR-independent induction of FGF19 with different selectivity for various BA. A novel G-protein coupled membrane receptor, BG37/TGR5, has been found, expressed in ileum and other tissues, which binds BA and increases intracellular cyclic adenosine monophosphate (cAMP) [148]. No link has been shown between TGR5 activation and

downstream FGF15/19 expression or for any other FXR target genes. Interestingly, one study [149] did report a possible ‘crosstalk’ scenario between FGF15 and TGR5 on gallbladder motility. It remains likely that intestinal TGR5 is closely linked to glucose and lipid homeostasis [150] (similarly to FXR and also FGF19 [151]) rather than BA regulation.

This study successfully validated previous reports [152, 153] regarding OCA as a potent ligand for FXR. OCA strongly induced FGF19 in all subjects at both of the studied concentrations. This supports the potential therapeutic role of OCA as a novel FXR agonist in gastrointestinal disease with defective FXR activation/FGF19 induction patterns. OCA enters the enterohepatic circulation [154] thus can be used to regulate hepatic BA synthesis. Previous reports have focused on cholestatic diseases [145] and, more recently, metabolic disorders [155, 156] but we would also anticipate a role in gastro-intestinal disorders.

The individual variability in ileal FGF19 induction by both CDCA and GCDCA may indicate different levels of BA homeostatic equilibrium. Results from this study indicate that individuals may vary in their capacity to produce ileal FGF19 at a transcriptional level. The FXR-FGF19 axis is likely to be influenced by factors upstream and down stream from the intracellular BA ligand binding and activation of FXR within the enterocyte. Upstream factors may involve the Ileal microbiota.

Microbiota will influence ileal FGF19 production via their effects on the composition of BAs within the intestinal lumen prior to absorption. Dihydroxylation into secondary BAs will lead to the less potent FXR activation upon entry into the enterocyte. Downstream effects on the FXR-FGF19 pathway have been shown to be influence by PPARα activation. PPARα activation can occur with certain dietary fats and promotes glucoronidation and disposition of intracellular BAs. This would reduce intracellular BA concentrations and reduced FXR-FGF19 activation [157]. A novel protein, DIET1 may also influence downstream secretion of FGF19 protein levels [158] and is discussed further in **chapter 8**.

The consequences of this variability in FGF19 production may not simply be confined to BA homeostasis. Serum FGF19 levels have also been associated with increased BMI, lipid and glucose homeostasis [87]. It is interesting to observe the higher median BMIs in patients diagnosed with excess faecal BAs or low seHCAT retention levels PBAD [71].

Our group has demonstrated that primary BA diarrhea patients have lower median serum FGF19 levels than controls, and proposed that primary BA diarrhea may be due to inadequate FGF19 expression, resulting in impaired negative feedback and consequent overflow of BA into the colon [159]. One previous study demonstrated a significant depletion in CA levels in primary BA diarrhea patients compared to controls [160]. This could have been important if our findings had suggested a higher potency for CA in inducing FGF19 compared to CDCA, but was not the case, as CDCA and CA were not significantly different. We did not recruit any patients with diagnosed primary BA diarrhea for this part of the study, but explant results are presented in Chapter 6.

In summary, this study has found that FGF19 expression is highly responsive at a transcriptional level with FXR activating BAs in human ileum. We have demonstrated this through an explant system, which in future can be used to investigate the role of FGF19 in disorders of BA homeostasis.

Chapter 6: Reduced bile acid stimulated expression of FGF19 is associated with conditions of bile acid diarrhoea

Abstract

Introduction: FGF19 is a gut peptide produced from ileal enterocytes in response to absorbed BA. FGF19 conserves the bile salt pool by inhibiting hepatic BA synthesis. Ileal disease is common in Crohn's disease. Impaired ileal FGF19 production may lead to excessive BA synthesis and contribute to increased fecal BA levels. Increased fecal BA is a causative factor in the pathogenesis of diarrhoea in CD patients, referred to as secondary bile acid diarrhoea SBAD. Primary BAD (PBAD) occurs in the absence of overt ileal disease or malabsorption. Impaired ileal FGF19 production may result in increased faecal BA levels due to excessive hepatic BA synthesis in PBAD. We aimed to investigate whether BA-stimulated expression of FGF19 in the ileum is reduced in patients with CD and PBAD.

Methods: Methods: Patients attending for colonoscopy (n=30) were prospectively recruited and gave informed consent to give additional ileal biopsies. 14 patients had CD (4 with previous right hemi colectomy and 10 without), 16 patients had unexplained diarrhea and had SeHCAT tests. Groups of 2-3 biopsies (explants) were incubated separately for 6h with either BA-free culture media (unstimulated controls), chenodeoxycholate (CDCA) or glyco-CDCA (GCDCA), both at 50uM. After RNA extraction and cDNA synthesis, FGF19 expression was quantified relative to GAPDH by RT-PCR.

Results: Results: In patients with unexplained diarrhoea SeHCAT 7d retention values were <15% in 7 patients, indicating primary BAD, and >15% in 9 who were normal diarrhea controls. A positive correlation was found between SeHCAT retention and the magnitude of the fold change in FGF19 expression stimulated by either CDCA (r=0.54, n=16, p=0.04) or by GCDCA (r=0.76, n=7, p=0.04). The

median CDCA stimulated fold change in FGF19 expression was significantly lower in CD compared to healthy controls (87, n=12 vs 351, n=24, p=0.03) and a similar non-significant trend was observed for median GCDCA stimulated expression (114, n=11 vs 161, n=12, p=0.65).

Conclusion: Median CDCA stimulated FGF19 transcript levels were lower in CD patients and PBAD patients. As uptake of CDCA is independent of carrier-mediated apical BA uptake this implies abnormalities in ileal FGF19 production rather than active BA uptake processes. A reduced capacity to induce ileal FGF19 expression in response to BA is proposed to lead to excessive hepatic BA synthesis and increased faecal BA levels rendering CD patients and PBAD patients more susceptible to BAD.

6.1 Background

The peptide FGF19 is produced from enterocytes in response to BA absorption and binding to the farnesoid X receptor FXR. FGF19 maintains homeostasis of the BA pool size by inhibiting hepatic BA synthesis. The most potent natural ligand for intestinal intracellular FXR activation is CDCA or its conjugated form. For FXR activation to occur, BA must first be absorbed from the luminal aspect of the enterocyte into the cell. Any defect in ileal FGF19 production could involve BA uptake, intracellular pathways (FXR activation and upregulated FGF19 transcription) or post transcriptional pathways (translation and secretion).

Using our ileal explant system to study BA induced FGF19 expression will identify defects arising from either BA uptake, FXR activation or FGF19 transcription. Any defect attributable to active BA uptake is more likely to affect conjugated BA explant stimulation which is dependent on the expression of ASBT [9]. Unconjugated BA uptake occurs via passive diffusion independent of BA transporter expression.

Other groups have shown that the basal expression of other FXR target gene transcripts SHP + IBABP are reduced in biopsies from patients with CD. The same group has demonstrated inhibitory effects

of inflammatory cytokines (TNF α and IL-1 β) on FXR activation and target gene transcription via Nf-KB pathways [55] in animal studies and human cell lines. GW4046 induced FGF19 transcription was shown to be completely abolished by co-incubation with TNF α in human cell lines over 24 incubation periods. This suggests potential defects in the BA induced FGF19 stimulation pathway at both the intracellular BA-FXR binding and activation stage in addition to BA uptake stage in CD.

Using our previously described ileal explant system to study BA stimulated FGF19 transcript levels we aimed to compare the magnitude of FGF19 fold change (calculated using the comparative Ct method, chapter 5) between patient groups. We hypothesized that reduced production of FGF19 in SBAD and PBAD may occur at a transcriptional level (the intracellular BA-FXR binding and activation stage). This would lead to significantly lower BA stimulated FGF19 transcript levels in mucosal explants from patients with these BAD conditions. Any impairment in FGF19 production resulting from reduced ASBT mediated BA uptake would lead to lower BA induced FGF19 transcript levels with conjugated BA (GCDCA) stimulation relative to unconjugated BA. CDCA will enter cells via passive diffusion as opposed to active transport process requiring expression of ASBT. If the defect involves intracellular BA-FXR activation then we would expect lower BA induced FGF19 transcript levels with both GCDCA and CDCA stimulation in BAD patients.

We have proposed that in PBAD, impaired ileal FGF19 production leads to excessive bile acid synthesis and increased faecal bile acids, producing secretory diarrhoea. In SBAD of CD, defective FGF19 production results in excessive hepatic bile acid synthesis, which in the presence of sub-optimal ileal reabsorption of bile acids, will further increase faecal BA levels.

6.2 Aims:

(1) Establish if BA stimulated FGF19 transcript levels in mucosal explants from BAD patients are significantly lower compared to healthy control patients.

(2) In CD patients establish if a difference exists in the BA stimulated FGF19 transcript levels when comparing conjugated and unconjugated BA stimulation.

(3) Establish if BA stimulated FGF19 transcript levels in mucosal explants from PBAD patients are significantly lower with both conjugated and/or unconjugated BA compared to healthy control patients.

(4) Establish if BA stimulated transcript levels from other FXR responsive genes are attenuated in mucosal explants from CD patients.

(5) Perform exploratory studies on co-incubation and/or pre-incubation of ileal explants with both TNF α and BA to establish if TNF α inhibits BA stimulated FGF19 transcript levels ex vivo.

6.3 Specific Methods

6.3.1 Patients with Crohn's disease

14 patients with a histologically confirmed diagnosis of Crohn's disease were recruited. Pre defined clinical phenotypes were recorded according to the Montreal classification. Drug history and demographics were also recorded. For patients that had undergone previous ileal resection, the length of ileum removed and type of anastomosis was recorded according to previous surgical specimen records

6.3.2 Patients assessed by SeHCAT with chronic diarrhoea

16 patients with normal colonoscopy and a long term history of persistent diarrhoea (an average of 3 loose, type 6 or 7 stools per day for greater than 3 months) were recruited and then further assessed with 7 day SeHCAT scanning. Drug history and demographics were also recorded.

SeHCAT results of <15%, <10% and <5% were used to diagnose mild, moderate and severe PBAD.

Patients with other causes of diarrhoea such as GI malignancy, infection, IBD and coeliac disease

were excluded. In the 30 patients studied, bile acid stimulated transcript expression was investigated using our established ileal explant system previously described in **chapter 2.3.5** [109]. A collection of 6-9 ileal biopsies were obtained and groups of 2-3 biopsies were incubated separately with a bile acid-free culture media (unstimulated control), or 50 μ mol/L of chenodeoxycholate (CDCA) or glyco-CDCA (GCDCA). After 6h, biopsies were stored in RNAlater. RNA extraction and cDNA synthesis following standard methods. BA stimulated expression of FGF19 and other transcripts (FGF19, IBABP, ASBT, OST α , SHP) were quantified relative to GAPDH by qRT-PCR and expressed as fold changes (RQ). Stimulated explant FGF19 transcript levels were quantified relative to the transcript levels in the unstimulated explants (the 'calibrator', or internal control) and fold change (RQ) calculated using the comparative Ct method as described in **chapter 5.3.2**.

6.4 Results: Crohn's disease

6.4.1 Patient demographics

We set out to study BA induced FGF19 transcript levels in mucosal ileal explants in patients with IR-CD and NR-CD. Patients with a diagnosis of Crohn's disease were prospectively recruited whilst attending for colonoscopy. Patient clinical phenotypes and characteristics are described in **table 6.1**.

	Healthy controls	Crohn's disease
<i>n</i>	28	14
Age (median + IQR)	48 (34-58)	44 (22-74)
Sex (%)		
Male	13 (46)	4 (28)
Female	15 (54)	10 (72)
Previous ileal resection	0	4 (28)
Distribution of CD		
Ileo-colonic	-	7 (50)
Ileal	-	4 (28)
Colonic	-	3 (22)
Medication		
No medication	20 (71)	5 (43)
Long term treatments	8 (29)	8 (57)
Bile acid sequestrants	-	2 (14)
Immunomodulators	-	5 (36)
Biologics	-	1 (7)
Budesonide	-	1 (7)
Indications for colonoscopy		
Disease assessment / new diagnosis	-	12/2 (83/17)
Change in bowel habit +/- form	10	7
PR bleed	4	-
Anaemia	5	-
FH of CRC	1	-
Polyp surveillance	7	-
PR mucous	1	-

Table 6.1 Patient demographics of healthy controls and Crohn's disease patients.

6.4.2 Ileal BA stimulated FGF19 transcript levels in CD

Mean GAPDH Cts were similar to those observed in healthy control explants. Mean GAPDH Ct levels were similar with low SD in both control and CD patients with unstimulated and BA stimulated explants suggesting a low variance in the expression of this control gene across experimental conditions. Median FGF19 transcript levels were significantly higher in BA stimulated explants from CD patients compared to the paired unstimulated control explants from the same patient suggesting that BA stimulation of FGF19 expression does occur in ileum from patients with CD (stimulated explants vs unstimulated explants $p=0.0001$, for C50 and G50, comparing dCt using Mann Whitney test, **table 6.2**)

	CD patients		
	Unstimulated explants (n=14)	C50 Stimulated explants (n=12)	G50 Stimulated explants (n=11)
GAPDH Ct	23.0 (0.3)	24.1(1.0)	23.4 (0.4)
FGF19 Ct	30.6 (0.6)	24.9 (1.0)	24.8 (1.1)
FGF19 dCt	7.6 (6-10)*	1.5 (-1 – 4)*	1.2 (-1.6 – 6)*

Table 6.2 Cts from explants in CD, Crohns disease patients. C50 = explants stimulated with 50µM chenodeoxycholic acid and G50 = explants stimulated with 50µM glycol-chenodeoxycholic acid. Mean Cts and SEM are shown unless marked with an * representing median and IQRs.

6.4.3 Median CDCA stimulated FGF19 transcript levels are lower in CD ileum compared to healthy controls

The median CDCA stimulated fold change in FGF19 expression was significantly lower in CD compared to healthy control patients (87, IQR 29 -316, n=12 vs 350 IQR 161 - 485, n=24, p=0.02). No significant difference in median fold change was seen in GCDCA stimulated expression although a trend towards lower levels was observed (114 IQR 20 - 332, n=11 vs 161 IQR 77-350 , n=12, p=0.62 **Figure 6.1**).

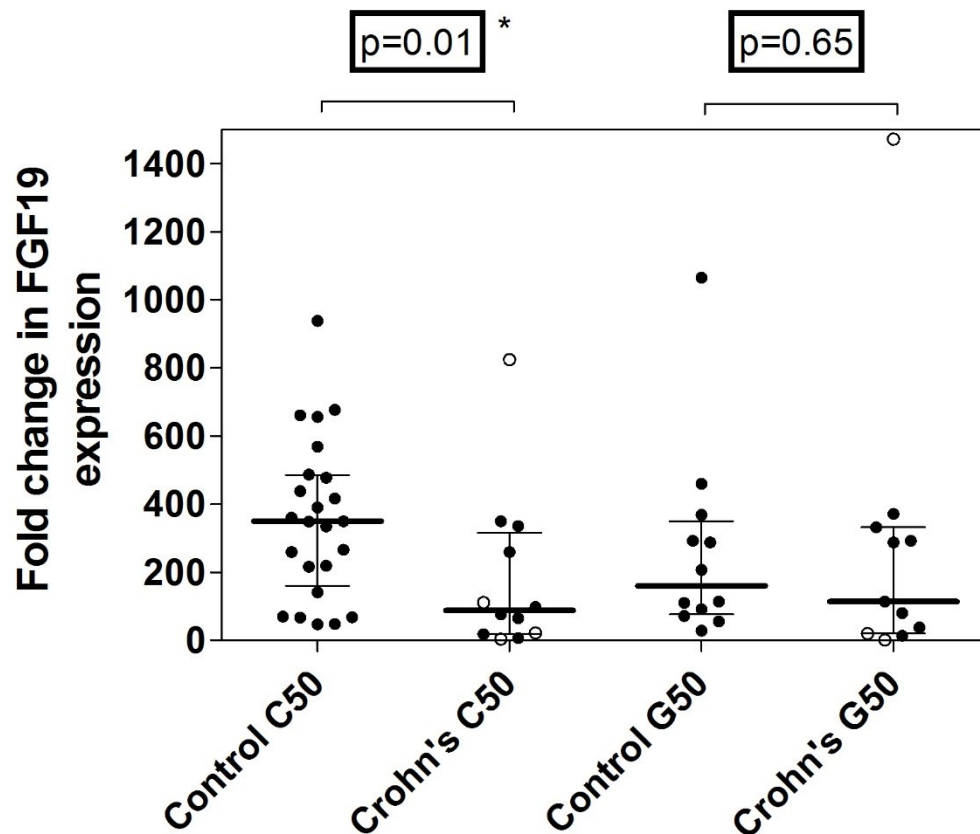


Figure 6.1 Fold change in BAstimulated expression in ileal explants in healthy controls and patients with Crohn’s disease. Open circles = ileal resected patients. Median and IQR shown.

No significant difference in median levels of BA induced FGF19 transcript levels were seen between GCDCA and CDCA stimulated explants ($p=0.69$, Mann Whitney). Similarly, there was no significant difference in median levels of CDCA induced FGF19 expression when compared to paired GCDCA stimulated explants from the same patient ($p=0.5$, Wilcoxon paired test, **Figure 6.2**). BA stimulated expression of FGF19 was not associated with age, or the presence of diarrhoea (Stool freq >3 , stool form type 5-7 BSFS) or any class of medication.

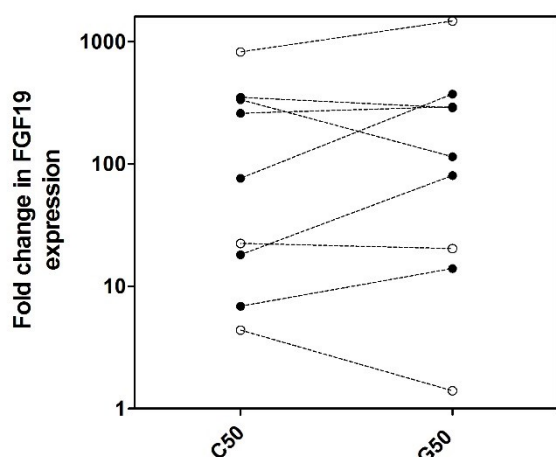


Figure 6.2: Paired fold changes in FGF19 expression from ileal mucosal explant incubations with both CDCA (C50) and GCDCA (G50) from 9 individual patients. Open circles: represent neo-terminal ileal explants (patients with previous right hemicolectomy and ileal resection). Lines represent paired ileal explants from the same patient.

Induction FGF19 expression was also demonstrated with OCA at doses of 1 and 20uM and CDCA at lower doses of 20uM in patients with CD.

6.4.4 Post-hoc analysis of BA stimulated FGF19 transcript levels in IR-CD, neo-terminal ileum

Observing BA stimulated transcript levels in the 4 patients who had undergone previous ileal resection revealed a trend towards a lower fold changes in BA stimulated expression compared to healthy controls with the exception of one outlier (red circles, **Figure 6.3 table 6.3**).

pt	Age	s	Phenotype	IR (cm)	Medication	Stool Freq	Stool form	C50	G50
1	66	f	Ileo-colonic	45	Colesevalam azathioprine	5	7	4.4	1.4
2	68	f	Ileal	51	Colesevalam	6	5-7	824	1473
3	45	f	Ileal	15		3	5	111	
4	68	f	ileal	15		3	6	22.4	22.3

Table 6.3 Patient demographics of IR, ileal resection patients with Crohn's disease studied with ileal explant studies.

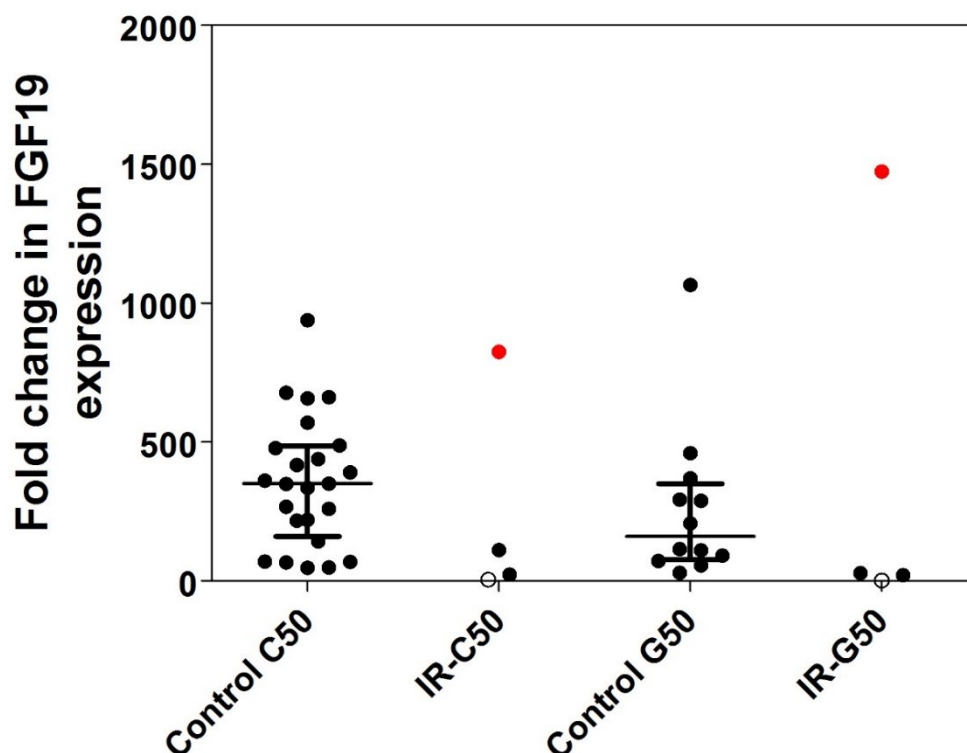


Figure 6.3. Comparison of fold change in BA stimulated FGF19 expression in neo-terminal ileal mucosal explants. Open circles = CDCA stimulated and GCDCA stimulated expression in a patient with 2 previous ileal resections (Pt 1). Red circles = FGF19 expression in BA stimulate explants with GCDCA and CDCA in a patient of 51cm ileal resection (Pt 2). C50 = Chenodeoxycholic acid at 50uM, G50 = Glycho-chenodeoxycholic acid, IR= Ileal resected patients. Median and IQRs are shown where appropriate.

6.4.5 BA stimulated Ileal SHP and IBABP transcript levels in patients with NR-CD

Fold change in CDCA and GCDCA stimulated SHP or IBABP expression were small but significant in CD patients ranging from 2 -20 fold. This is similar to fold changes in SHP and IBABP expression observed in healthy controls seen in **Chapter 5.3.6**.

There was no significant difference in median CDCA stimulated IBABP transcript levels comparing 8 CD patients to 6 healthy control patients (Stimulated IBABP median fold change in healthy controls 3.56 IQR 1.8-4.8 vs CD patients 1.87 IQR 1.3 – 4.0, $p=0.32$, $n= 8$). No significant difference in GCDCA stimulated transcript levels were seen (stimulated IBABP median fold change in healthy controls 1.9 IQR 1.7-3.3 vs CD patients 2.1 IQR 0.7-5.5, $p=1.0$). Likewise, fold change in SHP transcript levels

showed no significant differences with CDCA or GCDCA stimulated explants in 8 CD patients compared to 6 healthy control patients ($p = 0.70$ and $p = 0.72$ respectively).

6.4.6 Post-hoc analysis of Ileitis and BA stimulated expression of FGF19 transcripts in non ileal resected patients

Post hoc analysis of the effects of ileal inflammation on BA stimulated expression of FGF19 was assessed. Ileal inflammation was either endoscopically evident as inflammation or ulceration within the terminal ileum (in 3 patients with NR-CD) or found after histologic examination of biopsies (microscopic ileitis) taken from the terminal ileum at the time of research biopsies. Evidence of microscopic inflammation was part of the exclusion criteria for the healthy control cohort. Patient characteristics are shown in **Table 6.4**. Patients with previous ileal resections were excluded from this part of the analysis to avoid the confounding effect of resection may have on ileal inflammation.

Within this group of patients GAPDH transcript levels were within expected levels with low variability. FGF19 transcript levels were significantly induced in all patients with both macroscopic or microscopic ileitis. The median fold change in FGF19 transcript expression from CDCA stimulated explants was not significantly different in patients with ileitis compared to healthy controls although a possible trend towards lower induction may be present ($p=0.12$, **figure 6.4**).

Age	Sex	PMH	DHx	Ileal histology	Overt ileitis	C50
24	f	Obstetric cholestasis		Villous shortening and chronic inflammation	Nil	316
62	f	Renal transplant	Mycophenolate	Mild chronic inflammation lymphocytic infiltrate	Nil	220
25	m	UC	Asacol	Focal granuloma, chronic inflammation	Nil	70
57	f	Hyperthyroid	Carbimazole	Lymphocytic infiltrate and chronic inflammation	Nil	360
52	f	Crohns	Azathioprine	Chronic inflammation	Apthous ulcers	260
74	m	Crohn's and renal dialysis	Enalapril, irbesartan, atenolol, aspirin, adcal	Chronic inflammation	Inflammation + ulceration	98
42	f	Crohn's		Chronic inflammation	Apthous ulcers	65

Table 6.4. Patients demographics of patients found to have macroscopic ileal inflammation at the time of colonoscopy or microscopic inflammation on subsequent ileal histology.

Within this group of patients GAPDH transcript levels were within expected levels with low variability. FGF19 transcript levels were significantly induced in all patients with both macroscopic and microscopic ileitis. The median fold change in FGF19 transcript expression from CDCA stimulated explants was not significantly different in patients with ileitis compared to healthy controls although a trend towards lower induction may be present ($p=0.12$, **figure 6.4**).

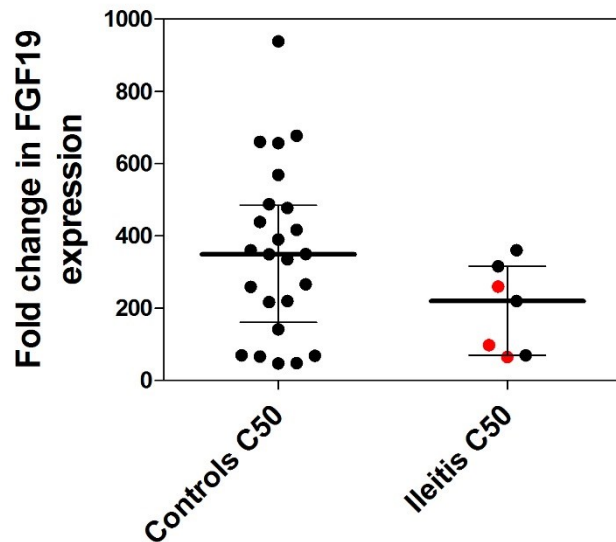


Figure 6.4. Comparison of fold change in FGF19 expression with 50uM CDCA (C50) in healthy controls (n=24) and non ileal resected patients with either endoscopically visible overt ileitis (red dots) or microscopically evidence on subsequent histological analysis of ileal biopsies (n=7).

6.5 Results: PBAD: Positive correlation between BA stimulated FGF19 transcript levels and SEHCAT retention levels.

FGF19 expression was studied in 16 patients with diarrhoea in ileal explants cultured for 6h with CDCA (50μmol/L), GCDCA or under control conditions using the ileal explant system described elsewhere. Seven patients had PBAD (SeHCAT <15%) and 9 were ID controls (SeHCAT >15%).

FGF19 transcript expression was stimulated in most subjects, with a median of 184-fold with CDCA, similar to values found previously [109]. The lowest fold changes in FGF19 expression were observed in biopsies from patients with the lowest SeHCAT retention values (**Figure 6.5**). Positive correlations were found between SeHCAT retention and the magnitude of the fold change in FGF19 expression stimulated by CDCA ($r_s = 0.54$, $p = 0.04$, $n = 16$) and by GCDCA ($r_s = 0.61$, $p = 0.07$, $n = 7$). Confirming our previous findings, IBABP, OSTα and SHP transcripts were also significantly stimulated

to lesser degrees (all $p < 0.002$) but ASBT was not affected (**Table 6.5**). Apart from FGF19, only the degree of stimulation of IBABP correlated with SeHCAT ($r_s = 0.47$, $p = 0.04$, $n = 14$).

Transcript	Stimulation (RQ) by CDCA	Correlation with SeHCAT	p
FGF19	184	0.54	0.02
IBABP	2.2	0.47	0.04
OST α	2.8	-0.10	NS
SHP	2.6	-0.16	NS
ASBT	1.0	-0.05	NS

Table 6.5 Stimulation of ileal transcripts by chenodeoxycholic acid (CDCA) and relationship to SeHCAT. Median values for stimulation with CDCA 50 μ mol/L for 6h in ileal explant system shown. Spearman rank correlation coefficients and p values are shown for the relationships between stimulation and SeHCAT values. N=12-16 patients. NS, $p > 0.05$

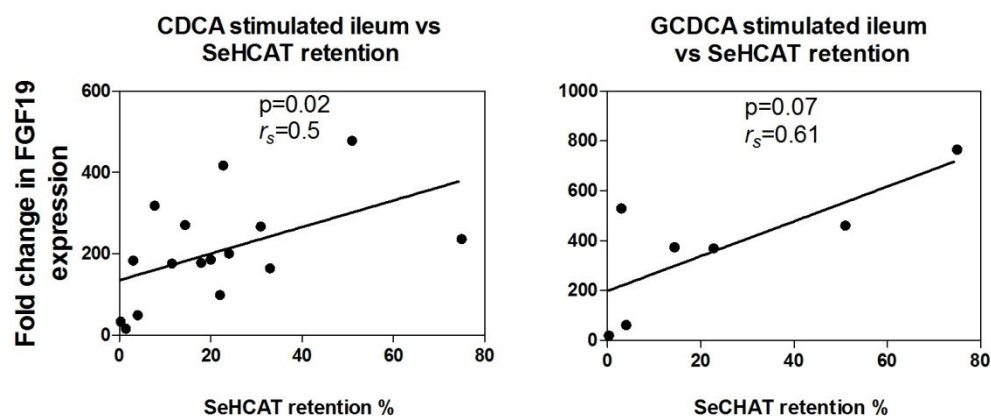


Figure 6.5. Stimulation of ileal FGF19 transcript expression related to SeHCAT 7d retention. The fold-change in RNA expression is shown with CDCA in (a) and with GCDCA in (b). The linear regression lines are shown.

6.6 Pilot data on the effects of TNF α on BA stimulated FGF19 transcript levels

Preliminary data was obtained investigating the effects of TNF α on BA stimulated transcript levels. BA stimulated FGF19 transcript levels were lower in the 2 patients whose explants were incubated with CDCA and TNF α compared to explants incubated in parallel with CDCA only from the same patient.

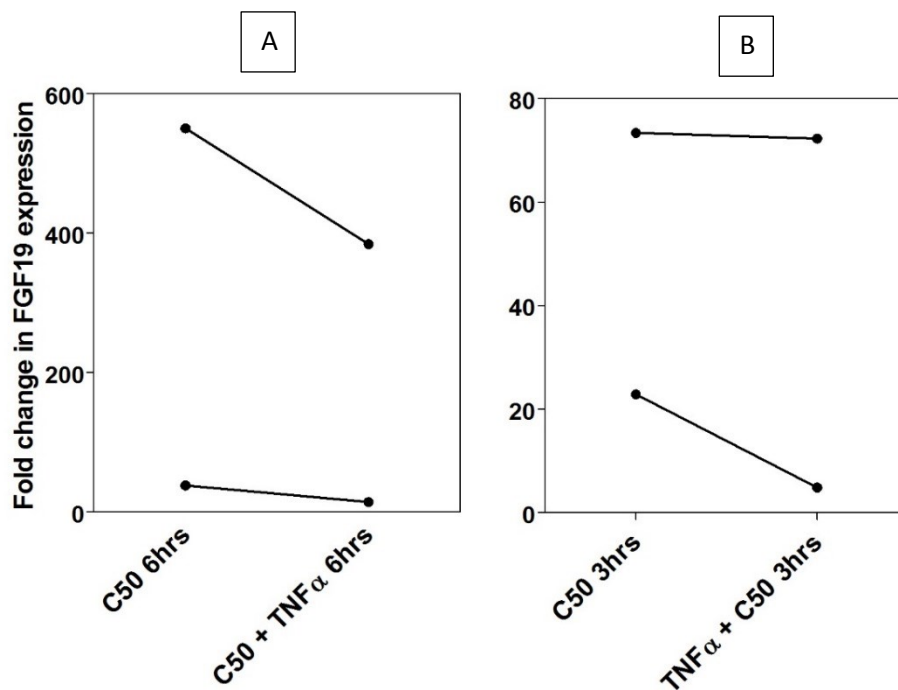


Figure 6.6 Fold changes in BA stimulated FGF19 expression with and without exogenous TNF α . **A.** (C50 6hrs) Mucosal explants incubated 50uM CDCA or (C50 + TNF α 6hrs) 50uM CDCA + TNF α for 6 hours. **B** (C50 3hrs) Mucosal explants pre- incubated in BA free media for 3 hours then moved to media containing 50uM CDCA (TNF α + C50 3hrs), Mucosal explants incubated in BA free media + TNF α for 3 hours, then moved to media containing 50uM CDCA and TNF α for 3 hours. Each fold change is calculated relative to an internal BA free control incubation in the standard way (chapter 5).

6.7 Discussion

The hypothesis of this chapter was supported by demonstrating that lower ileal BA stimulated FGF19 transcript levels are found in patients with BAD. Median CDCA stimulated FGF19 transcript levels were significantly lower in explants from patients with CD than healthy controls. Patients with chronic diarrhoea and low SeHCAT levels also had low levels of BA stimulated FGF19 expression. This points towards a potential defect in the FXR-FGF19 pathway, in response to BAs, occurring at a transcriptional level in some of these patients.

Lower FGF19 transcript levels in GCDCA rather than CDCA stimulated explants were expected in CD due to previously described reduced ASBT expression and presumed reduced conjugated BA uptake. However we observed the converse (significantly lower levels of BA stimulated FGF19 transcript levels in explants stimulated with CDCA rather than GCDCA). It may be that defects in the FXR-FGF19 pathway occurring post uptake of BA into the ileal enterocyte play a greater role in the pathogenesis of BAD than BA malabsorption.

It is possible that medications taken by some of the patients with CD may have influenced GCDCA stimulated FGF19 expression. In Jung et al's study on CD patients (where ASBT protein expression was found to be 70% that of healthy controls) it is important to note that these patients were not on any medication 5 days prior to the study or during the study. Whereas 7 of the 14 CD patients in our study were on medications (1x receiving IFX, 5 immunomodulators and 1x budesonide). It is possible that medications may restore ASBT expression as observed with budesonide and other glucocorticoids. Two glucocorticoid response elements were found in the ASBT promoter conferring inducibility by glucocorticoids. Although only one of our CD patients studied was taking budesonide ASBT expression may have been influenced by other pathways modulated by other immunomodulatory medication or biologic therapy.

However, no significant differences were found in FGF19 transcript levels in the patients taking medication compared to those on no medication. The contribution of ASBT expression on FGF19 responses could have been controlled for further by analysing ASBT transcript levels in these mucosal explants and expressing FGF19 levels as a proportion to ASBT levels.

To separate any confounding effects from anatomical differences in expression of carrier mediated BA uptake or FGF19 expression that may result from ileal resection it was important to consider patients with previous ileal resection separately in post-hoc analysis. (table 6.2). Out of the 4 IR CD patients studied, 3 demonstrated relatively low fold changes in BA stimulated FGF19 expression. This included one patient (Pt 1) with 2 previous ileal resections and a cumulative TI resection length of 45 cm. This patient was one of the only patients that did not demonstrate a significant fold change in ileal transcript expression levels in GCDCA stimulated explants. This patient only had a fold change of 1.4 in BA stimulated expression compared to the paired unstimulated mucosal explants.

One of the other patients (pt 2) who also had undergone an extensive ileal resection in the past (51cm) displayed the highest observed fold change in the GCDCA stimulated FGF19 expression. Pt 2 had been taking a BA sequestrant (cholesevalam) up to 48 hours prior to the colonoscopy, whereas pt 1 had stopped her BA sequestrant (Questran) 1 week prior to the colonoscopy. Previous studies on serum FGF19 levels have shown dramatic decreases in levels after and during oral administration of BA sequestrants [22] . What has not been described so well is what happens to FGF19 levels after stopping such treatments. Pattni had performed a pilot study on 2 healthy control subjects and demonstrated a significant rebound phenomenon after stopping BA sequestrants with fasting serum levels increasing 2 -3 times higher than baseline pre-treatment levels for up to one week after treatment cessation (Sanjeev Pattni, Imperial College MD).

If the outlier with the high FGF19 transcript levels is excluded then this exploratory post-hoc data shows very low levels of BA induced FGF19 stimulation in these patients suggesting a reduced capacity to induced FGF19 transcription in response to BA in neo-terminal ileum. As the finding is

observed with both G-CDCA and CDCA this reduced capacity may not simply be due to reduced ASBT expression in neo-terminal ileum. Again this could have been controlled for by analysing ASBT transcript levels in each set of explants and expressing FGF19 as a proportion to ASBT levels.

We know that the capacity for active BA uptake is highest at the terminal ileum just upstream to the ICV . However it is not known how the capacity to produce FGF19 varies along the small intestine. We know that the basal expression of FGF19 transcripts are higher at the TI compared to duodenum. One would expect a gradient of increasing expression of FGF19 or capacity as one moves from proximal to distal small intestine as with ASBT but this has not yet been confirmed.

The low or absent BA FGF19 response in the pilot studies on neo-terminal ileum from patients with resection lengths ranging from 15-51cm could indicate that BA induced FGF19 response may be restricted to a limited portion of TI. If the expression of FGF19 is more restricted than the distribution of ASBT expression (and carrier mediated BA uptake) then FGF19 signalling rather than BA malabsorption could be a predominant factor in the aetiopathogenesis of BAD in some patients with CD. Relatively limited ileal resections or inflammation could significantly disturb FGF19 production with minimal effects on BA absorption. It is not known to what extent BA absorption changes as part of the intestinal adaptation that can occur after resection. Further studies on neo-terminal ileum and the distribution of FGF19 and ASBT expression along the small intestine are required.

Regarding PBAD, the positive correlation with both GCDCA and CDCA stimulated FGF19 expression with SeHCAT values is also indicative of a defects in FXR-FGF19 activation that may occur pre- and post BA uptake. Unstimulated ASBT transcripts are expressed at lower levels in biopsies from patients with low SeHCAT levels (Chapter 4). This could in itself result in reduced bile acid uptake and consequently reduced bile acid/FXR-dependent stimulation of FGF19 transcription in Low SeHCAT patients. However, again, this appears not to be the only reason, as incubation with CDCA also gives SeHCAT-dependent FGF19 transcript stimulation. Unconjugated CDCA, unlike glycine-

conjugated GCDCA, is not dependent on transport by ASBT and will produce FXR-mediated transcription in cells not expressing ASBT [9, 137].

If the defect in FGF19 production in PBAD does lie with BA stimulated FXR activation then one might expect to see a relationship with SeHCAT values and other FXR target genes. A similar positive correlation was observed with BA stimulated IBABP transcript levels and SeHCAT values. However the BA stimulated responses in other target genes (OSTa, ASBT, SHP) studied here is very small, as in chapter 5 (median twofold difference in expression), compared to FGF19 and it may be difficult to see a relationship with such small BA stimulated expression levels.

The mechanism of a potential defect in BA stimulated FXR activation of FGF19 transcription is not clear in PBAD. Cell cultures incubated with FXR agonists and inflammatory cytokines (TNF α , IL-1B, IL-6) have implicated Nf-KB suppression of FXR activation and FGF19/15 transcription. However PBAD has not been associated with ileal inflammation and all of the PBAD patients studied here had histologically normal TI biopsies. It is possible that PBAD is associated with sub-clinical upregulation of inflammatory cytokines. Increased cytokine secretion (TNF α , IL-1B, IL-6) has been demonstrated upon LPS activation of PBMC in patients with IBS-D compared to healthy controls [161]. Enhanced immune activation is thought to play a role in symptoms of IBS, particularly those suffering from chronic diarrhoea [162, 163].

Other transcription factors may affect FXR activation and FGF15/19 transcription. Cross talk between transcription factors is well described (ref). Transcription factors with an inhibitory influence on FXR have recently been described such as SIRT1[131, 164] or Yin Yang-1 [165]. FXR activity is also affected indirectly by other pathways; for instance, intestinal peroxisome proliferator-activated receptor α -UDP-glucuronosyltransferases signalling has recently been suggested. Mouse models Dextran sulphate sodium (DSS)-induced colitis demonstrate activated intestinal peroxisome PPAR α -UGTs pathway in inflamed colonic mucosa. UGTs normally accelerate the metabolic elimination of BAs, and thereby decrease their intracellular levels in the small

intestine. Activation of UGTs mediated by inflammation therefore lead to reduced intracellular BAs resulting in repressed FXR-FGF15 signalling [132].

In conclusion, results from this chapter have demonstrated that patients with primary or secondary bile acid diarrhoea may have a reduced capacity to produce FGF19. Increased faecal BA may occur as a result of BA malabsorption in CD but malabsorption has never been demonstrated in PBAD patients. The relationship between both conjugated and unconjugated BA stimulated FGF19 and SeHCAT retention levels also supports our hypothesis.

Low seHCAT retention levels represent increased faecal BA loss from the enterohepatic circulation. Increased BA loss can occur through other mechanisms other than ileal malabsorption. Low FGF19 levels would lead to increased synthesis of new BA. The bile acid pool size would be larger and with ileal reabsorption at saturation point a new steady state would ensue with greater levels of BA entering the colon and being lost in the faeces.

Chapter 7: The response of patients with secondary bile acid diarrhoea to the farnesoid X receptor agonist obeticholic acid

Abstract:

Background & Aim: Bile acid diarrhea (BAD) is a common yet unrecognized cause of chronic diarrhea secondary to ileal disease or resection in Crohn's disease. Many patients have reduced levels of the ileal hormone fibroblast growth factor 19 (FGF19), a negative regulator of hepatic bile acid synthesis, secreted in response to farnesoid X receptor (FXR) agonists. We hypothesized that obeticholic acid (OCA), a potent FXR agonist, could increase FGF19 in BAD patients and produce clinical benefits.

Methods: After a 2-week run-in when bile acid sequestrants were discontinued, patients with previously diagnosed Crohn's disease and secondary BAD (n=10) or idiopathic chronic diarrhea (n=8), received oral OCA 25mg daily for 2 weeks. Serum FGF19, total bile acids and 7 α -OH-4-cholesten-3-one (C4) were measured fasting and for 6h before and after 2w OCA treatment. Symptom diaries were recorded.

Results: In the group of patients overall OCA increased median fasting FGF19 from 43 to 104 pg/mL ($P=0.03$) and significantly reduced fasting C4 and bile acid responses. In the secondary BAD group, improvements occurred in stool form ($P=0.04$) and in a diarrhea index ($P=0.03$) and this was associated with increases in FGF19 and reductions in C4 and BA levels in patients with shorter ileal resections (resections < 45cm, n=7/10). Symptoms of abdominal pain and urgency also improved. FGF19, C4 and bile acids changed in the idiopathic diarrhea control group, but no significant clinical improvements occurred. Total and LDL cholesterol increased. OCA treatment was well tolerated.

Conclusions: This proof-of-concept study has shown that OCA stimulates FGF19, reduces bile acid synthesis and produces clinical benefits in most patients with secondary BAD. FXR agonists have therapeutic potential in diarrhoea in symptoms of CD.

7.1 Background

FGF19 production in the ileum is stimulated by BA binding to the farnesoid X receptor (FXR) and activating transcription [7]. Using explants of human ileum, we showed that FGF19 transcripts and protein levels were potently stimulated by chenodeoxycholic acid (CDCA), the most active natural FXR agonist [109]. More potent FXR agonists have been synthesized with the aim of treating liver and other disorders [152]. Obeticholic acid (OCA, also known as 6 α -ethyl-CDCA and INT-747) is about 100-fold more potent as an FXR agonist than CDCA [166]. Clinical trials of OCA in primary biliary cirrhosis and non-alcoholic steatohepatitis (NASH) have been published or are in progress [87, 167, 168].

We hypothesized that, if potent FXR agonists such as OCA were to stimulate FGF19 in patients with BAD, this would reverse the impaired regulation of BA synthesis and, by reducing BA production, lead to clinical improvement. We anticipated that the effect would be most marked in primary BAD but that impaired BA absorption could limit the effect in secondary BAD. To test this hypothesis, we have performed a proof-of-concept study in two groups of patients, with secondary BAD, and idiopathic controls with chronic diarrhea.

Methods are outlined in Chapter 2.8

7.2. Results

7.2.1 Study patients

A total of 25 patients entered the study but seven patients (three in the secondary BAD group and four in the chronic idiopathic diarrhea control group) withdrew for personal reasons before the first dose of OCA. None of these patients received OCA. Consequently, 18 patients (10 secondary BAD and 8 idiopathic diarrhea controls) received OCA and had blood sampling. Further details of these groups are shown in **Table 1**.

	Secondary BAD	Idiopathic controls
Number	10	8
Sex (%)		
Male	3 (30)	5 (63)
Female	7 (70)	3 (37)
Age (y)		
Median	45	39
Range	27 – 71	25 – 68
SeHCAT (7d retention %)		
Number	6	8
Median	0.6	25.7
Range	0 – 10.4	16.0 – 40.0
Previous ileal resection (n)	8	0
Median length (cm)	34	-
Range (cm)	0 – 71	-
0 – 45 cm (n)	7	-
>45cm (n)	3	-
Previous BA sequestrant use (n)		
Yes	4	0
No	6	8

Table 1. Demographics of the two patient groups in this study

7.2.2 FGF19

The baseline (D0) median fasting FGF19 in the secondary BAD group (32 vs 57ng/L) was significantly lower than the idiopathic control group (0.004, Mann-Whitney test). The changes in fasting FGF19 after 2 weeks of OCA treatment (D14) were variable in the secondary BAD group (median % increase over baseline = 25%, IQR -6 – 140; $P=0.11$) with some patients having large increases and others having only small changes. Serial FGF19 samples post OCA and after meals revealed a generally ‘flat’ response, **figure 1**. In the idiopathic control group, FGF19 increases were also variable and did not reach significance (median % change = 130%, IQR -14 – 304; $P=0.12$).

As expected, FGF19 increased in the serial blood samples taken for 6h after the OCA doses. The groups differed in D0 AUC (Mann Whitney, $P=0.002$) and peak FGF19 values ($P=0.0005$) with the responses being higher in the idiopathic controls than the secondary BAD groups. There were no significant differences between the responses on D0 compared to D14 in FGF19 AUC or 6h peak in either group.

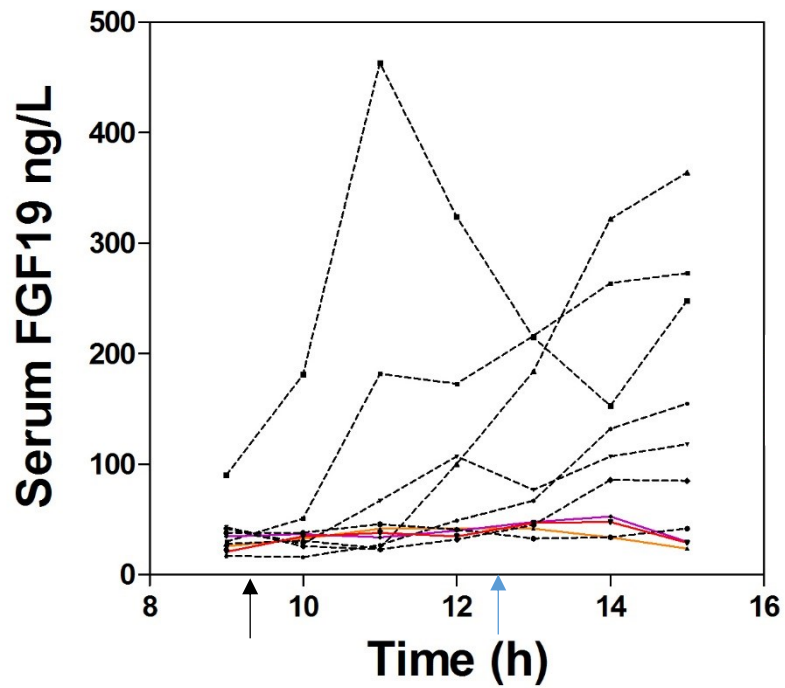


Figure 7.1. Serial FGF19 levels in SBAD patients following an overnight fast on the first day of the study D0, serial FGF19 measurements taken before and after OCA + breakfast administration (black arrow) and lunch (blue arrow). The 3 coloured lines represent the values from the 3 patients with ileal resection >45cm.

	Secondary BAD (n=10)			Idiopathic controls (n=8)			Overall (n=18)		
	D0	D14	P	D0	D14	P	D0	D14	P
FGF19 (ng/L)									
Fasting	32 24–42	46 24–72	0.11	116 57–186	194 126–344	0.12	43 29–114	104 41–205	0.03
6h response AUC	401 267–1086	457 316–1016	0.51	1644 1247–2776	2099 1972–2341	0.13	1105 324–1675	1120 389–2097	0.07
6h peak	102 47–296	126 58–229	0.96	530 392–805	588 480–800	0.40	319 78–528	313 108–580	0.6
C4 (µg/L)									
Fasting	104 48–134	56 14–122	0.11	9 3–14	1 1–3	0.02	26 8–112	9 1–74	0.01
Bile Acids (µmol/L)									
Fasting	2.5 2.0–6.0	2.5 1.0–4.0	0.12	1.5 1.0–2.8	1.0 0.9–1.8	0.12	2.0 1.0–3.5	1.5 1.0–3.3	0.1
6h response AUC	32.0 20.5–39.0	22.0 15.8–30.0	0.04	29.0 23.2–44.7	17.0 10.6–20.0	0.02	31 22.0–39.0	20.0 14–30.0	0.002
6h peak	6.5 3.8–9.3	4.5 3.0–6.3	0.21	8.0 7.0–11.5	4.0 3.0–4.0	0.02	7.0 6.0–10	4.0 3.0–6.0	0.004

Table 2. FGF19, C4 and Bile Acids: fasting , 6h AUC and peak values on Day 0 and Day 14. Values are medians and IQR. AUC = area under the curve. D0 and D14 values were compared by Wilcoxon paired rank tests. Significant values ($P < 0.05$) are in bold

7.2.3 7 α -hydroxy-4-cholesten-3-one (C4)

Fasting C4 values were significantly different between the groups (**Table 2**), with those with secondary BAD being much higher than the controls. OCA treatment reduced C4 in both groups, but this did not reach significance in the entire secondary BAD group.

7.2.4 Total bile acid values

Fasting BA values were not different between the groups (**Table 2**). After 2w OCA treatment, median fasting values were lower overall but this did not reach significance. The BA increases after OCA and meals during the 6h serial sampling showed significant changes after 2w OCA treatment.

7.2.5 Changes in clinical symptoms

There were changes in clinical parameters during the 6 weeks of the study; In the secondary BAD group there were significant changes with OCA therapy in stool form ($P=0.04$) and in the stool index ($P=0.03$, **table 3**). The median % index change was -29%. These secondary BAD patients' symptoms were variable, with some being more severely affected than the primary patients. One patient had a weekly stool number of over 100 type 6-7 stools. Of the 4 patients who needed loperamide rescue therapy, none was able to stop it.

The patients with long resection responded less well than those with short or no resection. Limiting the analysis to the seven patients with an ileal resection length < 45cm produced significant changes in stool number ($P=0.03$), stool form ($P=0.02$) and index ($P=0.02$). This group also had significant changes in FGF19 (38 to 50, $P=0.03$) and C4 (90 to 32, $P=0.02$).

Patients in the idiopathic diarrhea control group showed no significant changes in median stool number, form or index. Changes were generally small; the median % index change was +6%.

Associated symptoms showed improvement in many patients. There were significant median reductions in urgency score in secondary BAD with resection < 45cm (% change -56%, $P=0.03$). Pain

scores in hours (% change -64%, $P=0.03$) and severity (% change -71%, $P=0.04$) both improved in the secondary BAD group. None of these symptoms changed significantly in the idiopathic control group. Bloating scores were unaffected.

	Weekly number of stools			Mean stool form (BSFS)			Stool index ^a		
	W2	W4	<i>p</i>	W2	W4	<i>p</i>	W2	W4	<i>p</i>
Secondary BAD	23	17		6.0	5.6		132	127	
n=10	14–44	17–42	0.17	5.3–6.9	4.6–6.7	0.04	72–473	47–321	0.03
Resection <45cm	17	12		5.7	5.2		95	62	
n=7	15–29	10–27	0.03	4.4–6.2	4.2–6.0	0.02	71–202	42–166	0.02
Idiopathic	15	18		4.9	4.9		96	83	
n=7 ^b	11–17	13–19	0.31	4.3–5.9	4.1–5.0	0.74	69–100	65–101	0.61
Overall	16	17		5.7	5.0		96	88	
n=17	12–29	12–27	0.82	4.5–6.4	4.3–6.0	0.27	72–186	60–171	0.44

Table 3. Values are medians and IQR. Week 2 (W2) and 4 (W4) values were compared by Wilcoxon paired rank tests. BSFS = Bristol Stool Form Scale. Significant values ($P < 0.05$) are in bold.

^a The stool index = [(weekly stool frequency x mean BSFS] + loperamide use [weekly mg x 3]).

^b One patient did not return diaries that could be analyzed

In the secondary BAD group, the change in stool number was associated with the % change in fasting FGF19 ($r=-0.56$, $P=0.05$). The resection length was associated overall with % changes in stool form ($r=0.54$, $P=0.05$), stool number ($r=0.46$, $P=0.09$) and in urgency ($r=0.77$, $P=0.01$). BA D0 AUC and percentage change in stool number correlated ($r=0.56$, $P=0.05$), but changes in BA were not significantly related to clinical symptoms overall in this group.

7.2.6 Ileal resection length

SeHCAT retention was inversely related to ileal resection length ($r=-0.97$, $P=0.001$), positively to FGF19 D0 six hour responses (AUC $r=0.99$, $P<0.001$; peak $r=0.93$, $P=0.004$) and C4 ($r=0.76$, $P=0.04$), and inversely with BA changes (AUC $r=-0.99$, $P<0.001$; peak $r=-0.91$, $P=0.01$). The percentage change in fasting C4 was associated with the percentage change in stool number ($r=0.80$, $P=0.003$).

Resection length was also inversely associated with FGF19 fasting, AUC and peak values ($r=-0.54$, -0.91 , -0.81 , $P=0.06$, <0.001 , 0.002 respectively); BA peak on D0 and the percentage change

in BA AUC ($r=-0.54, 0.63, P=0.05, 0.03$ respectively); and C4 for D0 and D14 fasting values and percentage change ($r=0.73, 0.84, 0.71, P=0.008, 0.001, 0.01$, respectively).

7.2.7 Idiopathic diarrhoea controls

In the idiopathic chronic diarrhoea controls, although there were no significant clinical median differences, age was associated with the percentage changes in index and urgency ($r=-0.68, -0.77, P=0.05, 0.04$, respectively), so the older patients had bigger improvements. Fasting FGF19 on D0 and D14 and the index were positively associated (D14, $r=0.75, P=0.03$), meaning in this group that the lower FGF19 values had the larger decreases in the index. Clinical changes (stool frequency and form) were negatively associated with the percentage change in BA AUC ($r=-0.75, -0.77, P=0.04, 0.04$ respectively), so that bigger reductions in the BA response were associated with smaller changes in the symptoms.

7.2.8 Tolerability, liver enzymes and lipid analyses

OCA was well tolerated and only a few minor adverse effects were reported of limited clinical significance. Three patients (2 in the secondary BAD group and 1 in the idiopathic group) reported mild headaches during OCA treatment in week 3 or 4. One patient recorded that she was suffering from “a viral infection” at the time of the headache. In the absence of a placebo-treated group, it is uncertain how these relate to OCA treatment. Another patient with Crohn’s disease experienced worsening of abdominal pain after starting OCA and did not have a bowel movement for 24h. Her symptoms settled on omitting OCA for 1d and then continuing with OCA 25mg on alternate days.

Lipid measurements showed higher values for total and LDL-cholesterol (median % increases of 10.4 and 19.7 respectively, both $P<0.001$ overall) and a reduction in triglycerides (median % decrease of 11.8, $P=0.06$) after 2w OCA. Similar magnitudes of changes were found in each group and are

comparable to those published previously [87]. There were no significant changes in median values of LFTs including alkaline phosphatase, alanine transaminase, bilirubin and albumin.

7.3 Discussion

Results from this work contributed to the first study of an FXR agonist, OCA, being used as a treatment for diarrhoea [96]. It is the first study to demonstrate that FGF19 levels increase after 2 weeks of OCA in patients with chronic diarrhoea. Previous work (chapter 3) had shown very low levels of FGF19 in patients with CD and that reduced levels were associated with the severity of symptoms of diarrhoea and the length of ileum resected [169]. In this study FGF19 was shown to significantly increase after 2 weeks of OCA in CD patients with limited ileal resections (excluding 3 patients with resections > 45m), resulting in significant reductions in BA synthesis and improvements in symptoms of diarrhoea. Clinical improvements were not seen in the idiopathic control patients despite similar changes in FGF19 levels and BA synthesis.

The group of patients (not part of this thesis) that benefited the most symptomatically in this study were the primary BAD patients [96]. These patients had the largest increase in fasting FGF19 and reductions in C4 and BA levels. It is likely that these patients achieve the greatest reductions in faecal BA levels that drive the secretory diarrhoea in this condition. Patients with SBAD are usually observed to have the highest concentrations of faecal BA concentrations and may have the potential to gain most from treatments aimed at reducing faecal BA. However it appears that OCA administration is more effective at stimulating FGF19 in PBAD than SBAD probably due to the intact ileum in the former condition.

Idiopathic diarrhoea patients did not demonstrate any significant clinical improvements with 2 weeks of OCA treatment despite significant reductions in C4 and BA levels. It may be that faecal BA levels are reduced in this group of patients but this may not have any beneficial effects if the faecal BA concentrations are not already present at pathological concentrations.

In the SBAD group, a number of correlations were observed between ileal resection length and the biochemical and symptomatic changes induced by 2 weeks of OCA. This underlines the importance of the terminal ileum in the expression of circulating FGF19 levels. We had considered excluding patients with more extensive ileal resections prior to recruitment based upon the observed 'flat' post prandial FGF19 response observed in 3 CD patients (Chapter 3). However extensive ileal resected patients can be the most severely affected patients with SBAD so we felt it was important to include them.

The increase in median fasting levels of FGF19 in conjunction with the decrease in median C4 levels after 2 weeks of OCA administration suggests that OCA has been retained within enterohepatic circulation of BA despite limited ileal resections in most patients. However, in patients with more extensive ileal resection, increases in fasting FGF19 were not seen after 2 weeks of OCA. The 3 patients with resections >45cm were 3 of only 4 patients in which the post-OCA, post prandial FGF19 levels revealed a completely flat response similar to the post prandial responses observed in chapter 3. This implies that the greater potency for FXR binding and activation has not overcome the flat post prandial FGF19 response in similar patients in this study.

There are two possible explanations for the lack of post OCA and meal induced rise in FGF19 with OCA administration in resected patients: Either OCA is not being absorbed or FGF19 is only secreted in response to BA absorption from a limited portion of terminal ileum. Previous bile acid kinetic studies suggest that the latter is more likely. OCA tends to follow the kinetics of any other orally administered BA. Peak plasma concentrations occur within 90-120 minutes. OCA is a derivative of CDCA, 6 ethyl-CDCA. Post prandial rises in CDCA generally tend to be greater than cholic acid. This may relate to the faster jejunal rates of absorption of unconjugated CDCA compared to other unconjugated bile acids [140]. Previous studies on patients with ileal resection have shown that the post prandial rise in serum CDCA are generally preserved despite involving patients with resection lengths greater than our patients ranging from 50 - 280cm [170]. It was also noted that having an

ileo-transverse, rather than an ileo-ascending colon anastomosis may attenuate the post prandial rise also.

It is important to point out that a lack of FGF19 stimulation does not necessarily imply a lack of FXR activation. OCA induced FXR binding and activation has been proposed to promote anti-inflammatory and protective intestinal pathways [86, 171]. FXR expression appears to be more widespread than FGF15/19 across the intestine [118]. FXR agonists may also have direct roles on the colonic epithelium, regulating chloride and water secretion [172]. Results from this study would suggest that the results of FXR activation on ileal FGF19 secretion predominate on colonic function in the context of chronic diarrhoea. OCA may be best placed for use in patients with ileal Crohn's disease or limited ileal resection as both an oral anti-inflammatory drug and a symptomatic treatment.

OCA stimulated blood levels of FGF19 as has been shown before [87]. We expected to find increases in FGF19 with OCA in the control subjects in this study, but were uncertain whether patients with secondary BAD would be able to respond. The responses (not part of this thesis), both in the short term after the first OCA dose, and after 2 weeks treatment, show that in primary BAD, a potent FXR agonist like OCA is able to enter the ileal enterocyte and stimulate FGF19 transcription and blood levels to median values similar to those found in healthy controls. In SBAD patients median levels of FGF19 were stimulated in patients excluding the 3 >45cm ileal resection patients but levels still did not reach the same levels achieved in the idiopathic control or PBAD patients.

OCA was well tolerated, confirming findings in other trials. A recently completed phase IIB trial into the use of OCA in non-alcoholic steatohepatitis involving 25mg OCA therapy for 72 weeks has recently been published providing some data on long term safety [168]. The increases in total and LDL-cholesterol found here are similar to those found before in patients with nonalcoholic fatty liver disease and type 2 diabetes treated with OCA[87], and also in gallstone patients treated with CDCA, the natural FXR agonist [173]. This may be an expected consequence of lowering bile acid synthesis.

In our overall study population, new bile acid synthesis (as measured by fasting C4 levels) is reduced by 80% from the pretreatment values, and as bile acid excretion is the main means of cholesterol loss from the body, the 10-20% increase in serum cholesterol levels is not surprising. The time course of these changes and any long term effects are unknown.

A clear limitation of this study is that recording symptoms in diaries is subjective and could easily have been influenced by placebo effects. However, symptomatic responses were considered together with the operator-blinded biochemical measures (FGF19, C4 and BA) and the primary outcome of this proof-of-concept study (fasting FGF19) was a quantitative endpoint. Future trials of the clinical effects of OCA will utilize a double-blind, randomized, placebo-controlled study design. The present study also compared symptoms on OCA therapy to symptoms without specific therapy and future trials should include comparisons of OCA to treatment with bile acid sequestrants.

In conclusion, this proof-of-concept study provides the first evidence that FXR agonists produce reductions in bile acid synthesis and clinical symptom responses in patients with BAD. Targeting FXR to stimulate FGF19 is a novel therapeutic approach for many patients with secondary BAD, and for the large group of patients with primary BAD, currently receiving other treatments for functional diarrhea or D-IBS.

Chapter 8: Variation in *DIET1* may modify the PBAD phenotype by influencing serum FGF19 levels

Abstract

Background: Serum fasting FGF19 is positively correlated with SeHCAT retention in patients with chronic diarrhoea. The phenotype of PBAD is associated with low median levels of FGF19, excessive BA synthesis and low SeHCAT retention due to excessive faecal BA loss. A novel protein DIET1 promotes FGF15/19 secretion from cell lines. A common non-synonymous SNP in the *DIET1* gene has recently been shown to enhance FGF19 secretion. It has been hypothesized that this *DIET1* variant may be a modifier of BAD severity.

Methods: We performed a case-control, candidate gene study for this SNP on 78 patients with chronic diarrhoea who had been assessed with fasting FGF19 levels and SeHCAT testing. We genotyped each patient for this SNP using an allelic discrimination assay. We hypothesized that the SNP would have a reduced frequency in the PBAD patients defined by seHCAT retention levels.

Results: The SNP occurred with a minor allele frequency of 0.25 in our cohort of patients which is similar to findings from the human genomes project. The SNP was not found to be associated with either control patients or PBAD patients defined by SeHCAT. However in the PBAD patients defined by a SeHCAT retention of <10%, the variant was associated with a significantly higher mean fasting FGF19 (253 ± 53 vs 119 ± 19 , $p=0.02$). Significant positive correlation between SeHCAT retention and FGF19 levels were present in patients expressing the major allele but this relationship was not present in patients expressing the minor allele.

Conclusion: The association between fasting FGF19 levels and Faecal BA losses may be modified by this *DIET1* variant in patients with chronic diarrhoea. This variant may also contribute to the large variability of fasting FGF19 levels in human studies in general.

8.1 Background

Given the similarity between the phenotype of Diet1-deficient mice and the clinical features of BAD (described in the introduction, [158]), Karen Reue et al had sequenced the coding region of *DIET1* in a case-control study sample (provided by our research group) of primary BAD (unpublished work, personal communication). This preliminary study had genotyped 44 patients including 22 patients with PBAD and 22 control patients. Although no causative variants were found, a common non-synonymous SNV *rs12256835* was found exclusively in the controls. The SNV leads to an amino acid substitution of glutamine at position 1721 in place of a histidine residue, H1721Q. Functional studies revealed that the Diet1–1721Q isoform promotes FGF19 secretion in a human intestinal cell line HT-29 using the FGF19 R+D Quantikine assay also used in this thesis [174]. It is hypothesized that this variant may modify the phenotype of PBAD by enhancing FGF19 secretion.

Using a candidate gene approach we went on to perform a genotype study of this SNV on a further larger replication case-control cohort of patients. PBAD can be regarded as mild moderate or severe according to SeHCAT retention values. We aimed to look for associations of this SNV according to commonly used SeHCAT ‘cut-off’ levels at 5, 10 and 15% to define mild, moderate or severe phenotypes.

8.2 Specific methods genotyping

Previously extracted genomic DNA was studied for the *rs12256835* SNP in a cohort of patients who have been previously described [71]. This cohort consisted of 78 prospectively recruited patients with chronic diarrhoea who underwent SeHCAT testing and fasting FGF19 serum sampling. Other intestinal diseases such as IBD or coeliac disease had been excluded from this cohort and the ethnic origin was predominantly caucasian. The 1000 genome project had revealed an *rs12256835* minor allele frequency (MAF) of 0.28 [100]. We assumed a similar MAF of 0.28 in the diarrhoea control patients from this study. On the basis of recent findings of lower prevalence of the minor allele in

PBAD patients, power calculations were therefore designed to detect a low frequency of 0.05. For 90% probability at detecting a significant difference between a prevalence of 0.28 and 0.05 we would need 64 alleles (32 patients) in each group (diarrhoea controls vs PBAD). Diarrhoea controls were defined as patients with symptoms of chronic diarrhea who did not demonstrate excessive faecal BA loss with SeHCAT retention levels of >10%. Other causes of chronic diarrhea such as coeliac disease or inflammatory bowel disease had been excluded. The Allelic discrimination assay used to genotype the patients is described in Chapter 2.7

8.3 Results: The minor allele may attenuate the reduced FGF19 levels associated with PBAD

Dominant genetic models were used to compare patient phenotypes according to the presence or absence of the minor 'G' allele (the *rs1225683* SNV). The allelic discrimination assay assigned a genotype for all 78 patients (figure 1). 34 patients had a genotype containing the minor allele (5 homozygous, 29 heterozygous) and 44 without. Thirty nine minor alleles (the *rs1225683* SNV) were observed out of the total 156 alleles giving an overall minor allele frequency in this cohort of 0.25.

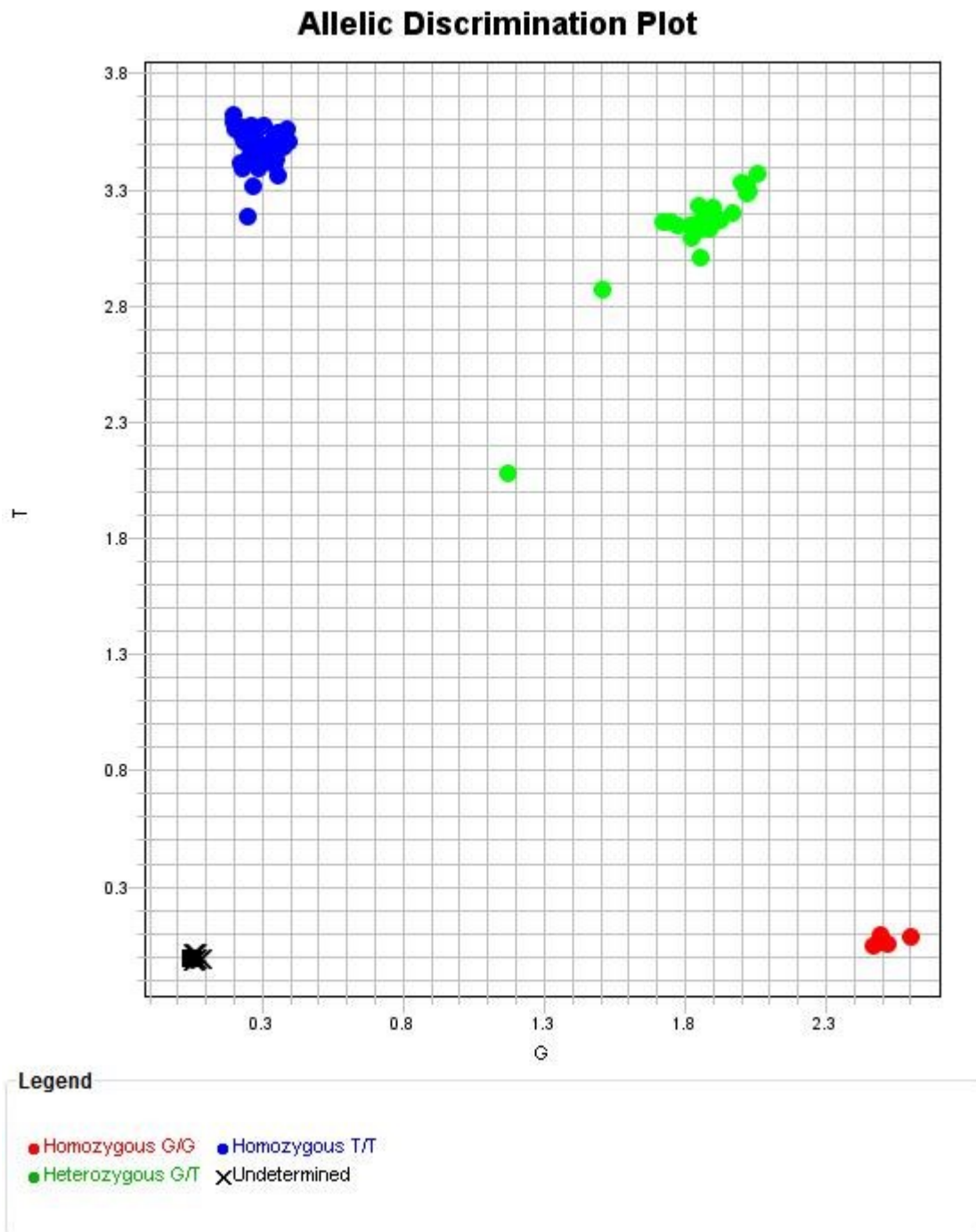


Figure 2. Allelic discrimination plot from Taqman rs12256835 genotype assay, StepOne software V2.3. The blue dots signify samples where only the VIC dye was detected (T/T genotype). The red dots signify the small number of patients where only the FAM dye was detected ie the patients homozygous for the minor allele (G/G genotype). The green dots represent samples where a mixture of the two dyes were detected ie heterozygous, genotype G/T .

The median SeHCAT retention value from all 78 patients studied was 16.5 % (IQR 8 – 29). Basic patient characteristics according to SeHCAT retention levels < or >10% are shown in **table 1**. In this larger replication cohort of patients there was no significant association between the SNV and groups of patients according to the seHCAT definition of mild, moderate or severe PBAD. Unlike the preliminary case-control study the SNV was not found to have significantly increased expression in control patients. Further analysis of the cohort of 78 patients revealed that the *rs1225683* SNV was associated with significantly higher mean fasting FGF19 values in patients with moderate PBAD (seHCAT < 10%) **table 2**. A similar trend revealed a higher mean fasting FGF19 level in patients expressing the *rs1225683* SNV when comparing patients from the upper quartile to the lower quartile of SeHCAT values (≤8% SeHCAT retention vs ≥29%) **table 2**.

		T/G & G/G	T/T
SeHCAT ≤10%	n	12	14
	Age	48 ± 5	43 ± 4
	M:F	7:5	5:9
	Ethnicity		
	W	8 (67%)	10 (71%)
	AI	1 (8%)	2 (14%)
	AC	1 (8%)	0
	ME	1 (8%)	1 (7%)
	Other	0	1
SeHCAT >10%	n	22	30
	Age	43 ± 4	50 ± 3
	M:F	8:14	14:16
	Ethnicity		
	W	15 (68%)	26 (90%)
	AI	3 (14%)	2 (7%)
	AC	3 (14%)	0
	ME	1 (14%)	0
	Other	0	1 (3%)

Table 1. Demographics table: Showing age, male:female ratios and ethnicity according to genotype and SeHCAT retention < or > 10%.

		FGF19 (pg/ml)	
		T/G & G/G	T/T
SeHCAT ≤10%	n	12	14
	Mean ± SEM	253 ± 56	119 ± 19 *
SeHCAT >10%	n	22	30
	Mean ± SEM	215 ± 28	246 ± 24

Table 2. 2x2 table comparing mean FGF19 values +/- SEM in patients with T/G or G/G genotype to those with genotype T/T in patients with SeHCAT >or <10%. * p=0.022, T/T vs. T/G & G/G, SeHCAT ≤10%, independent t-test.

		FGF19 (pg/ml)	
		T/G & G/G	T/T
Lower Quartile	n	7	12
SeHCAT ≤8%	Mean ± SEM	202 ± 62	116 ± 20.73
Upper Quartile	n	5	14
SeHCAT ≥29%	Mean SEM	255.5 ± 42	245.0 ± 42

Table 3. 2x2 table comparing mean FGF19 values +/- SEM in patients with T/G or G/G genotype to those with genotype T/T in patients with SeHCAT values in the upper and lower quartiles (≤8% SeHCAT retention and ≥29% SeHCAT retention).

Linear regression analysis demonstrated FGF19 levels were related to SeHCAT retention levels in T/T genotype patients without the minor G allele ($n = 44$, $P = 0.034$) but this relationship was not present in the patients with the genotypes containing the G allele ($n = 34$, $P = 0.732$, **Figure 3**). Multiple regression analysis of age, BMI, FGF19 and *rs1225683* genotype revealed that SeHCAT values were significantly dependent upon the presence and number of minor alleles ($P = 0.036$).

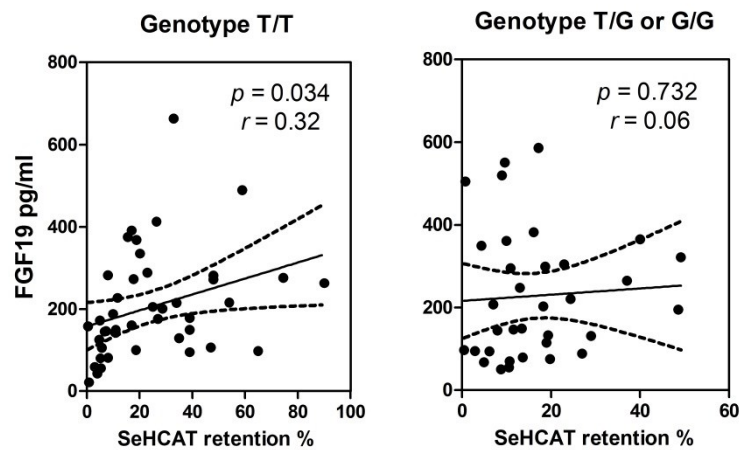


Figure 3. Linear regression analysis of % SeHCAT retention at 7 days vs fasting FGF19 levels taken at the time of SeHCAT testing according to *rs1225683* genotype. Lower SeHCAT retention signifies greater fecal loss of bile acids from the enterohepatic circulation. A positive relationship between SeHCAT retention and FGF19 levels was present in the patients with a T/T genotype but not in those containing the G allele. By potentially enhancing FGF19 secretion, this SNV may protect against the reduced FGF19 production associated with PBAD. It may be that some patients with PBAD still have excessive BA production and losses due to FGF19 receptor defects within the liver (FGFR4 / β Klotho) [175]. (Pearson's correlation coefficients shown).

8.4 Discussion

This SNP has a frequency of 0.2816 in the 1000 Genomes project, suggesting that it is a common source of polymorphism in the Diet1 protein. Interestingly, in 1000 Genomes project[100], the allele frequency for *rs12256835* is similar in European and Asian individuals (MAF 0.17, 0.24 respectively). However there is an increased frequency in the Luhya of Webuye, Kenya (MAF 0.57), the Yorubans of Ibadan, Nigeria (MAF 0.63), and in individuals of African ancestry in the southwestern United States (MAF 0.43). Individuals of African descent were not present in our study sample. Our cohort consisted of patients of mainly white European ethnicity and we found a MAF of 0.25 in our cohort as a whole which is consistent with the 1000 Genomes project.

The preliminary results from the small case-control candidate gene study on the SNV had revealed a complete absence on the minor allele in patients with PBAD. All of the PBAD in this cohort had SeHCAT's less than 10%. This finding was not borne out in our larger replication cohort. There were no significant associations in cases vs control despite analysing according to mild, moderate or severe phenotypes based on SeHCAT testing. Although this SNP may not completely protect against the PBAD phenotype, our findings suggest that it may have an ameliorating influence. The SNV was associated with a relatively higher mean fasting FGF19 than those without the SNV in the group of patients with PBAD. This *DIET1* variant may therefore modify FGF19 levels in PBAD. The positive correlation that is usually observed between SeHCAT levels and FGF19 levels is not found in the cohort of patients carrying the SNP. It would be interesting to characterize these patients further to see if they have less severe symptoms or differences in post-prandial FGF19 rather than fasting FGF19. In the earlier mouse models, diet1 deficient mice had reduced fgf15 expression, increased BA synthesis, were protected against atherosclerosis and had reduced circulating cholesterol levels. It would be interesting to see if this SNV is associated with increased FGF19 levels in other patient groups and whether this may be associated with dyslipidaemia.

8.5 Conclusion

Defective ileal FGF19 secretion is a putative mechanism in PBAD. The H1721Q variant resulting from this SNP may enhance FGF19 secretion. This study demonstrates that this SNV may have clinical relevance. In a condition associated with low median levels of FGF19, patients expressing this variant had significantly higher median levels of FGF19 than those that did not. The variant may modify The phenotype of BAD by influencing FGF19 production. However it is recognised that this is only a candidate gene study in a small cohort of patients. A full genome wide association study would be required to produce more statistically meaningful results.

Chapter 9: Conclusion and future work

The results from this thesis include the first study to demonstrate an association between reduced median levels of serum FGF19 levels and symptoms of diarrhoea in CD, a condition strongly associated with bile acid malabsorption and SBAD. It is also the first to demonstrate reduced basal and BA stimulated FGF19 transcripts in ileum from patients with PBAD and low SeHCA levels. Reduced median BA stimulated FGF19 expression was also found in ileum from CD using unconjugated CDCA. Reduced expression of FGF19 transcripts has therefore been observed in both BAD conditions, PBAD and SBAD and supports the role of an impaired FXR-FGF19 pathway in the pathogenesis of BAD. The two conditions of BAD are contrasted throughout the thesis by the fundamental difference that SBAD is associated with BAM yet in PBAD BA absorption is thought to remain preserved.

In chapter 3 in patients with CD we have demonstrated an inverse relationship between fasting serum FGF19 levels and the extent of ileal resection or the severity of diarrhoea symptoms suggesting a semi-quantitative relationship between FGF19 levels and the amount of remaining functioning ileum. However there is also a suggestion from the results in chapter 3 that FGF19 levels may be more dependent on the degree of ileal inflammation than remaining functional ileum as the lowest levels of FGF19 were actually observed in non-resected patients with relatively short segments of ileal inflammation. In ileal resected patients with quiescent disease the relationship between FGF19 and resection length may not be linear. There seems to be a minimum basal level of circulating FGF19 which is low but does not become undetectable despite more extensive ileal resections. Other work has demonstrated low FGF19 levels in short bowel patients but the levels observed were within a similar range to the cohort of patients studied in this thesis with much shorter resections.

Although detectable basal fasting levels of FGF19 may be preserved despite extensive small bowel resections, results from this thesis has suggested that the post prandial rise in FGF19 levels is

dependent on a relatively short section of terminal ileum. Exploratory studies on serial post-prandial FGF19 levels revealed a complete lack of meal induced rise in 3 patients from chapter 3 with relatively limited ileal disease or resection. In chapter 7, serial post-prandial levels also lacked a post-prandial rise in levels in 3 patients with slightly more extensive ileal resections (45 – 78 cm) despite the administration of the potent FXR agonist (obeticholic acid) with meals. Chapter 6 refers to a small pilot study on BA stimulated FGF19 transcript expression in ileal explants from neo-terminal ileum in previous ileal resected CD patients which revealed a trend towards very low levels of expression.

The CD study of chapter 3 suggests that low levels of this hormone may have a high sensitivity for predicting BAD. As virtually all non-resected patients with diarrhoea and known ileal disease, undoubtedly associated with increased faecal BA losses, had low levels of FGF19. We were unable to properly assess sensitivity and specificity in this study without a parallel gold standard assessment of faecal BA losses (Faecal BA levels, SeHCAT levels, or C4, or response to BA sequestrants).

Reduced FGF19 levels may be less specific for SBAD as a number of CD patients with low levels did not experience diarrhoea symptoms. Despite this potential lack of specificity, a low FGF19 may have clinical utility as a marker of BAD and may predict a response to BA sequestrants in CD patients suffering from diarrhoea with unknown disease distribution. To confirm this a larger study of FGF19 which includes other markers of BAD and / or a subsequent trial of BAD specific therapy. If the study included prospective data on the presence of ileal inflammation (colonoscopy, or small bowel MRI) then one could assess if low levels were more sensitive as a biomarker ileal inflammation rather than BAD. BAD does not always appear to be associated with ileal inflammation or resection. The reasons for this have not been elucidated and also requires further study. Factors involved are likely to encompass a number of factors such as: the degree of associated colonic inflammation, microbial de-toxification of BA, colonic adaptation to increased faecal BAs.

The individual variability in ileal FGF19 induction by both CDCA and GCDCA may indicate different levels of BA homeostatic equilibrium. Results from Chapter 5 indicate that individuals vary significantly in their capacity to produce ileal FGF19 at a transcriptional level.

In BA physiology, the main variable that must be maintained for efficient fat digestion is a high concentration of BAs within biliary secretions and small intestine. The enterohepatic circulation of a pool of recycled BA provides a high efficiency, low energy process, where the liver does not have to synthesize large quantities of new BA. There seems to be a great deal of redundancy in this process as patients can usually maintain nutritional status without the need for parenteral nutritional support with as little as 50cm of remaining small bowel in continuity with colon, or as little as 100cm of small bowel as a jejunostomy. The same can be said for patients that have undergone cholecystectomy. This is due to the liver's capacity to increase BA synthesis up to 15 fold the normal baseline levels if required [176].

With this level of redundancy in the levels of BA synthesis, there may be a great deal of variability in the BA pool size between individuals under normal conditions. This would be largely dictated by levels of hepatic BA synthesis. Some individuals will therefore have larger pool sizes and higher levels of BA entering the colon, being lost in faeces and vice versa. Levels of FGF19 expression is likely to be a key factor in the set point at which the bile acid pool size is maintained between individuals. The wide variability in ileal BA stimulated FGF19 expression observed in chapter 5 may underlie this variability in levels of BA synthesis and BA pool size.

Some patients may have low ileal BA-FGF19 responsiveness. This would lead to a steady state with low basal and/or post prandial FGF19 levels, higher Hepatic BA synthesis rates, large BA pool size, increased faecal BAs and convey a degree of susceptibility to BA diarrhoea and possibly gallstones or even obesity. Conversely, others may have high levels of ileal BA-FGF19 responsiveness maintaining a smaller BA pool size, lower faecal BAs and be more susceptible to constipation. Consistent with the

central role of FXR-FGF19 and BAs in the balance between diarrhoea and constipation are studies that have observed Low faecal BA levels in patients with constipation and irritable bowel syndrome[177]. Elobixibat is an ASBT antagonist which has been shown to block the uptake of BA at the terminal ileum and resolve constipation by increasing faecal BAs [178, 179]. This would also be expected to reduce ileal BA induced FGF19 expression and further increase BA synthesis and faecal BA levels. This represents a converse approach to stimulating FGF19 in the treatment paradigm described in chapter 7. A new emerging treatment paradigm is to treat IBS related constipation or diarrhoea by manipulating faecal BA excretion [127, 180-182]. Most clinical trials are at phase II status in this respect as in Chapter 7 of this thesis. Further phase III trials are eagerly awaited.

This thesis has demonstrated that in PBAD FGF19 transcripts are significantly lower in basal, unstimulated, fasting samples from patients with low SeHCAT values. However ASBT transcripts are also lower in basal expression samples (chapter 4); if ASBT protein levels are also reduced, it could be that this results in reduced bile acid uptake and consequently reduced bile acid/FXR-dependent stimulation of FGF19 transcription. This appears not to be the only reason, as incubation with CDCA also gives SeHCAT-dependent FGF19 transcript stimulation. Unconjugated CDCA, unlike glycine-conjugated GCDCA, is not dependent on transport by ASBT and will produce FXR-mediated transcription in cells not expressing ASBT. Significantly lower CDCA stimulated FGF19 transcript expression was also found in CD patients compared to healthy controls. This suggests that both SBAD and PBAD may be associated with defects in BA stimulated FGF19 production at a transcriptional level that is not simply a result of impaired active BA transporter defects. BA stimulated FXR transcription of target genes has been shown to be inhibited by a number of other intracellular pathways.

This thesis supports the role of an impaired FXR-FGF19 pathway in the pathogenesis of BAD in some patients in even in the absence of BAM. In CD, an impaired FXR-FGF19 may contribute to the BAD experienced by these patients in addition to BAM[169]. We know that the last 100cm of terminal

ileum is important in maintaining the BA pool size as resections beyond this will lead to a depleted BA pool and steatorrhoea [183]. This distance relates to a gradient of ASBT expression and is likely to represent the length of terminal ileum that is critical to the reabsorption of luminal BAs. There is a suggestion from exploratory data in this thesis that post-prandial FGF19 expression may be expressed over a shorter length of 50cm of terminal ileum . Older BA kinetic studies in CD have confirmed that despite the presence of sub-optimal ileal reabsorption of BA with limited ileal resection or ileal inflammation, the BA pool size is maintained. More recent studies have confirmed a rise in FGF19 levels post oral CDCA administration CD patients in the absence of known ileal involvement or ileal resection [117]. However if post prandial FGF19 response is lost then this could lead to disproportionately high levels of hepatic BA synthesis which is in excess of what is required to maintain the BA pool size. The new steady state would be associated with higher faecal BA levels. Larger prospective studies of post-prandial FGF19 measurements in ileal CD would be required to confirm this.

Exploratory studies of certain functional genetic variants have been undertaken in PBAD patients but none have been shown to specifically associate significantly with this seHCAT defined phenotype. The association of the variant in the DIET1 gene with FGF19 levels in moderate PBAD (SeHCAT<10%) presented in this thesis is the first study to show a potential association between genetic variation and clinical features of the condition (FGF19 levels in SeHCAT < 10%). The H1721Q variant of the DIET1 protein is thought to enhance the secretion of FGF19 protein. This would not be related to the findings of reduced FGF19 transcription that we have found in ileal explant studies. However, the variant may modify the expression of serum levels of FGF19 protein. Our explant protocol could be used to investigate this aspect further by studying FGF19 culture fluid levels from patients that have been genotyped for the H1721Q variant. An association between BA stimulated culture fluid FGF19 levels and genotype could be examined in relation to the BA stimulated FGF19 transcript levels. This would require analysis of the explant culture fluid from the cohort of patients studied in chapter 6.

Patients could be genotyped with future recall for blood sampling and genotyping for the DIET variant. An alternative approach would be to PCR the cDNA from the explant samples with the primers used for allelic discrimination assay in chapter 8. This would be possible as the variant is within the coding sequence of the gene and hence would be part of the mRNA and represented within the cDNA. The presence or absence of amplification with the primers would allow a crude assessment of the expression of the variant. It would not accurately determine if a heterozygous or homozygous expression of the genotype is present however. Also, some genes show allelic imbalance in their expression which would need to be taken into consideration[184]. The primers would also have to be carefully optimized for use with different SYBRgreen chemistries.

Summary

In summary, this thesis presents results that supports the original hypothesis. The results demonstrate impairment in the ileal FXR-FGF19 pathway at multiple levels in patients with a diagnosis of PBAD (diagnosed by SeHCAT) or SBAD. We have demonstrated that serum FGF19 levels may have clinical utility as a marker of BAD or ileal inflammation in patients with Crohn's disease. The thesis also supports the possibility that disturbances in the FXR-FGF19 pathway may not simply occur at the BA absorption or FGF19 transcription level but may also relate to FGF19 secretion influenced by genetic variation in the DIET1 gene. This may further impact upon hepatic FGF19 responses to secreted FGF19. The relationship between ileal FGF19 expression and SeHCAT levels in chapter 6 strengthens the role of SeHCAT as a diagnostic tool for defining the PBAD group of patients. The observation of low FGF19 expression in patients with low seHCAT retention confirms and further characterizes the specific phenotype of this subgroup of patients who might otherwise be diagnosed as diarrhoea-predominant irritable bowel syndrome. As BA have emerged of as modulators of (postprandial) lipid, glucose and energy metabolism (via FXR and TGR5 receptors) further associations of the PBAD phenotype with other metabolic diseases [185-187] is being elaborated further. Finally the proof-of-concept OBADIAH1 study provides the first evidence that

FXR agonists produce reductions in bile acid synthesis and clinical symptom responses in patients with BAD. This study supports a new treatment paradigm aimed at manipulating faecal BA levels to improve functional GI symptoms.

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